PYRIDOXINE #124

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P Y R I D O X I N E

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PYRIDOXINE

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PYRIDOXINE

Summary

Pyridoxine, or vitamin B_6, is a beta-hydroxypyridine derivative, which was first identified as a separate member of the vitamin B complex in 1934 by P. Gyorgy. Gyorgy has been active in B vitamin research since 1927. He was originally concerned with seborrhoid dermatitis and acrodynia in infants. Goldberger and Lillie found that when rats were fed diets deficient in the whole vitamin B complex, supplemented with riboflavin (B_2) and thiamine (B_1), they developed a pellagra-like dermatitis. This led to the realization that there was another B vitamin (978). In fact eight other B vitamins plus various submembers were identified in the 1930's and 40's. The B_6 deficiency symptoms in rats are referred to as rat acrodynia. In 1932, a Japanese researcher, S. J. Ohdake, isolated pyridoxine HCl from rice bran and determined the empirical formula not knowing that it was a vitamin (2668). Lepkovsky was the first to report the isolation of pure crystalline vitamin B_6 in 1938. The exact structure of pyridoxine was derived by R. Kuhn et al. in 1939 at the Merck laboratory. Gyorgy was responsible for the name "pyridoxine" in 1939 (978).

A water-soluble vitamin, pyridoxine, is an essential body constituent. It is a coenzyme for a long series of decarboxylase enzymes for amino acid transformations (including lyases, oxidoreductases, transferases, isomerases, and many others). Pyridoxine is not synthesized by mammals, as is the case with vitamins in general, and is obtained from dietary sources. Horses and ruminants do not normally need pyridoxine in their diet (1721). Only small amounts of pyridoxine are needed by man. NAS/NRC sources state that the dietary requirement of vitamin B_6 for adult humans is 2 to 2.5 mg/day. The best sources of pyridoxine are yeast and rice bran; others are liver, molasses, milk, egg yolk, spinach, other green vegetables, and seed vegetables, such as corn. The total annual usage reported for 1970 was 16,834 pounds (1846).

There are 3 free and 3 phosphorylated forms of pyridoxine - pyridoxine, pyridoxamine, pyridoxal, pyridoxine phosphate, pyridoxamine phosphate, and pyridoxal 5-phosphate. The latter form is the most significant biologically, since this form acts as the coenzyme for the many reactions previously mentioned.

The following diagram indicates the biological conversions of pyridoxine inside the body (2294):

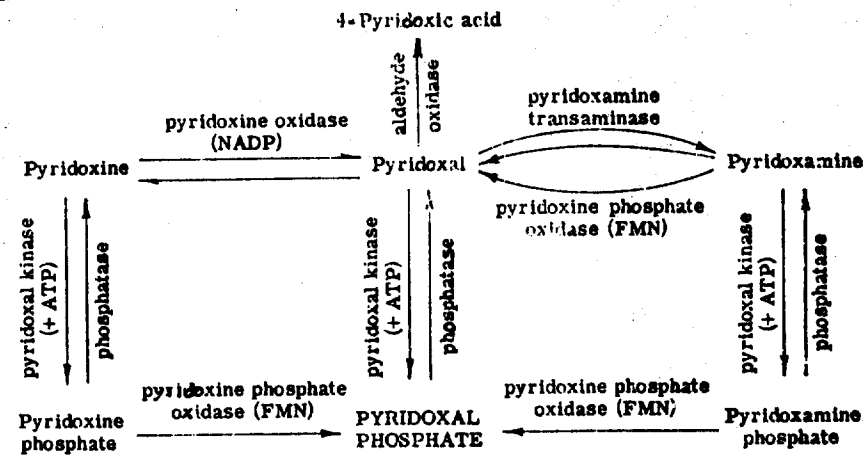


FIG. 1. Metabolic interconversions of the various forms of vitamin B_6.

Hillman reported that vitamin B_6 was found by Turner and Reynolds, 1955 and by Tower, 1956 to be synthesized in the lower bowel by microorganisms (1092).

Booth and Brain (301) stated that pyridoxine is not absorbed to a significant extent in the stomach of black and white rats. Most of the absorption occurs in the jejunum, smaller amounts in the ileum, and very small amounts in the colon. It was hypothesized that pyridoxine is absorbed by passive diffusion rather than by active transport, since a linear relationship exists between the dose given and the amount absorbed. Over 90% of the pyridoxine given was absorbed within 60 minutes, regardless of the size of the dose.

Booth (300) stated that the ileum acts as a reserve to take up excess pyridoxine. Contractor and Shane (511) found that high levels of pyridoxal phosphate, pyridoxal, and pyridoxic acid were detected in the blood of 2 healthy women 2 hours after oral administration of 100 mg pyridoxine. Pyridoxine was elevated in the urine within 2 hours; however, no pyridoxine was recovered in the blood. These findings indicate that pyridoxine is rapidly absorbed from the intestine and rapidly metabolized, excreted, or stored. Only 30% of the administered pyridoxine was recovered in its various forms in the urine and blood after 48 hours, indicating a large tissue uptake. No significant variation was found in the levels of B_6 and its other forms in women at various stages of the menstrual cycle.

Johansson et al. (1235) studied the distribution of 20 mcg $^3H_8$ - labeled pyridoxine injected i.v. into male mice for up to 7 days. Labeled, free pyridoxine was gone from the liver within 60 minutes, indicating its rapid phosphorylation. During the first hour, the main labeled forms of pyridoxine recovered in the liver were pyridoxine-5'-phosphate and pyridoxal-5'-phosphate. One to 7 days after the pyridoxine was administered, the two main forms were pyridoxal-5'-phosphate and pyridoxamine-5'-phosphate; the other forms were recovered in small amounts. In the carcass, the main forms recovered were pyridoxine and pyridoxine-5'-phosphate the first hour and about equal recoveries of the other 4 forms from 1 to 7 days later.

Dahlkvist et al. (563) did a similar distribution study in mice,

including more organs. The dose of $^{3}H_{8}$-pyridoxine (22 mcg i.v.) was the same. In all of the tissues studied (carcass, liver, intestine, kidneys, heart, lungs, spleen, skeletal muscle) except for the brain, a high initial uptake of labeled pyridoxine was followed by a rapid decrease during the first day. The permeability of the blood-brain barrier is low, since the maximum uptake was observed after 24 hours. The three organs with the highest initial uptake were the liver, the heart, and then the skeletal muscle. After a period of equilibration, the tissue concentrations were about equal. Most of the total recovered isotope was in the carcass and liver. During a 30 day period, 75% of the isotope recovered was in the carcass, 10 to 15% was in the liver, and the rest in other organs.

McCoy and Colombini (1678) injected C^{14}-pyridoxine into male Swiss mice and found that the main initial reactions were phosphorylations of the three free forms of pyridoxine in the liver and the oxidation of pyridoxine to pyridoxal in the brain and carcass. The predominant forms of pyridoxine in the liver, brain, and carcass were pyridoxal phosphate and pyridoxamine phosphate. The carcass was considered to be a storage site for pyridoxine, since 7% of the total isotope given was found there after 24 hours. Only 1.6% was in the liver, and only 0.1% was in the brain. The carcass also contained larger amounts of pyridoxal (0.9%) pyridoxine phosphate (0.7%) and pyridoxine (0.2%) than the liver.

After the injection of 20 mcg ^{3}H-pyridoxine i.v. into rats, Tiselius (2593) found that pyridoxine easily passed through the blood-brain barrier and was evenly distributed between the cerebrum and the cerebellum. Male mice injected with 20 mcg ^{3}H-pyridoxine i.v. converted pyridoxine to pyridoxal phosphate, (61% of total isotope) within 40 min. This was the major form of pyridoxine recovered, the level remaining at about 50% of the total isotope for the 13 day period studied. Pyridoxamine phosphate varied from 28 to 56% and pyridoxal was usually less than 10% for the 13 day period. The pyridoxine phosphate form reached a peak at 20 minutes and then dropped to below 10%.

Scudi et al. (2291) found that vitamin B_6 was rapidly absorbed from the gastro-intestinal tract. Evidence for this was the rapid appearance of B_6 in the urine of dogs and humans. Maximum recovery occurred in 1 hour after the oral dose of 4.5 to 45 mg/kg vitamin B_6 to fasting dogs, and in 2 or 3 hours (20% recovery) in nonfasted dogs. When 50 mg i.v. (about 0.68 mg/kg) or 100 mg orally were administered to 24 nonfasted men, recoveries were 8.7% after 1 hour and 7.6% after 4 hours respectively.

Johnson et al. (1237) found that when daily doses of 8 mg pyridoxine were given orally to 4 healthy men (in addition to a normal diet), an average of over 50% of the added pyridoxine was recovered in the sweat and urine as pyridoxine and its metabolites. The pyridoxine supplement increased the total excretion (sweat and urine) by 4.3 mg. Of this increase, 5.5% was pyridoxine, 9.5% was pseudopyridoxine (or pyridoxal) and 85% was 4-pyridoxic acid.

In normal urine samples, collected from 3 men, Rabinowitz and Snell (2060) found that the main metabolite of pyridoxine excreted was 4-pyridoxic acid (91 to 98% of the total excreted). This has been confirmed repeatedly by other investigators, Johansson et al., (1232) and

Cox et al. (539). Some pyridoxamine and pyridoxal were detected, but no measurable pyridoxine was found. After the men ingested 100 mg pyridoxine HCl, pyridoxine was the main form of B_6 excreted; however, less than half of the administered dose was recovered as pyridoxine. The main metabolite was again 4-pyridoxic acid (69% of the total excreted); 4 to 5% was pyridoxamine, and 14 to 15% was pyridoxal. Maximum amounts of the compounds excreted occurred in 2 to 5 hours. The low recoveries of the administered pyridoxine may have been due to the formation of unidentified metabolites or to the storage of pyridoxine in the body tissues.

Cox et al. (539) found that the fecal excretion of ^3H activity in rats after the administration of ^3H-pyridoxine HCl (616 mcc) was very small. The total excreted in the feces in 96 hours was 0.03%. The half life of ^3H activity in the blood and urine was 45 minutes (after the first 15 minutes); 70% of the pyridoxine was excreted unchanged.

Johansson et al. (1232) found that the half-life of ^3H in the body following administration of 250 to 400 mcg ^3H-pyridoxine in saline i.v. to 3 men was 18 to 38 days. About 15 to 20% of the ^3H was excreted the first day. The total amount of B_6 in the body was from 40 to 150 mg. The turnover rate of vitamin B_6 was calculated to be 2.2 to 4.4% per day or 1.7 to 3.6 mg per day, a relatively slow rate. The amount of pyridoxine excreted per day was 6.5% per day in the urine and 7.9% per day from the body reservoir, according to a study in mice by Dahlkvist et al. (563). The dose of $^3H_8$-pyridoxine was 22 ug i.v. in water.

Contractor and Shane (513) identified another metabolite of pyridoxine, 4-pyridoxic acid 5'-phosphate, which may explain the poor recoveries of administered pyridoxine in previous studies. Doses of ^3H- and C^{14}-labeled pyridoxine were injected i.p. into rats and TLC, electrophoresis, and fluorescence were used to identify a new metabolite, which accounted for approximately 20% of the total radioactivity given. The authors believe that the major metabolic pathway is the oxidation of pyridoxal phosphate to 4-pyridoxic acid 5'-phosphate which is hydrolyzed to 4-pyridoxic acid. Another possible pathway is pyridoxal phosphate to pyridoxal (by hydrolysis) which is then oxidized to 4-pyridoxic acid by aldehyde oxidase.

Contractor and Shane (512) compared the levels of pyridoxine, all forms, and its metabolites in the urine and blood of men, pregnant women, and nonpregnant women. A vitamin B_6 deficiency was noted in pregnant women, due to an increased requirement for this vitamin by the fetus.

Flexner and Chassin (761) found that the amount of pyridoxine excreted tends to decrease with increasing age in humans following intravenous administration of 50 mg pyridoxine.

In the review of Sakurazi (2213) on the function of pyridoxine in fat metabolism, it was suggested that pyridoxine is involved in the conversion of carbohydrates and proteins to fats. Pyridoxine is also necessary for the conversion of linoleic acid to arachidonic acid and may act as a cofactor for the conversions of both palmitic acid to palmitoleic acid and stearic acid to oleic acid. Other evidence for

the relationship between pyridoxine and essential fatty acids (EFA) is that the deficiency symptoms of each of them are similar and that the symptoms of one can be relieved by the administration of the other. The function of pyridoxine may be as a physiological antioxidant, such as the stabilization of vitamin A in vivo by pyridoxine.

Pyridoxine has been found by Failey (728) to have a slight but significant serum cholesterol-lowering effect in 6 nondiabetic hospitalized patients (male) and 5 diabetic patients (female). Pyridoxine was given orally, 400 mg/day, for 36 days in the nondiabetic subjects and 7 days for the diabetic subjects. Lupien (1585) found that pyridoxine did not produce significant cholesterol-lowering effects in the serum, liver, and heart in rabbits given doses of 6 mg/kg/day i.p. or as 1% of the diet for up to 8 weeks. Moreover, there was a temporary increase in serum and liver cholesterol levels compared to the control group after 3 weeks of treatment.

Kawanable (1295) reported that the injection of 10 mg/kg pyridoxine i.v. into fasted rabbits appeared to inhibit the elevation of the blood sugar level within 3 hours, when compared to a saline-injected control group. Pyridoxine given in combination with vitamin B_2, with nicotinamide, or with both of the other vitamins produced elevated blood sugar levels.

The administration of excess levels of pyridoxine in the diet (0.2 mg%) of male white rats for 4 weeks by Kato (1288) temporarily suppressed the biosynthesis of protein-bound lipoic acid within 2 weeks.

Since pyridoxine serves as a coenzyme for many enzymes catalyzing amino acid transformation, there are many studies on the enzyme inducibility of pyridoxine. Two studies are included on the induction of tyrosine transaminase in rats. Hamamoto et al. (1001) reported that the oral or i.p. dosage of 500 mg pyridoxine to male rats increased the hepatic activity of this enzyme 2- to 3-fold. This enzyme induction was thought to be mediated by adrenal glucocorticoids. Similar results were found by Susten and Kirksey (2502) in maternal and fetal rat livers with a dose of 1 g pyridoxine HCl/kg i.p. Another study on alanine aminotransferase activity was performed by Driskell et al. (662). At dietary levels of 10 to 30 mcg pyridoxine, the enzyme activity was increased in rat erythrocytes and in maternal and fetal livers in rats.

Many studies on the effects of pyridoxine in combination with other compounds are in the literature. In 1947, Lecoq et al. (1495) found that 50 ug pyridoxine injected at the same time as adrenaline into rats antagonized the augmentation of nerve chronaxia, which is the effect of adrenaline alone. Cheldelin and King (457) reviewed some pyridoxine antagonists, such as 4-desoxypyridoxine, 5-desoxypyridoxine, 5-desoxypyridoxal, and 5-desoxypyridoxamine which induce the symptoms of vitamin B_6 deficiency.

Goldner and Vallan (909) stated that 3 mg pyridoxine/day orally significantly potentiated the serum cholesterol-lowering effect of 3 g niacin in 53 hypercholesterolemic patients on both normal and low cholesterol diets.

Teyeira and Martinez-Lage (2562) found that pyridoxine antagonized the adverse effects of high doses of ethyl alcohol in rats, guinea pigs and cats. The alcohol intoxication was induced in doses of 10 to 40 ml/kg i.p. or i.v. Pyridoxine was given either before or after the alcohol in doses of 50 to 300 mg/kg i.p. or i.v. Pyridoxine reduced the mortality, slowed the cardiac frequency, and caused EEG recovery in a higher portion of the animals compared to control groups.

Attempts have been made to use pyridoxine along with L-DOPA (levodopa) in treating the symptoms of Parkinsonism in humans. Pfeiffer and Ebadi (1994) stated that each of these drugs alone increases the level of dopamine in the basal ganglia of rat brains. Parkinsonism is believed to be the result of low dopamine levels. When given together, pyridoxine caused the level of dopamine to decrease, possibly by the formation of a condensation product between L-DOPA and pyridoxal phosphate. Pyridoxal phosphate is the coenzyme for the degradation of dopa to dopamine. Leon et al. (1514) reported the same antagonism between the 2 drugs in human patients with parkinsonism.

Pyridoxine has been found to have a beneficial interaction with isoniazid (INH), used in treating tuberculosis. Evidence for this in white mice was reported by Chertkova and Udilova (464). Doses of 3 to 10 mg per os or s.c. given concurrently with 3 mg. INH, a lethal dose, prevented the death of the mice. Goldman and Braman (907) reported that pyridoxine (10 mg per 100 mg INH) was effective in preventing the peripheral neuropathy and optic neuritis in humans given high doses of INH or low doses for long periods of time.

An interesting effect on pyridoxine was reported by McCoy and Colombini (1678). They found that the oxidation of pyridoxine by pyridoxine oxidase in the brain is inhibited by the chronic administration of marijuana in mice.

Pyridoxine is considered to be of very low toxicity. An acute toxicity table can be found on page 12. There is much chronic toxicity data available for pyridoxine. Weigand et al. (2775) found no toxic effects in 5 mice injected with 100 mg/kg/day i.v. for 2 weeks. Both Unna and Antopol (2667) and Unna (2666) reported no adverse changes in weight or pathology when pyridoxine was given orally 0.5 to 25 mg daily to young rats for up to 100 days.

Kato (1288) found that weight gain was temporarily suppressed in 40 day old male rats fed excess pyridoxine (0.2 mg%) in their feed for over 4 weeks. Even with the continued administration of excess vitamin B_6, for 4 weeks, these rats gained as much weight as the controls.

De Bastiani and Batti (591) found that various histological changes occurred as an adaptation syndrome, when excess pyridoxine was given repeatedly to male white rats. With loses of 50 mg i.m. daily for 28 days, the adrenals were enlarged and showed evidence of adrenal cortex stimulation. The adrenals returned to normal size by the end of the experiment. The thymus glands became atrophied, and the number of

leucocytes was high (neutrophil leucocytosis) during the first 10 days.

Unna (2666) found no abnormal effects of 20 mg pyridoxine/kg/day on the growth, hematology, or histology of 3 puppies treated for 8 days. The same author gave one monkey 10 mg pyridoxine/kg/day orally for 39 days and the same dose s.c. to another monkey for 106 days with no ill effects. Hematology and bone marrow tests were normal.

Pyridoxine has been the subject of numerous special studies. Unna (2666) found no in vivo effects on respiration, heart rate, and blood pressure in rabbits and cats when pyridoxine HCl was injected i.v. in doses of 1 to 50 mg/kg. No in vitro stimulation of rabbit smooth muscle from the intestine or uterus was produced in concentrations up to 1:10,000.

Four studies on reproduction are included, which concluded that excess dietary pyridoxine does not have adverse effects on reproductive success in various species of rats. Unna and Antopol (2667) gave male and female rats 2.5 mg pyridoxine HCl daily for 87 days. Normal litters were produced. Morrison and Sarett (1803) fed groups of female rats pyridoxine levels 50 times higher (about 15 mg/kg/day) than that of the controls for 12 weeks, after which they were mated to untreated males. More young were born per litter in the excess pyridoxine groups, and there were no adverse effects on reproduction or on the weights of the kidneys, livers, or adrenals. Excess pyridoxine intake by the mothers did not increase the pyridoxine requirement of the young, although their liver stores were greatly increased. Schumacher et al. (2275) and Driskell et al. (662) had similar results with feeding 4 mg/kg/day and 32 mg/kg/day respectively.

Simionescu et al. (2368) found that the administration of pyridoxine HCl, i.p., at a dose of 8 mg/kg/day for 66 days induced changes in the estrus cycle of pubescent female rats. The changes included the absence of a diestrus period a transient proestrus, and a prolonged estrus. The pyridoxine appeared to stimulate the hypophysio-utero-ovarian axis as indicated by the enlargement of the uterus and ovaries and the numerous follicles and corpora lutea in the ovaries.

Pyridoxine (600 mg/day orally) does have an antilactogenic effect in women. Foukas (781) reported its 95% successful use in suppressing the post-partum lactation within 1 week after delivery and in relieving painful breast engorgement within 10 to 12 hours.

Tugrul (2642) administered pyridoxine s.c. to groups of breeding rabbits, which were also receiving glutamic acid, a teratogen. The pyridoxine seemed to prevent abortions and resorptions, but did not affect the malformations. Conti and Milio (508) concluded that pyridoxine is not teratogenic to the chick embryo at doses of up to 1 mg. Karnofsky et al. (1283) found that the LD_{50} of pyridoxine HCl in the chick embryo is 5 to 10 mg per egg.

Miller et al. (1744) found that rats raised on low pyridoxine diets, which also contained the carcinogen p-dimethylaminoazobenzene, had fewer tumors than those raised on higher levels. Weir et al. (2781) reported that excess pyridoxine HCl, administered parenterally, had no effect on

the number or pattern of leucocytes in mice injected with leukemic transplants.

A number of articles on the effect of pyridoxine on dental caries have appeared. Wynn et al. (2867) found that the addition of pyridoxine (1 to 100 ppm) to diets high in sucrose did not reduce their carcinogenicity in the albino rat. These results contradict much of the published data, as reviewed by Hillman in 1964 (1092). Hillman stated that much of the data supporting claims that pyridoxine has a protective effect on cavity production was based on poorly-controlled studies with school children in the U.S. Pyridoxine appears to have been effective in reducing cavity production in pregnant women, but pregnant women often have a relative vitamin B_6 deficiency. Hillman presented much of the supporting data; however, he concludes that no definite evidence exists either way.

Attempts have been made to use pyridoxine in countless types of diseases and injuries. Jolliffe (1242) reviewed its use in treating neuro-psychiatric syndromes, such as paralysis agitans, muscular dystrophy, and amytrophic lateral sclerosis. A number of recent articles have been published about using pyridoxine HCl to eliminate depression, a common side effect in women using oral contraceptives. Adams et al. (18) reported that the pyridoxine is effective only in those women who have absolute vitamin B_6 deficiencies (present in 20% of women using oral contraceptives).

Two other clinical uses of pyridoxine are in relieving nausea and vomiting from radiation sickness in cancer patients undergoing radiation therapy (2098) and in decreasing the healing time of corneal erosions in rabbits (2153). As is often the case in clinical uses of pyridoxine, Reeves stated that pyridoxine may be effective in radiation sickness, because radiation induces a vitamin B_6 deficiency.

Cordelli et al. (520) found that pyridoxine treatment in rats appeared to cause administered Sr^{85} to disappear more rapidly when compared to that in saline-treated controls.

PYRIDOXINE HYDROCHLORIDE

Chemical Information

I. Nomenclature

A. Common Names

1. Vitamin B_6 hydrochloride
2. Pyridoxine hydrochloride
3. Pyridoxinium chloride
4. Pyridoxol hydrochloride
5. Adermine hydrochloride
6. Hexabione hydrochloride
7. Antiacrodynia factor

B. Chemical Names

1. 5-hydroxy-6-methyl-3,4-pyridinedimethanol hydrochloride
2. 2-methyl-3-hydroxy-4,5-bis(hydroxymethyl)pyridine hydrochloride
3. 5-hydroxy-6-methyl-3,4-pyridinedicarbinol hydrochloride
4. 3-hydroxy-4,5-dimethylol-alpha-picoline hydrochloride

C. Trade Names

1. Beclian
2. Benadon
3. Campoviton 6
4. Hexabetalin
5. Hexavibex
6. Hexermin
7. Hexobion
8. Pyridipca

D. Chemical Abstracts Registry Number

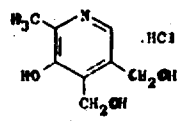
ADF 000058-56-0

II. Empirical Formula

$C_8H_{11}NO_3 \cdot HCl$

C	46.72%
H	5.88%
N	6.81%
O	23.34%
Cl	17.24%
HCl	17.73%

III. Structural Formula



IV. Molecular Weight

205.64

V. Specifications

Food Chemicals Codex

Assay	Not less than 98.0% of $C_8H_{11}NO_3 \cdot HCl$ (computed on dried basis)
Chloride content	Not less than 16.9%; not more than 17.6% of Cl (computed on a dried basis)
Limits of impurities	
Heavy metals (as Pb)	Not more than 30 ppm (0.003%)
Loss on drying	Not more than 0.5%
Residue on ignition	Not more than 0.1%

VI. Description

A. General Characteristics

Pyridoxine hydrochloride occurs as colorless or white crystals or as a white, crystalline powder.

B. Physical Properties

Pyridoxine hydrochloride sublimes and has a melting point of about 206 degrees C (a range of 205 to 212 degrees C). Its free base melts at 160 degrees C. One gram of pyridoxine hydrochloride is soluble in 5 ml of water or in 100 ml of alcohol. It is also soluble in propylene glycol and in acetone; it is insoluble in ether and in chloroform.

C. Stability

Pyridoxine hydrochloride should not be exposed to sunlight, but is stable in air. Keep in tightly closed containers.

VII. Analytical Methods

The Food Chemicals Codex gives one assay method for pyridoxine hydrochloride. A 170 mg sample is dissolved in 5 ml glacial acetic acid plus 5 ml acetic anhydride and then boiled, after which 30 more ml of

acetic anhydride are added and the mixture is boiled a second time. After the mixture is cooled, 30 ml of benzene and 5 drops of neutral red T.S. are added and it is titrated with 0.1 N perchloric acid from red to blue. Each 1 ml perchloric acid corresponds to 20.56 mg pyridoxine hydrochloride.

A number of microbiological assay methods for pyridoxine have been developed, using the mold Neurospora. After the culture is incubated, it is steamed and the mycelium removed, dried, and weighed. Frobisher describes the general method, in which the dry weight of the mycelium is directly proportional to the concentration of pyridoxine in the sample of food being assayed (803).

VIII. Occurrence

A. Plants

1. Yeast
2. Cereals
3. Fresh vegetables

B. Animals

1. Liver from domestic animals
2. Fresh meat
3. Egg yolks
4. Milk

C. Synthetics

None

D. Natural inorganic sources

None

Biological Data

I. Acute Toxicity

Substance	Animal	No.	Route	Dosage mg/kg Body Weight	Measurement	Ref.
Pyridoxine HCl	Mice	23	i.v.	545.3 ± 42.9	LD_{50}	2775
Pyridoxine base	Mice	169	s.c.	50-1500	LD_0	464
Pyridoxine base	Mice	169	p.o.	50-1500	LD_0	464
Pyridoxine HCl	Rats	23	i.v.	657 ± 18.3	LD_{50}	2775
Pyridoxine base	Rats	10	s.c.	3100	LD_{50}	2667
Pyridoxine HCl	Rats	10	s.c.	3700	LD_{50}	2667
Pyridoxine base	Rats	10	Oral	4000	LD_{50}	2667
Pyridoxine HCl	Rats	10	Oral	6000	LD_{50}	2667
Pyridoxine base and HCl	Rats	14	Oral	3000-7000	LD_{50}	70
Pyridoxine base and HCl	Rats	1	s.c.	3000	LD_{50}	70
Pyridoxine base	Rats	10	s.c.	3100	LD_{50}	2666
Pyridoxine HCl	Rats	10	s.c.	3700	LD_{50}	2666
Pyridoxine base	Rats	10	Oral	5500	LD_{50}	2666
Pyridoxine HCl	Rats	10	Oral	1	LD_0	2666
Pyridoxine	Rats	Unk.	Oral	5000	LD_{50}	1777
Pyridoxine	Rats	5	i.p.	1600	LD_0	1554
Pyridoxine	Rats	5	i.p.	1675	LD_{16}	1554
Pyridoxine	Rats	5	i.p.	1925	LD_{50}	1554
Pyridoxine	Rats	5	i.p.	2195	LD_{84}	1554
Pyridoxine	Rats	5	i.p.	2200	LD_{100}	1554
Pyridoxine	Rats	5	s.c.	2600	LD_0	1554
Pyridoxine	Rats	5	s.c.	2750	LD_{16}	1554
Pyridoxine	Rats	5	s.c.	3130	LD_{50}	1554
Pyridoxine	Rats	5	s.c.	3600	LD_{84}	1554
Pyridoxine	Rats	5	s.c.	3800	LD_{100}	1554
Pyridoxine HCl	Rabbits	Unk.		1000	LD_0	2666
Pyridoxine HCl	Rabbits	Unk.		> 1000	LD	2666
Pyridoxine HCl	Dogs	Unk.		1000	LD_0	2666
Pyridoxine HCl	Dogs	Unk.		> 1000	LD	2666

Substance	Animal	No.	Route	Dosage mg/kg Body Weight	Measurement	Ref.
Pyridoxine base and HCl	Dogs	3	Oral	2000-2500	LD_{100}	70
Pyridoxine base and HCl	Dogs	1	Oral	1000	LD_{100}	70

Mice

Chertkova and Udilova reported the maximum permissable doses for pyridoxine, in acute administration, for 169 white mice were from 1 to 30 mg per animal, about 50 to 1500 mg/kg body weight s.c. or per os (464).

Weigand et al. found that the LD_{50} of a 1% solution of pyridoxine hydrochloride in 23 mice was 545.3 plus or minus 42.9 mg/kg i.v. The mice usually were dead within 5 minutes following tonic and clonic convulsions (2775).

Rats

The acute toxicity of a 5% solution of pyridoxine hydrochloride in 23 rats was found by Weigand et al. to be 657.5 plus or minus 18.3 mg/kg i.v. after tonic and clonic convulsions, the rats usually died within 5 minutes (2775).

Unna and Antopol administered various dose levels of pyridoxine, as the hydrochloride on a 20% solution or as the base, to groups of 10 rats. No toxic symptoms were produced by doses up to 1 g/kg within 24 hours after receiving higher doses, the rats underwent tonic convulsions. These effects lasted for 3 weeks with non-lethal doses, and death occurred within 36 to 72 hours with lethal doses. The dead animals had enlarged adrenals and hemorrhages in the cortex. The s.c. LD_{50} of the free base was 3.1 g/kg and 3.7 g/kg for pyridoxine HCl. The oral LD_{50} was 4 g/kg for the free base and 6 g/kg for the hydrochloride (2667).

Antopol and Tarlov studied the acute toxicities of pyridoxine base and pyridoxine hydrochloride, administered by different routes, in 15 adult male rats. An additional 3 rats served as controls. Very large doses, in excess of 3 g/kg, caused impaired coordination and hypotonia at the joints. No convulsions were observed. High doses of pyridoxine caused adverse changes in the nervous system of all but 2 of the rats. A summary of the histological changes and survival periods is shown in the following chart (70).

CHART

Summary of pathologic changes produced by excessive amounts of vitamin B6

ANIMAL	DOSAGE	ROUTE OF ADMINIS-TRATION	SURVIVAL PERIOD	HISTOLOGIC CHANGES
Rat 1	3 gm/kg*	Oral	4 days?	Marked rarefaction of posterior columns
Rat 2	3 gm/kg*	Oral	4 days?	Slight rarefaction of posterior columns
Rat 3	3 gm/kg*	Oral	4 days?	Marked rarefaction of posterior columns
Rat 4	3 gm/kg*	Subcutane-ous	25 days	1. Moderate rarefaction of posterior columns. 2. Vacuolization of posterior roots. 3. Focus of necrosis in anterior grey horn consisting of chromatolysis of ganglion cells with infiltration by lymphocytes.
Rat 5	5 gm/kg*	Oral	10 days	Slight rarefaction of posterior columns
Rat 6	5 gm/kg*	Oral	10 days	Slight rarefaction of posterior columns
Rat 7	3 gm/kg*	Oral	20 days	1. Slight rarefaction of posterior columns 2. Focus of necrosis of same type as in Rat 4 at posterior root entry zone
Rat 8	5 gm/kg+	Oral	4 days	No abnormality
Rat 9	5 gm/kg+	Oral	4 days	No abnormality
Rat 10	6 gm/kg*	Oral	2 days	Very slight rarefaction of posterior columns
Rat 11	6 gm/kg*	Oral	2 days	1. Marked rarefaction of posterior columns 2. Moderate chromatolysis of posterior root ganglion cells
Rat 12	7 gm/kg*	Oral	16 days	1. Moderate rarefaction of posterior columns 2. Vacuolization of posterior roots
Rat 13	6 mg/kg*	Oral	4 days	1. Marked rarefaction of posterior columns 2. Marked chromatolysis of posterior root ganglion cells
Rat 14	6 gm/kg*	Oral	37 days	1. Moderate rarefaction of posterior columns 2. Slight chromatolysis of posterior root ganglion cells
Rat 15	5 gm/kg*	Oral		Moderate rarefaction of posterior columns

* Vitamin B6 base

\+ Vitamin B6 HCl

The vitamin was administered in one dose unless otherwise stated.

Unna reported early studies with rats which showed that pyridoxine is a substance of very low toxicity. Rats (120-150 g) were given pyridoxine at various levels, (10 rats/level) (see Figure 1). The LD_{50} for pyridoxine administered s.c., was 3.1 g/kg for the base and 3.7 g/kg for the hydrochloride. The oral LD_{50} for pyridoxine hydrochloride was 5.5 g/kg. Toxic symptoms included tonic convulsions, opisthonus, and death. The dead animals were found to have enlarged adrenals with some hemorrhage in the cortex. Rats were able to tolerate doses of 1 mg/kg or less without untoward effects (2666).

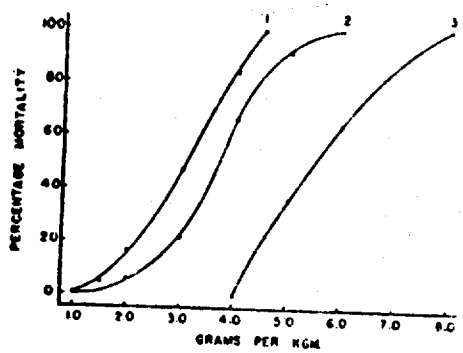


FIG. 1. TOXICITY OF VITAMIN B_6 IN RATS
1. Vitamin B_6 (base) subcutaneously. 2. Vitamin B_6 hydrochloride subcutaneously.
3. Vitamin B_6 hydrochloride orally.

Molitor discussed the great difference between the daily requirement for pyridoxine and the LD_{50} in animals, using the rat as test animal. The daily requirement in the rat for vitamin B_6 is 10 gamma and the LD_{50} (oral) is 1 g/animal weighing 200 g or 5 g/kg; the ratio between these 2 values is 100,000 (1777).

Lipkan computed the toxicity of pyridoxine in white rats using the probe-analysis method. See table page 12. The pyridoxine was given in solutions of distilled water to groups of 5 rats. The deaths of the animals were recorded for 2 to 3 days. Also included were the maximum tolerated dose levels or LD_0's - 2600 mg/kg s.c. and 1600 mg/kg i.p. (1554).

Rabbits

Unna found that rabbits could withstand doses of pyridoxine hydrochloride up to 1 g/kg with no ill effects. Larger dose levels caused impaired coordination within 2 or 3 days, followed by convulsions, paralysis, and death (2666).

Dog

Unna reported that doses up to 1 g/kg of pyridoxine hydrochloride were tolerated by dogs with no suffering or permanent damage. The temporary effect of 1 g/kg was an ataxic gait, which disappeared within a week. Higher doses were lethal in 2 to 3 days after impaired coordination, convulsions, and paralysis (2666).

Antopol and Tarlov studied the acute toxicities of pyridoxine base and hydrochloride in 4 adult dogs (male and female). Two control dogs were given no pyridoxine. One of the dogs died 14 days after receiving 2.5 g/kg in 5 oral doses (0.5 g/kg each) over a 48 hour period. The other animals were sacrificed during severe ataxia, the main toxic symptom of the large doses of pyridoxine. A summary of the histological changes in the nervous systems of the dogs is in the chart below (70).

CHART

Summary of pathologic changes produced by excessive amounts of vitamin B6

ANIMAL	DOSAGE	ROUTE OF ADMINIS-TRATION	SURVIVAL PERIOD	HISTOLOGIC CHANGES
Dog 1	2 gm/kg+ in 4 doses over period of 36 hours	Oral	8 days	1. Moderate degeneration of axis cylinders and myelin sheaths of posterior columns 2. Marked chromatolysis of posterior root ganglion cells
Dog 2	2 gm/kg+ in 4 doses over period of 36 hours	Oral	13 days	Slight rarefaction of posterior columns
Dog 3	1 gm/kg+	Oral	14 days	Slight rarefaction of posterior columns
Dog 4	2.5 gm/kg+ in 5 doses over period of 48 hours	Oral	14 days	1. Marked degeneration of myelin sheaths and, to a less extent, of axis cylinders of posterior columns and posterior roots: few degenerated fibers seen in Marchi preparations in anterior and lateral columns. 2. Occasional chromatolysis in posterior root ganglion cells

* Vitamin B6 base

\+ Vitamin B6 HCl

The vitamin was administered in one dose unless otherwise stated.

Man

Weigand et al. administered in one dose pyridoxine hydrochloride to 12 male subjects using various routes. The oral doses were 100 mg in gelatin capsules; the i.v. (100 to 200 mg) and i.m. (50 mg) doses were given in 2.5% solutions. The only effect was pain at the injection site, probably due to the acid. The results are summarized in the following table (2775).

Table 1

Action of Vitamin $B_6 \cdot HCl$ in Men by Various Routes of Administration.

Subject No.	Age	Dose, mg: Oral	Dose, mg: Intramuscular	Dose, mg: Intravenous	Reactions noted
1	22	100			None
			50		"Sore" at site of injection for 1½ hours
				100	None
2	42	100			"
3	35	100			"
4	40	100			"
5	24	100			"
			50		"Aching" for 15 min
				100	None
6	33		50		"Burning" for 15 min
				100	None
7	28		50		"Sore" for 2 hrs
				100	None
8	30		50		"Sore" for 15 min
				100	None
9	28		50		"Aching" for 2 hrs
				100	None
10	21			200	"
11	29			200	"
12	21			200	"

II. Short Term Studies

Mice

Weigand et al. injected 5 mice i.v. with 100 mg/kg pyridoxine hydrochloride daily for 2 weeks (except weekends) and found no adverse effects (2775).

Rats

Groups of 15 three week old rats were given daily oral doses of pyridoxine HCl for 87 days by Unna and Antopol. No differences were noted between the rats given 0.25, 1.0, or 2.5 mg/kg and the control group (2667).

Unna fed groups of 15 young rats 0.5, 1.0, or 2.5 mg pyridoxine per day (0.005, 0.01, or 0.025% of a 10 g/day diet) for 80 days. The highest dose level produced no adverse changes in weight or pathology when compared to the controls. The 2.5 mg pyridoxine dose was continued into the second and third generations with no ill effects (see Table 1). No toxic effects were observed in 20 young rats fed 25 mg pyridoxine daily for 100 days (equivalent to 0.25% of a 10 g/day diet) (2666).

TABLE 2

Growth of young rats receiving 2.5 mgm of vitamin B6 daily by mouth

	AVERAGE WEIGHT							
	at start		20 days		50 days		80 days	
	Males	Females	Males	Females	Males	Females	Males	Females
	grams	grams	grams	grams	grams	grams	grams	grams
Stock diet only. 14 rats: 6 males, 8 females. Control..	27	27	79	72	193	136	267	186
2.5 mgm vitamin B6 daily. 13 rats: 9 males, 4 females. 1st generation.....	29	28	86	76	177	137	262	177
2.5 mgm vitamin B6 daily. 13 rats: 7 males, 6 females. 2nd generation.....	32	32	81	74	183	136	252	181
2.5 mgm vitamin B6 daily. 19 rats: 8 males, 11 females. 3rd generation.....	34	32	85	79	193	144	267	184

Kato gave 40 day old male Wistar white rats, (average weight, 50 g), a standard solid feed for 4 to 6 days. At this time, vitamin B_6 was added to the feed - 0.2 mg% (10 to 20 g feed consumed per day) - for over 4 weeks. A control group was fed the standard feed for the experimental period. Weight gain was temporarily suppressed in the rats fed excess pyridoxine. By the fourth week, the weights were similar to that of the controls, even with continued administration of excess pyridoxine (1288).

De Bastiani and Zatti injected 14 male white rats, weighing 70 g, with 50 mg pyridoxine i.m. daily for 28 days. Every 4 days from day 1, 2 rats were sacrificed and autopsied. Another group of 14 untreated rats was used as a control. Te weights and histology of the adrenals and thymus and changes in the leucocytes were studied. For the first 2 weeks of pyridoxine treatment the adrenals weighed slightly more than those of the control animals. The adrenals returned to control weights during the last 2 weeks of treatment. In rats sacrificed at 8, 12, and 16 days, the adrenals exhibited a moderate hypertrophy of the fasiculated part and had an increase in the spaces between the cellular trabeculae containing many red blood cells. These changes indicate a stimulation of the adrenal cortex. During the first 12 days, the sudanophil lipid granules were reduced. The thymus glands became atrophied with the weights being below the normal levels throughout the study. For the first 10 days of the study, there was a moderate neutrophil leukocytosis with relative lymphopenia. This leucocyte alteration was at a maximum 4 to 5 hours after the first dose of pyridoxine. The authors stated that the histological changes in the pyridoxine-treated animals represented an adaptation syndrome to stressful doses of pyridoxine (591).

Dog

When 3 puppies from the same litter were fed 20 mg pyridoxine/kg/day for 80 days, their growth, hematology (hemoglobin, erythrocyte, leucocyte, differential blood count) at weekly intervals, and histology were no different from the controls (2666).

Monkey

A daily oral dose of 10 mg pyridoxine/kg was given to one 3 kg monkey for 39 days. A second monkey weighing 2.8 kg was given the same dose of pyridoxine s.c. daily for 106 days. A third monkey given no pyridoxine served as a control. No toxic effects were observed. Hematology and bone marrow tests were normal (2666).

III. Long Term Studies

None Available

IV. Special Studies

Physiological Effects

Pyridoxine hydrochloride did not change respiration, heart rate, or blood pressure of rabbits and cats under urethane and chloralose anesthesia, when injected i.v. with doses of 1 to 50 mg/kg. No effects of in vitro stimulation with pyridoxine hydrochloride were found on smooth muscles of the rabbit intestine (with concentrations up to 1:10,000) or on rabbit uterus (2666).

Reproduction

Unna and Antopol fed 3 male and 3 female rats a daily oral dose of 2.5 mg pyridoxine HCl for 87 days. Two litters were produced with 5 and 8 pups having an average birth rate of 5 g (2667).

Morrison and Sarett studied the effects of excess dietary pyridoxine on the growth and reproduction of female weanling rats of the McCollum-Wisconsin strain. Groups of 12 rats, 19 to 21 days old and weighing about 50 g, were given experimental diets for 12 weeks. The control group was given an adequate supply of pyridoxine, 150 mcg/100 g diet (about 0.3 mg/kg/day), and the test groups were given 50 times the control level of pyridoxine (about 15 mg/kg/day) or of both pyridoxine and thiamine. No significant effects on weight gain or food consumption were found (see following table).

TABLE 3

Data on weight gains, food and water intakes and food efficiencies of female rats fed diets containing various levels of thiamine and pyridoxine for 12 weeks

	Diet 1 Adequate thiamine, pyridoxine	Diet 2 Excess thiamine	Diet 3 Excess pyridoxine	Diet 4 Excess thiamine, pyridoxine
No. of survivors[1]	11	12	12	11
Initial weight, gm	49	49	49	49
6 weeks				
Weight gain, gm	106 ± 112	109 ± 12	112 ± 9	116 ± 16
Food intake, gm	372	380	387	393
Water intake, ml	557	658	664	583
Food efficiency[3]	28.5 ± 2.1	28.8 ± 1.8	29.0 ± 2.6	29.5 ± 2.8
12 weeks				
Weight gain, gm	145 ± 19	146 ± 15	153 ± 14	158 ± 19
Food intake, gm	811	816	827	827
Water intake, ml	1354	1702	1620	1443
Food efficiency[3]	17.9 ± 1.4	18.0 ± 1.1	18.6 ± 1.8	19.2 ± 1.8

1 Each group contained 12 animals initially.

2 Standard deviation.

3 Average grams gain per 100 gm food intake.

After the 12 week period (while still receiving the test diets) the females were mated with untreated males of the same stock. The adult females were sacrificed 21 days after parturition; the kidneys, livers, and adrenals were studied. There were no adverse effects of excess pyridoxine on reproduction. More young per litter were born to females fed diets with excess of both pyridoxine and thiamine (see following table).

TABLE 4

Data on reproductive performance of female rats fed diets containing various levels of thiamine and pyridoxine

No. and description of diet	No. of animals	No. of litters	No. of young	Av. birth weight of young	Survival data				Av. weight of young, 21 days
					5 days		21 days		
				gm	no.	%	no.	%	gm
1. Adequate thiamine, pyridoxine	10	6	44	6.5	43	93	32	73	37
2. Excess thiamine	12	11	75	6.3	72	96	57	76	43
3. Excess pyridoxine	11	9	75	6.1	67	89	56	75	41
4. Excess thiamine, pyridoxine	10	9	87	6.2	78	90	56	64	39

The excess pyridoxine diet was also found to have no significant effects on the weights of the organs studied or on the level of solids, total lipids, riboflavin, pantothenate, thiamine, pyridoxine and vitamin B_{12} in the livers of the adult females. The young were given pyridoxine deficient diets for 5 weeks. The young whose mothers had been fed the excess pyridoxine diet gained significantly more weight than the young of females given the control diet. The authors concluded that the intake of excess pyridoxine by pregnant mothers does not increase the pyridoxine requirement of the young. The level of pyridoxine in the livers of the young reflected maternal pyridoxine intake, since the liver stores of pyridoxine were greatly increased in the young at weaning (1803).

Schumacher et al. studied the effects of pyridoxine on reproduction in Long-Evans rats. A control group of 15 female rats was fed a basal diet containing 0.25 mg pyridoxine per 100 g of diet (0.167 mg/kg/day). The test group of 5 female rats was given a diet containing 25 times the amount of pyridoxine in the basal diet (4.175 mg/kg/day). After 2 weeks the test and control females were mated with untreated males. The same test and controls diets were continued through gestation and lactation. No significant effects of the high pyridoxine level were observed on the average birth weight, the number of young per litter or the average weight of the young at weaning. The high pyridoxine diet significantly increased the pyridoxine level in the newborn, but this was normal at weaning - 21 days of age. The maternal diet did not affect the growth response of the young until weaning. In experiments with pyridoxine depleted young, no increased need for pyridoxine after weaning was observed in the offspring of females fed the high pyridoxine diet during the reproductive period (2275).

Driskell et al. studied the relationship between dietary pyridoxine and reproductive capacity in female Sprague-Dawley rats. In one experiment, female Sprague-Dawley rats weighing 250 g were fed a basal diet supplemented with various levels of pyridoxine for 1 week, (12 animals per group) for which 6 of the rats in each group were mated. The experimental diets were continued until the 21st day of gestation; when the animals were sacrificed. An equal number of nonpregnant animals served as controls. In a second experiment, weanling females were fed diets supplemented with pyridoxine at 6 levels, 14 rats per group. At

80 to 110 days old, 7 females per group were mated as in the first experiment. Reproductive capacity was not impaired by high levels of pyridoxine-8000 mcg/15 g diet (the daily consumption of pregnant animals) or 32 mg/kg/day of pyridoxine (662).

Simionescu et al. divided 30 pubescent female rats into 2 groups - one given pyridoxine HCl treatments i.p. the other controls. The estrus cycles of the controls were studied by examining vaginal smears for 29 days. The test group was first given 400 ug/100 g body weight 4 mg/kg/day daily for 3 weeks, after which the dose was increased to 8 mg/kg/day for 66 days. The dose levels used induced no toxic effects and no changes in behavior, weight, or appearance were observed. Compared to the untreated control group, the estrus cycle was shortened (after about 20 days) no diestrus period was observed, the estrus phase was prolonged, and proestrus was transient. Hypertrophy of the uterus and ovaries was found, especially during estrus. Numerous follicles and corpora lutea were observed in the ovaries. Pyridoxine treatment appeared to stimulate the hypophyseo-utero-ovarian axis (2368).

Foukas reported the highly successful use of pyridoxine in suppression of post-partum lactation. The results of a placebo-controlled, double-blind test of 254 women divided into 4 groups are reported in the following table.

TABLE 5

Time taken for lactation to cease

Drug	Number of patients	Per cent of patients in whom lactation ceased within: 1 week	2 weeks	3 weeks	4 or more weeks
Placebo	86	17	45	28	10
Stilboestrol	68	83	12	5	--
Pyridoxine (blind)	75	93	7	--	--
Pyridoxine (known)	25	100	--	--	--

Three 200 mg pyridoxine tablets/day for 6 days were administered to 100 patients beginning the second or third day after delivery. Pyridoxine usually relieved the painful breast engorgement within 10 to 12 hours after treatment. Pyridoxine also relieved symptoms faster than stilboestrol, and had none of its adverse effects, such as excess vaginal bleeding or delayed menstruation. It is suggested that pyridoxine probably suppresses lactation by its pyridoxal phosphate form, which is the coenzyme of DOPA decarboxylase. This enzyme is stimulated by pyridoxine to convert DOPA to dopamine in the hypothalamus. Then the increased dopamine level inhibits the lactogenic hormone, prolactin (781).

Teratology

Tugrul administered 25 mg/kg/day of glutamic acid hydrochloride orally and 25 mg/kg/day of pyridoxine s.c. for 40 days to a group of 6 breeding rabbits (4 females; 2 males), all of which were confined in the same cage. In 2 other groups, glutamic acid was administered alone to either the females only or to both the males and the females. A control group of 28 untreated breeding rabbits (20 females; 8 males) was observed throughout the experiments. The treated animals and their offspring were autopsied to determine adverse effects and teratogenesis, since glutamic acid is a precursor to Thalidomide (gamma-phthalimidoglutarimide). Glutamic acid, which is administered clinically for hypoacidity, epilepsy, and other mental disorders, was found to be teratogenic, to cause abortions and resorptions, and also to cause hyperplasia of the uterus and inhibition of spermatogenesis in the treated adults. The group which received vitamin B6 in addition to glutamic acid did not have abortions and resorptions, but the teratogenic effects were similar to those of the other groups. The author concluded that pyridoxine may have prevented the arrested pregnancies, but that it offered no protection against the malformations of glutamic acid (2642).

Conti and Milio injected 622 fertilized Leghorn chick eggs with 0.1, 0.2, 0.4, 0.6, or 1.0 mg pyridoxine 24 hours after incubation. The pyridoxine, dissolved in 0.1 ml liquid Tyrode, was injected into the albumin (PX). Control groups of eggs were given no treatment (C), injected with 0.1 ml Tyrode (T), or the albumin was pricked (P). The incidence of teratogenic effects and early embryo deaths were observed. About 6.9% of the control embryos died prematurely, regardless of treatment. The frequency of embryonic deaths, underdeveloped embryos, or deformities (about 5%) was about the same for the pyridoxine treated eggs as for the controls at all dosage levels (see table). The authors concluded that pyridoxine is not teratogenic to the chick embryo at the doses used (508).

	Control Group (%)			Embryos treated (%) with PX after 24 hours incubation				
	C	P	T	1 mg	0.6 mg	0.4 mg	0.2 mg	0.1 mg
Early death	6.9	4.5	6.5	6.7	5.7	6.8	7.8	8.6
Late death	11.1	16.9	7.9	19.0	21.7	14.6	9.7	13.3
Living the 21st day	82.0	78.6	85.6	74.3	72.6	78.6	82.5	78.1
Underdeveloped	2.5	3.4	2.1	3.1	5.0	5.2	2.1	2.1
Malformed	5.5	4.8	3.5	7.1	6.0	5.2	2.1	4.2

Karnofsky et al. injected various doses of pyridoxine hydrochloride into fertile White Leghorn eggs incubated 0 to 13 days at 38 degrees C and 75%

relative humidity. The doses were dissolved in 0.05 to 0.2 ml saline, and observations were made 10 days after injection. Using 6 to 20 embryos per dosage level, the approximate LD_{50} of pyridoxine was 5 to 10 mg/egg. Pyridoxine was found to protect embryos from the effects of antagonists of vitamin B_6, such as methoxypyridoxine and desoxypyridoxine (1283).

Carcinogenicity

Miller et al. raised weanling male and female albino Sprague-Dawley rats on synthetic diets containing either 1.5 or 6.0 mg pyridoxine hydrochloride/kg diet for 8 to 9 weeks. The rats were then divided into groups of 15 to 17 and fed an additional 4 months on diets containing 0.06% p-dimethylaminoazobenzene, a substance which induced liver tumors. The carcinogenic diets were varied in pyridoxine levels - either 0.2 (a low level) or 2.5 mg/kg diet and in casein levels - 12 or 48% of diet. After 4 months, laparotomies were performed to examine the livers and the diets minus the carcinogen were continued for 2 more months. The rats raised on the low pyridoxine diets had fewer tumors than those raised on the higher levels (1744).

Weir et al. studied the effect of excess pyridoxine administration on the production of leucocytes in normal mice and then in mice injected with a leukemic transplant. The normal mice were injected with pyridoxine HCl in divided daily doses of 8 or 16 mg for 48 days. No effect on the number or pattern of leucocytes was observed. The parenteral administration of 2 or 4 mg pyridoxine HCl, once daily to groups of 5 AK mice injected i.v. with leukemic transplants did not affect the leukemia. The results were compared to the control group given the leukemic transplant only. The authors also gave pyridoxine HCl injections of 8 mg twice daily to 5 of 10 adult Swiss mice in which granulocytosis had been induced. The remaining 5 mice served as controls, without pyridoxine treatment. The pyridoxine did not have a significant effect on the polymorphonuclear count. Although pyridoxine deficiency appeared to cause increased granulocyte production in mice (normal or leukemic), the results of these studies do not indicate that pyridoxine excess affects leucocyte production (2781).

Dental Caries

Wynn et al. found that the addition of pyridoxine to diets high in sucrose did not reduce their carcinogenicity in the albino rat. The study was conducted using either a caries-resistant strain, Emory-Wistar, which had been sialoadenectomized to cause caries susceptibility or using a caries-susceptible strain with intact salivary glands. The rats were all given the Harvard high-sucrose diet containing 3.5 ppm pyridoxine. The sialoadenectomized rats were divided into 3 groups of 8 litter-mates each soon after weaning. These rats were fed 1, 10, or 100 ppm additional pyridoxine for 20 days. The number of caries was not lowered by the additional pyridoxine (see Table 1). The intact rats were divided into 3 groups of 20 litter-mates each right after weaning and were 0, 10, or 100 ppm pyridoxine in addition to the 3.5 ppm already in the basic diet for 120 days. Pyridoxine supplementation did not reduce the cariogenicity in this study either (see Table 2). The authors stated that their results differed from that of Strean et al. and did not believe that the difference in pyridoxine levels accounted for the reduction of caries in one of 2 high-sucrose diets containing equal amounts of sucrose (2867).

Table 6

Carious lesions and caries scores of sialoadenectomized caries-resistant albino rats fed a cariogenic diet with different concentrations of pyridoxine

No. animals	Days on diet	Additional pyridoxine	Carious lesions[1]	Caries score[1]
		ppm		
8	70	1	24	59
8	70	10	24	61
8	70	100	22	55

[1] Average per animal.

Table 7

Carious lesions and caries scores of intact caries susceptible albino rats fed a cariogenic diet with different concentrations of pyridoxine

No. animals	Days on diet	Additional pyridoxine	Carious lesions[1]	Caries score[1]
		ppm		
20	120	0	17	40
20	120	10	18	35
20	120	100	18	33

[1] Average per animal.

In 1964, Hillman reviewed some of the data on the effect of pyridoxine on cavity production in humans. He stated that many of the studies done on humans were inadequate, poorly controlled, and were for too short a duration to provide definite evidence for the protective role of pyridoxine in tooth decay. Various studies with 11 to 15 year old schoolchildren, done by Boyd and Wessels, 1951; Dunning, 1962; Cohen and Rubin, 1958; Strean et al. 1958, indicate that the ingestion of 3 mg pyridoxine lozenges 3 times per day reduced the number of cavities in 1 year. Studies in both fluoridated and non-fluoridated water areas showed similar results. Pyridoxine may be useful on subjects living under stressful physiological conditions, such as adolescents and pregnant women. Hillman's study in 1962 on 540 pregnant women, living in a city without fluoridated water, showed that significantly fewer cavities appeared in the women who took a 6.67 mg pyridoxine lozenge 3 times per day. This was compared to a group taking placebo lozenges and to a group taking 20 mg pyridoxine in the vitamin-mineral capsule which all 3 groups took. The duration of the study was from 4 months of pregnancy until 6 weeks postpartum.

Other evidence for the anti-cavity effect of vitamin B_6 was observed in Cuban children, who chew sugar cane and have poor dental hygiene, but have fewer caries and a higher concentration of B_6 in their blood than a comparable group of children in New York City (reported by Goodhart, 1957; Boxer et al., 1957). Americans usually ingest refined sugar, from which much of the vitamin B_6 is removed in the refining process. Hillman stated that pyridoxine may reduce cavities locally by favoring the growth of noncariogenic or less acid-producing bacteria in the mouth or systemically by increasing tooth resistance (1092).

Therapy

Jolliffe reviewed the various uses of pyridoxine in the treatment of neuropsychiatric syndromes in humans. Pyridoxine is described as one of the more important vitamins in maintaining a normal nervous system. Its value in the therapy of paralysis agitans, in which the muscles become rigid resulting in paralysis, was demonstrated in 4 out of 15 patients. The doses used ranged from 50 to 100 mg daily or every other day for a month. Treatment had to continue several times per week for the improvement to remain. Pyridoxine treatment has also been reported to be beneficial in amytrophic lateral sclerosis and in muscular dystrophy (1242).

Adams, et al. discussed the clinical use of pyridoxine HCl to eliminate depression in women using oral contraceptives. Data from other studies show that oral contraceptives may induce a relative vitamin B_6 deficiency in about 80% of the women taking them and an absolute B_6 deficiency in 20% of all the women (shown by decreased urinary excretion of 4-pyridoxic acid). The mechanism for this is probably connected with altered tryptophan metabolism, which depends on pyridoxal-5-phosphate for several enzymatic reactions. The result seems to be a reduced formation of 5-hydroxytryptamine, which leads to depression. A double-blind crossover study was conducted on 22 depressed women using oral contraceptives to see if pyridoxine eliminated depression. Either pyridoxine HCl or a placebo was given orally in 20 mg tablets twice daily for 2 months. The results were compared to a group of 31 non-depressed women also using oral contraceptives and to a control group not using oral contraceptives. The oral contraceptive, used by the various groups, was the same preparation. Results confirmed previous reports that pyridoxine administration to depressed vitamin B_6-deficient (absolute) women on oral contraceptives relieved the depression. Depressed women who were not vitamin B_6-deficient did not respond to pyridoxine administration (18).

Reeves discussed the therapeutic use of pyridoxine HCl for radiation sickness due to radiation therapy in cancer patients. The author reported the results of 100 cases, in which, 100 mg pyridoxine HCl were administered daily in 3 divided oral doses of 50 mg and 25 mg. Some cases which did not respond to oral doses, did respond to i.v. doses. The main symptoms of radiation sickness relieved by pyridoxine are nausea and vomiting. Pyridoxine was also found to be beneficial prophylactically, when treatment was begun before the symptoms appeared. For prophylactic use, 25 mg pyridoxine was given orally 3 times daily with the inception of radiation treatment. These patients did not develop severe symptoms of radiation sickness. It was stated that radiation treatment may induce a vitamin B_6 deficiency, accounting for the disappearance of symptoms when pyridoxine is administered (2098).

A 0.5% solution of pyridoxine was found by Rokhlina and Zubareva to decrease the healing time of corneal erosions in rabbits. The corneal erosions were produced in both eyes of each rabbit studied. The pyridoxine solution was applied to the right eye 3 times per day; the left eye remained untreated as control. The time required for healing was 94 hours - 10-12 hours shorter than the untreated eye in 6 of 7 rabbits (2153).

Cordelli et al. investigated the effect of pyridoxine on the turnover of Sr^{85} in the bone of young Wistar rats (120 to 150 g). One group was given 20 mg pyridoxine in 0.5 ml saline, i.m., daily for 5 days. A control group was given 0.5 ml saline in the same manner. The animals were fed a diet of weighted fodder. At an unspecified time during the pyridoxine treatment, carrier-free Sr^{85} in saline was given i.p. to each rat. The amount of radioactivity recovered from the tail vertebrae for from 30 minutes to 7 days after the Sr^{85} was given was tested with a scintillation counter. It was found that the Sr^{85} disappears from the bone more rapidly in the pyridoxine-treated rats than in the controls (520).

Biochemical Aspects

I. Breakdown

Williams states in his book on The Biochemistry of the B Vitamins (2816) that pyridoxine has a fairly good degree of stability in food. Pyridoxine is sensitive to sunlight; 21% loss can occur after an 8 hour exposure. Pyridoxine is fairly stable during the pasteurization of milk, but a 50 to 65% loss occurs during the heat processing and canning of evaporated milk. About 80% of the pyridoxine is retained in sweetened condensed milk. In wheat milling, 80 to 90% of the pyridoxine is lost, and in wheat braking 1 to 17% is lost. No loss of pyridoxine occurs during the preparation of corn bread. About 70 to 80% pyridoxine is retained during the cooking of vegetables and canned goods. Its retention in meat on cooking is 45 to 80%. A 50% loss of pyridoxine is found in macaroni after cooking.

II. Absorption - Distribution

Booth and Brain studied the absorption of ^{3}H-labeled pyridoxine HCl in black and white rats (200 to 250 g). Oral doses of 0.05 or 5 mg of ^{3}H pyridoxine HCl were given to 2 groups of 12 rats. Three animals from each group were killed with ether 7-1/2, 15, 30, and 60 minutes later. The small intestine was divided into 4 equal segments for radioactive assay: (1) the duodenum and upper jejunum, (2) the lower jejunum, (3) the upper ileum, and (4) the lower ileum. Both doses were absorbed at similar rates. Within 60 minutes, over 90% of each dose had been absorbed. The distribution of radioactivity was studied in the remaining intact animals and in 5 rats in which 50 to 70% of the distal small intestine had been resected. The major part of the absorption is in the jejunum, although some absorption occurs in the ileum and a small amount in the colon. Intragastric administration of ^{3}H pyridoxine, in animals having the pylorus and esophagus ligated, showed that no significant pyridoxine absorption occurs in the stomach. Since a linear relationship between oral dose and amount absorbed was found, it was concluded that absorption of pyridoxine is by passive diffusion. A similar linear relationship was found between the amount fed and the amount excreted (301).

Booth reports that clinical testing with intestinal resection patients has shown that pyridoxine is absorbed in the jejunum (the proximal part of the small intestine). The ileum acts as a reserve when the proximal absorption is saturated. The type of absorption in the ileum is less active than that in the jejunum (300).

Contractor and Shane (1968) found no significant differences in the levels of pyridoxine and its various forms in the blood and urine of 4 healthy women at various stages of the menstrual cycle.

When 100 mg pyridoxine HCl were given orally to 2 healthy women, the levels of pyridoxal phosphate (PALP), pyridoxal (PAL), and pyridoxic acid (PIC) were elevated in the blood, and pyridoxine (POL) was elevated in the urine. No effect on pyridoxamine (PAM) as pyridoxamine phosphate (PAMP)

levels were observed. The high levels of PALP, PAL, and PIC observed 2 hours after the pyridoxine administration indicate that pyridoxine was rapidly absorbed from the intestinal tract. Pyridoxine was not recovered in the blood, even after 2 hours. It is believed that this may be due to the fact that pyridoxine is completely metabolized, excreted in the urine, or stored in the body tissues. Since only 30% of the pyridoxine administered was recovered in its various forms and metabolites in the blood and urine after 48 hours, there was probably a large tissue uptake. The following figure summarizes these results (511).

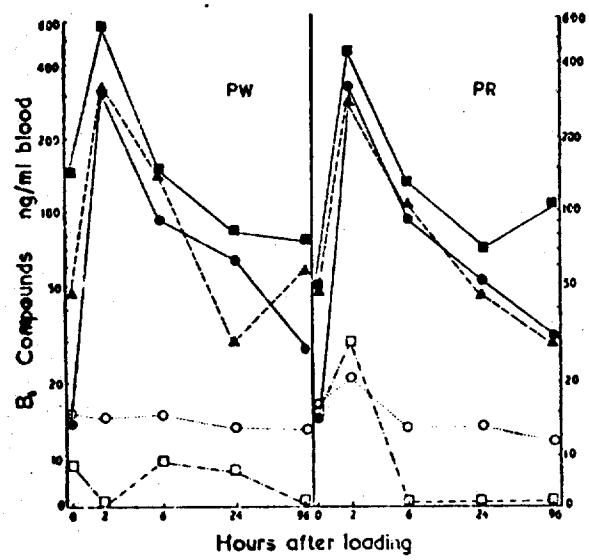


Fig. 1. Levels of B_6 compounds in blood of two normal females after oral dose of POL HCl (100 mg).
●——● PALP, ■——■ PAL, □------□ PAMP, ○·····○ PAM, △------△ PIC.

Dahlkvist et al. administered, in 0.2 ml water, 22 mcg $^3H_8$ pyridoxine i.v. to 20 g mice of the N.M.R.I. strain. Two mice were sacrificed at 2, 15, and 120 minutes and at 1, 7, and 30 days. The distribution of radioactivity was determined in the various organs and the urine. The excretion of isotope in the urine was comparable to that in other studies in rats and humans - a high rate of excretion at first and then, a slow excretion rate (see figure below). From day 1 to 30, pyridoxine was eliminated in the urine at a rate of 6.5%/day and from the body reservoir at 7.9%/day.

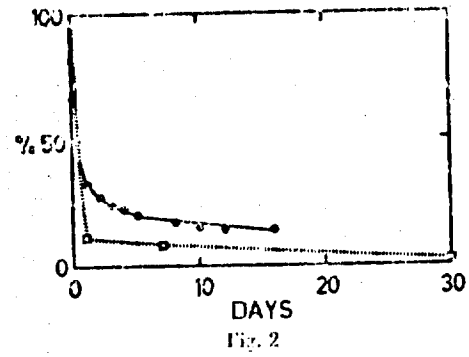


Fig. 2. Per cent of isotope remaining in the body at different times after the administration of ($^3H_8$) pyridoxine. The solid line has been calculated from the excretion of isotope in the urine of a series of 4 mice, and the dotted line from measurements of isotope in the tissues.

The variation of isotope concentration with time was similar in the carcass, liver, intestine, kidneys, heart, lungs, skeletal muscle, and spleen - an initial high concentration which rapidly decreased the 1st day and then became more gradual between day 1 and 30 (see Figures 3 and 4). In the brain, however, no initial high uptake was seen. The maximum isotope concentration was observed after day 1. The authors stated that the different results in the brain may have been due to a low permeability of the blood-brain barrier of the pyridoxine. The isotope concentration was highest in the liver, next in the heart followed by the skeletal muscle. After 1 day a high concentration occurred in the kidneys. The carcass and liver accounted for 80 to 90% of the total recovered isotope. The tissue concentrations of isotope were about equal after a period of equilibration. Figures 5 and 6 show the percent of total recovered isotope in the various organs 1, 7, and 30 days after pyridoxine administration. Between 1 and 30 days, 75% of the recovered isotope was in the carcass, 10 to 15% was in the liver, and the rest in the other organs (563).

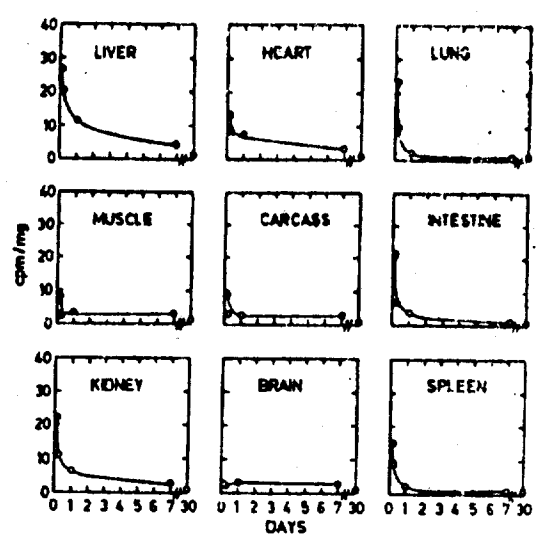


Fig. 3. Variation with time of isotope-concentration in different tissues of mice after the injection of ($^3H_8$)pyridoxine.

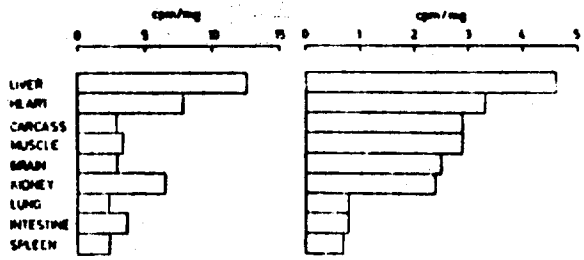


Fig. 4. Isotope concentration in different tissues of mice at different times after the injection of (^{3}H8)pyridoxine at 1 day (left) and 7 days (right) after the injection.

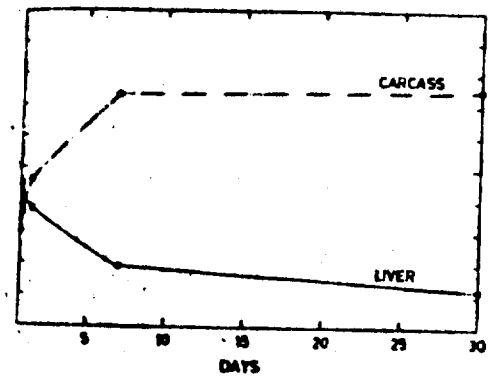


Fig. 5

Fig. 5. Per cent of total amount of recovered isotope in liver and carcass 1 to 30 days after the injection of (^{3}H8)pyridoxine.

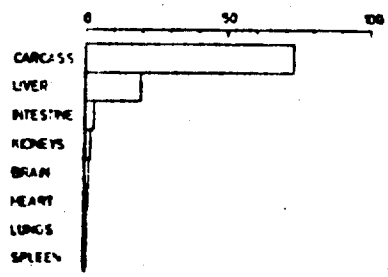


Fig. 6

Fig. 6. Per cent of total amount of recovered isotope in different organs of mice 7 days after the injection of (^{3}H8)pyridoxine.

Johansson et al. investigated the conversion of pyridoxine to its other forms in vivo by injecting $^3H_8$ pyridoxine, i.v., into 25 g male mice of the N.M.R.I. strain. One ml of a solution of 200 mcg pyridoxine per ml was injected (20 mcg) into the tail vein, after which the mice were sacrificed at various times. The livers and carcasses were tested for radioactivity in a scintillation spectrometer. It was found that labeled, free pyridoxine was gone from the liver 60 minutes after the injection, indicating its rapid phosphorylation. The labeled pyridoxine-5'-phosphate was at a maximum in 10 minutes, whereas 70% of the radioactivity was recovered as pyridoxal-5-'phosphate after 60 minutes. The isotope was recovered in other forms of pyridoxine in small amounts (see Figure 3).

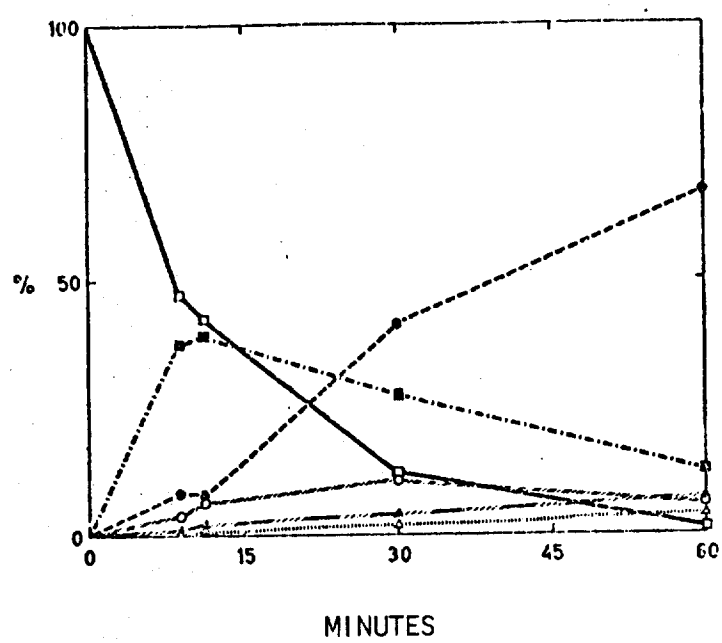


FIGURE 7: Distribution of radioactivity in mouse liver between different forms of vitamin B_6 during the first hour after intravenous administration of ($^3H_8$)pyridoxine. Pyridoxine (□), pyridoxine 5'-phosphate (■), pyridoxal (O), pyridoxal 5'-phosphate (●), pyridoxamine (△), and pyridoxamine 5'-phosphate (▲).

The recoveries in the mouse carcasses were as follows: 60% of the total isotope as pyridoxine after 60 minutes, 25% of the recovered isotope as pyridoxine-5'-phosphate after 60 minutes, and small amounts as other forms of pyridoxine: pyridoxal-5'-phosphate, pyridoxamine, and pyridoxamine-5'-phosphate - see Figure 8. Figure 9 shows that pyridoxal-5'-phosphate and pyridoxamine-5'-phosphate were the main labeled forms of the vitamin recovered in the liver from 1 to 7 days after the pyridoxine was given. Free pyridoxal, pyridoxamine, pyridoxal-5'-phosphate, and pyridoxamine-5'-phosphate were recovered in about equal amounts in the carcass (see Figure 10) 1 to 7 days after the pyridoxine was administered (1235).

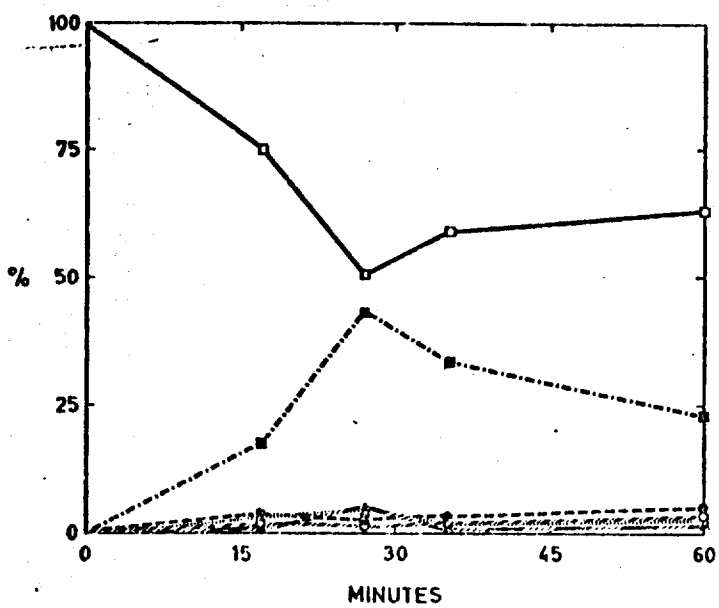


Fig. 8 : Distribution of isotope between the different forms of vitamin B_6 in mouse carcass during the first hour after the intravenous administration of [$^3H_6$]pyridoxine. Pyridoxine (□), pyridoxine 5'-phosphate (■), pyridoxal (○), pyridoxal 5'-phosphate (●), pyridoxamine (△), and pyridoxamine 5'-phosphate (▲).

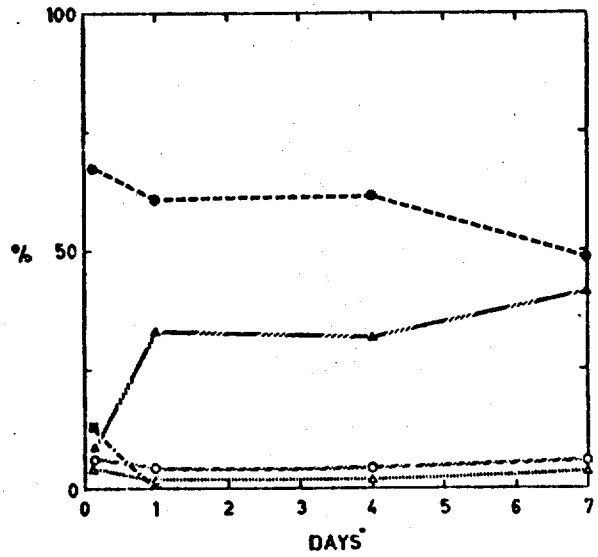


Fig. 9 : Distribution of isotope between different forms of vitamin B_6 in the liver from days 1 to 7 after the intravenous administration of [$^3H_6$]pyridoxine. Pyridoxine (□), pyridoxine 5-phosphate, (■) pyridoxal (○), pyridoxal 5-phosphate, (●) pyridoxamine (△), and pyridoxamine 5-phosphate (▲).

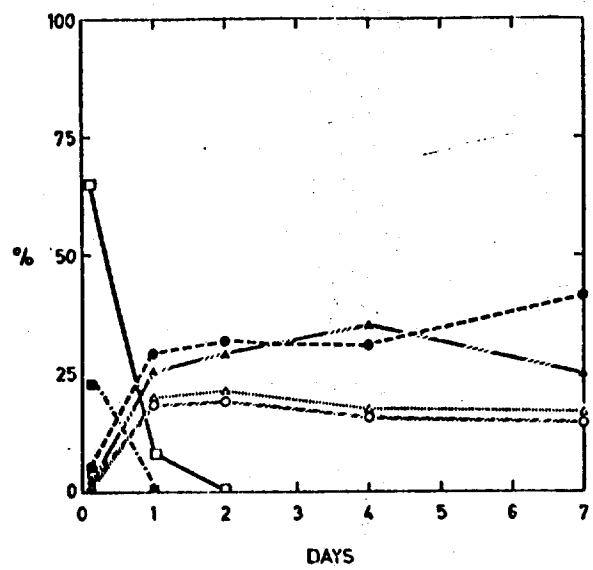


Fig. 10 : Distribution of isotope between different forms of vitamin B_6 in mouse carcass from days 1 to 7 after the intravenous administration of [$^3H_6$]pyridoxine. Pyridoxine (□), pyridoxine 5'-phosphate (■), pyridoxal (○), pyridoxal 5'-phosphate (●), pyridoxamine (△), and pyridoxamine 5'-phosphate (▲).

After C^{14}-pyridoxine administration to male Swiss mice, McCoy and Colombini found that the primary reactions in the liver are phosphorylations: pyridoxine to pyridoxine phosphate (PNP) to pyridoxal phosphate (PLP) and PLP to pyridoxamine phosphate (PMP). The main reactions in the brain are first oxidation to pyridoxal from pyridoxine (pyridoxine can be oxidized to pyridoxal or phosphorylated to PNP, which is oxidized to PLP). In the carcass, pyridoxine is mainly oxidized to pyridoxal. It was suggested that the carcass is a storage site for pyridoxine, since 7% of the total injected C^{14} was found there after 24 hours, compared to 1.6% in the liver and 0.1% in the brain. The predominant forms of pyridoxine in brain, liver, and carcass were PMP and PLP, but a larger proportion of PNP, pyridoxal, and pyridoxine was found in carcass (1678).

Snell reported, in his review of the metabolism of the B vitamins, that pyridoxine is not found in body tissues in amounts as high as are other forms of vitamin B_6. The various forms of vitamin B_6 are shown in Figure 11.

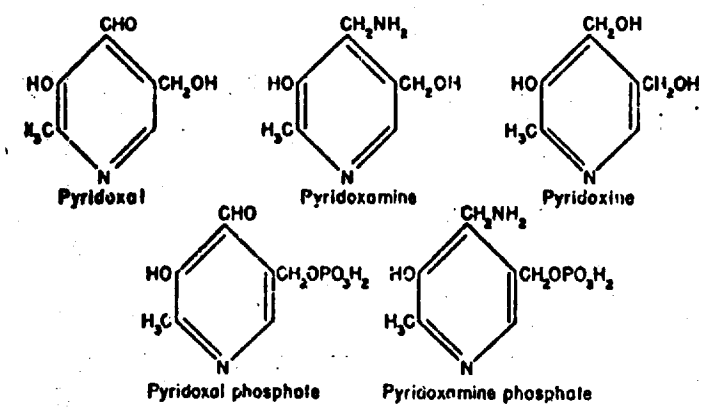


Fig. 11 CHEMICALLY IDENTIFIED free and bound forms of vitamin B_6.

The form of vitamin B_6 that is one of the most metabolically significant is pyridoxal phosphate. This form is the coenzyme for many types of amino acid transformations. Most of these transformations involve the non-oxidative degradation and interconversion of free amino acids (2390).

Hillman reported that vitamin B_6 was found by Turner and Reynolds, 1955 and by Tower, 1956 to be synthesized in the lower bowel by micro-organisms (1092).

Tiselius studied the passage of pyridoxine across the blood-cerebrospinal fluid (CSF) and the blood-brain barriers by injecting ^3H-pyridoxine i.v. into rats (200 to 250 g). The dose given was 20 mcg (20 mcCi) as a solution containing 100 mg/l pyridoxine (a physiological dose). The rats were sacrificed at varying times up to 3 hours after the injection and the isotope content of the CSF, brain tissue, and blood serum were determined. Initially, there was a higher isotope concentration

in the blood serum than in the CSF, but an equilibrium was reached after about 1 hour. Pyridoxine rapidly passed through the blood-brain barrier and was evenly distributed between the cerebrum and the cerebellum (see Figure 12A).

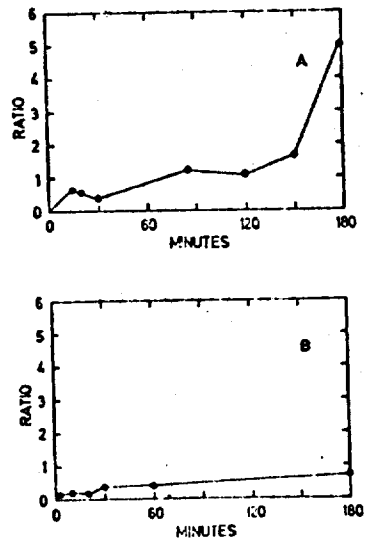


Fig. 12 —Ratio of isotope content of 1 mg of brain tissue (dry weight) to that of 1 μl of serum in the rat at different times after the intravenous administration of [^{3}H] pyridoxine (A) and [^{3}H]pyridoxine 5'-phosphate (B). Dosage and route as in Fig. 1.

A study with male mice of the N.M.R.I. strain determined the interconversions of the different forms of pyridoxine in the brain. The mice weighing 20 to 25 g, were given 20 mcg of 3H-pyridoxine i.v. The pyridoxine rapidly decreased to approximately 26% in 10 minutes. The pyridoxine phosphate reached a peak in 20 minutes. Ten minutes after the injection, 17% of the isotope appeared as pyridoxal phosphate; 61% after 40 minutes. Only small amounts of labeled pyridoxamine phosphate were detected in the first 50 minutes. From 2 to 13 days, this form varied from 28 to 56%. Pyridoxal was usually less than 10%, whereas pyridoxal phosphate stayed at around 50%. The results are summarized in Figures 13 and 14 (2593).

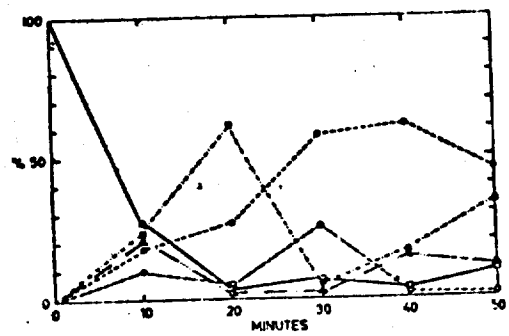


Fig. 13 —Distribution of isotope in mouse brain between the different forms of vitamin B_6 during the first 50 min after the intravenous administration of [^{3}H]pyridoxine (20 μg, 20 μCi). Pyridoxine (□), pyridoxal (O), pyridoxine 5'-phosphate (■), pyridocal 5'-phosphate (Θ), and pyridoxamine 5'-phosphate (▲).

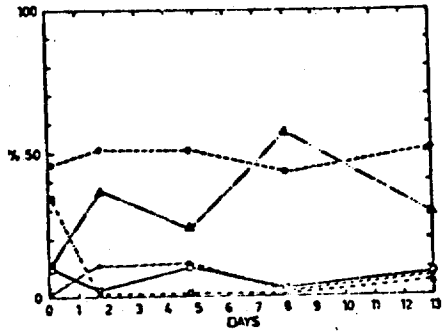


Fig. 14 -Distribution of isotope in mouse brain between the different forms of vitamin B_6 during the first 13 days after the intravenous administration of [^{3}H]pyridoxine (20 μg, 20 μCi). Key to symbols as in Fig. 4.

III. Metabolism and Excretion

Scudi et al. reported that vitamin B_6 is rapidly recovered in the urine of dogs and humans after its oral administration, indicating that it is rapidly absorbed from the GI tract. After the oral administration of 50 to 500 mg (per dog or 4.5 to 45 mg/kg), vitamin B_6 to 6 fasting dogs, weighing 7 to 15 kg, maximum recovery was observed in 1 hour (see Figure 1). Maximum recovery (18%) was observed 30 minutes after the same dose i.v. to the same dogs (see Figure 15). Only 20% recovery occurred after 6 hours; nonfasted dogs, maximum recoveries did not occur until 2 or 3 hours. Thirteen nonfasted healthy men, 60 to 85 kg, were given 50 mg vitamin B_6 i.v. (about 0.68 mg/kg). An average recovery of 8.7% of the dose occurred in 1 hour. When a similar group of 11 men were given 100 mg vitamin B6 orally, 7.6% was recovered in the urine after 4 hours (see Table 1) (2291).

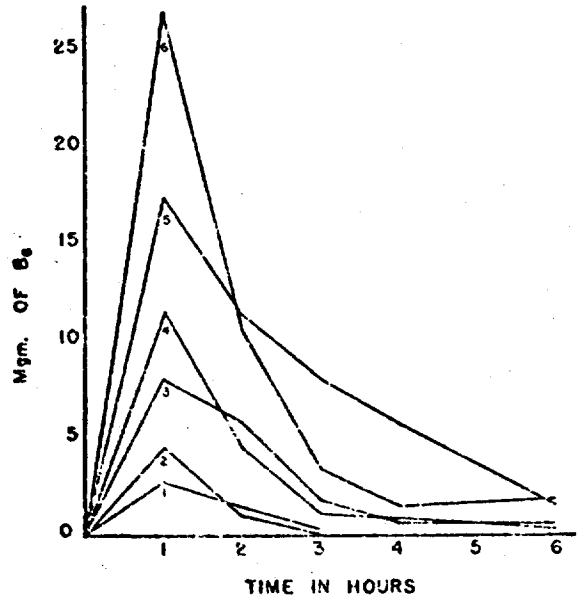


Fig. 15 Excretion of vitamin B_6 following oral administration. Curve 1, Dog 51, dose 50 mg.; Curve 2, Dog 58, dose 50 mg.; Curve 3, Dog 31, dose 100 mg., 6 hour output 16.8 per cent; Curve 4, Dog 97, dose 100 mg., 6 hour output 18.4 per cent; Curve 5, Dog 92, dose 200 mg., 6 hour output 22.0 per cent; Curve 6, Dog 31, dose 200 mg., 6 hour output 22.1 per cent, Dog 31, dose 500 mg., 6 hour output 20.3 per cent, Dog 97, dose 500 mg., 6 hour output 18.7 per cent. For Curves 1, 2, and 5 the urine samples were taken by catheter.

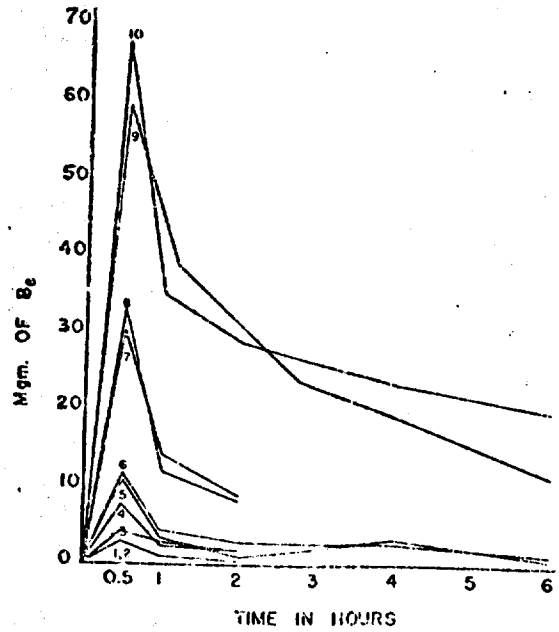


Fig. 16 Rate of excretion following the intravenous administration of vitamin B_6. Curve 1, Dog 31, dose 25 mg., 1 hour output 17.2 per cent; Curve 2, Dog 97, dose 25 mg., 1 hour output 16.8 per cent; Curve 3, Dog 97, dose 50 mg., 1 hour output 15.0 per cent; Curve 4, Dog 31, dose 50 mg., 1 hour output 20.8 per cent; Curve 5, Dog 97, dose 100 mg., 1 hour output 14.4 per cent; Curve 6, Dog 97, 100 mg., 1 hour output 16.5 per cent; Curve 7, Dog 31, dose 250 mg., 1 hour output 18.0 per cent; Curve 8, Dog 97, dose 250 mg., 1 hour output 18.0 per cent; Curve 9 (urine by catheter), Dog 66, dose 500 mg., 1 hour output 20.8 per cent; Curve 10, Dog 97, dose 500 mg., 1 hour output 20.5 per cent.

Table I

Vitamin B_6 Output in Human Subjects after Intravenous and Oral Administration

Subject No.	After intravenous administration of 50 mg. vitamin B_6					Total output 4 hrs. after oral administration of 100 mg. vitamin B_6
	15 min.	30 min.	45 min.	60 min.	Total output	
	mg.	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*
1	3.3	1.0	0.2	0.0	9.0	7.6
2	3.1	0.7	0.2	0.1	8.1	8.7
3	3.9	1.2	0.2	0.4	11.4	9.4
4	3.6			2.2	11.6	7.6
5	2.4			0.9	6.6	5.1
6	2.9			0.7	7.2	7.5
7	3.7			0.3	8.0	9.4
8	4.3			0.3	9.2	7.6
9	3.1			0.5	7.2	4.5
10	5.2			0.7	11.8	8.8
11	4.5			0.3	9.6	7.2
12	3.1			0.0	6.2	
13	3.5			0.1	7.2	
Average					8.7±1.6	7.6±1.1
Maximum deviation					3.1	3.1

The excretion of pyridoxine and its metabolites in human sweat and urine was studied in 4 adult males, 21 to 28 years old, by Johnson et al. The subjects were confined in an experimental chamber at 37.8 degrees C and relative humidity of 70% for 8 hours daily for two 5 day periods. No pyridoxine supplements were added to the diet in the first period. In the second period, 8 mg pyridoxine per day were added to the diet. The 8 mg pyridoxine supplement increased the total excretion by an average of 4.3 mg (to 8.67 mg). Pyridoxine was 5.5% of this, which is a 54% pyridoxine recovery. Most of the excretion was 4-pyridoxic acid (85%); the other 9.5% was identified as pseudopyridoxine or pyridoxal. The concentration ratio of pyridoxine metabolites was found to be similar in sweat and in urine (1237).

TABLE II

Concentration of Pyridoxine and Its Metabolites in Sweat

Post period No.	Subject	Pyridoxine	"Pseudopyridoxine" (pyridoxal)	4-Pyridoxic acid	Total
		γ per cc.	γ per cc.	γ per cc.	γ per cc.
I. No dosage	C	0.00055			
	D	0.00170	0.0225		
	E	0.00042	0.0043		
	F	0.00068	0.0105		
Average		0.00084	0.0324		
II. Dosage, 8 mg. pyridoxine per day	C	0.00049	0.0168	0.054	0.071
	D	0.0033	0.0145	0.048	0.066
	E	0.0016	0.0063	0.052	0.060
	F	0.0017	0.0090	0.055	0.066
Average		0.0018	0.0117	0.052	0.066
% of total		2.7	17.9	79.4	100

Rabinowitz and Snell collected 24 hour urine samples from 3 male subjects to establish base levels for the various excreted forms and products of vitamin B_6. The main excretion product, 91 to 98% of the total, was 4-pyridoxic acid. Pyridoxamine and pyridoxal were found, but the presence of pyridoxine could not be detected. The authors pointed out that at that time (1949), the detection methods were probably not sensitive enough for the small amounts of pyridoxine present. The subjects were each given one oral dose of 100 mg pyridoxine HCl with 200 ml water and their urine collected at various intervals for 36 hours. After 2 months the experiment was repeated. The results are summarized in Table II (2060).

Table 3

The Urinary Excretion of Pyridoxic Acid, Pyridoxal, Pyridoxamine and Pyridoxine.

Excretion products*	Mg excreted up to 24 hr after ingestion (a)	Mg excreted 24 hr before ingestion (b)	Mg recovered (c)	Mg excreted up to 24 hr after ingestion (d)	Mg excreted 24 hr before ingestion (e)	Mg recovered (f)
	Fed 70 mg pyridoxamine. Subject A			Subject C		
Pyridoxic acid lactone	24.60	6.48	18.12	22.37	2.59	19.78
Pyridoxal	1.57	0.102	1.47	0.985	0.058	0.93
Pyridoxamine	1.83	0.028	1.80	1.18	0.149	1.03
Pyridoxine	0.00	0.00	0.00	0.209	0.00	0.21
Total			21.39			21.95
	Fed 82 mg pyridoxine. Subject B			Subject A		
Pyridoxic acid lactone	28.40	1.97	26.43	25.27	3.91	21.36
Pyridoxal	1.69	0.074	1.62	1.38	0.052	1.33
Pyridoxamine	0.535	0.124	0.41	0.650	0.210	0.44
Pyridoxine	8.53	0.00	8.53	7.80	0.00	7.80
Total			36.99			30.93
	Fed 82 mg pyridoxal. Subject C			Subject B		
Pyridoxic acid lactone	59.79	3.84	55.95	53.22	2.54	50.68
Pyridoxal	1.53	0.084	1.45	1.28	0.049	1.23
Pyridoxamine	0.021	0.083	0.00	0.241	0.097	0.14
Pyridoxine	0.039	0.00	0.04	0.132	0.00	0.13
Total			57.44			52.18

* All products are expressed in terms of the free bases. Pyridoxic acid is determined and expressed the lactone. The molecular weight of the free bases are almost equal (pyridoxal = 167, pyridoxamine = 168, pyridoxine = 169, 4-pyridoxic acid lactone = 165). Expressed in this way, weights are directly comparable as molar quantities without significant error.

After the ingestion of 100 mg pyridoxine HCl, pyridoxine was the main form of vitamin B_6 excreted. Less than half (45%) of the administered dose was recovered. The authors explained that this low recovery of pyridoxine may have been because there were other metabolites formed, which have not yet been identified, or because pyridoxine was stored in the body. The other 2 forms excreted were pyridoxamine, 4 to 5%, and pyridoxal, 14 to 15%. Pyridoxic acid accounted for 69% of the excretion products. The pyridoxine excreted reached a peak after about 2 hours and was at a normal level within 8 hours (see Figure 17). Figure 2 expresses that similar results were obtained with the 4-pyridoxic acid levels after pyridoxine feeding.

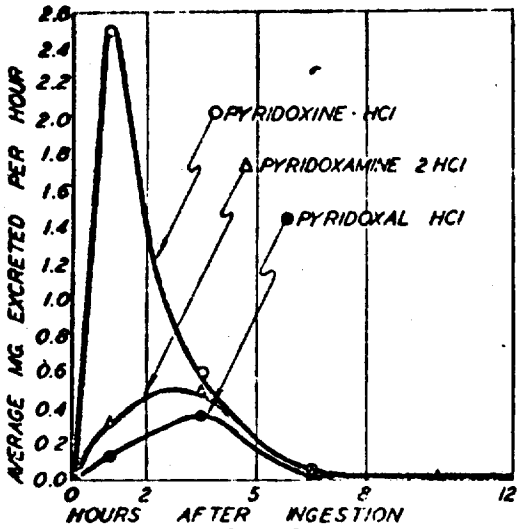


FIG. 17

The Excretion of Vitamin B_6. The total amount of the indicated form of the vitamin found in the urine samples is plotted against the average time interval, after ingestion of the dose, during which the sample was collected.

○ Fed 100 mg of pyridoxine HCl.
△ Fed 100 mg of pyridoxamine 2HCl.
● Fed 100 mg of pyridoxal HCl.

The levels of the forms of the vitamin other than the one fed are not shown.

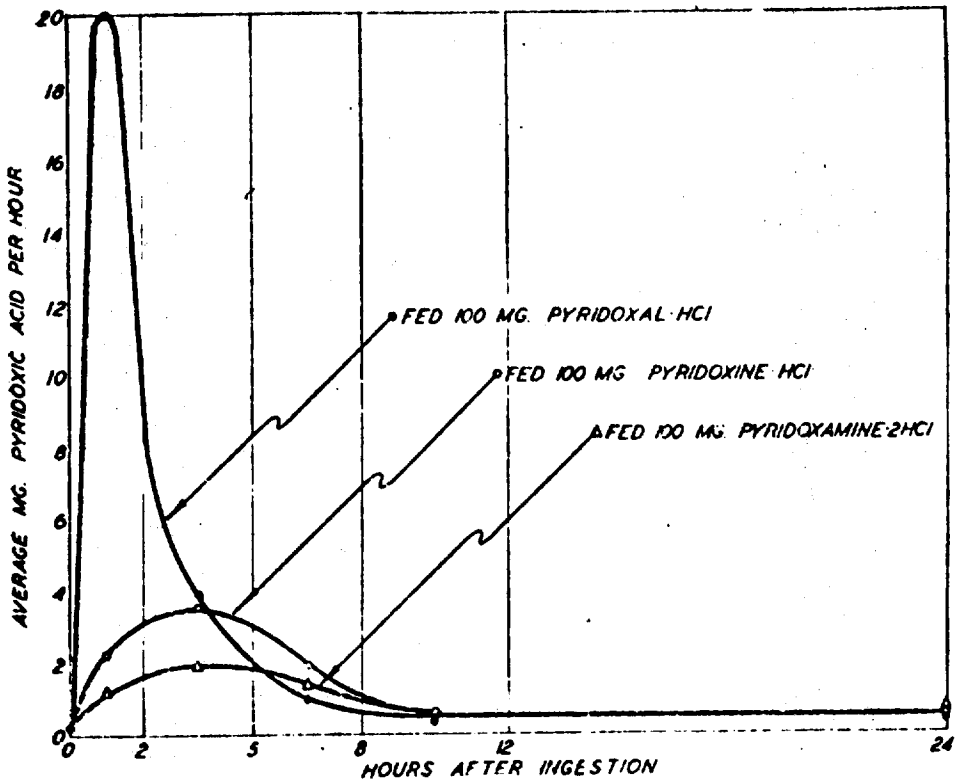


Fig. 18

The excretion of pyridoxic acid (expressed as 4-pyridoxic acid lactone) following ingestion of various forms of vitamin B_6.

The authors stated that the small amounts of vitamin B_6 normally ingested by humans in the diet may be metabolized differently from large doses, as those in this study (2060).

Cox et al. administered various doses of tritium-labeled pyridoxine hydrochloride i.v. to 11 rats weighing 250 to 350 g. The injection solution contained 2 mg (616 mcg) labeled and 18 mg unlabeled pyridoxine hydrochloride per ml. The doses given were 184.8 mcg (rat A), 369.6 mcg (rats B & C), and 616 mcg (rats D-K) H^3-pyridoxine hydrochloride. Blood, urine, and fecal samples were measured from tritium in a liquid scintillation counter; the urine samples were also chromatographed. The half-life of H^3 activity in the blood was 45 minutes, excepting the first 15 minutes, after the injection. Most of the pyridoxine given (70%) was excreted unchanged in the urine in a half-life of 45 minutes. The one degradation product, 4-pyridoxic acid, was excreted for 5 hours in small amounts. About 28% of the tritium was retained with a half-time of 17 days, which may represent what was stored in the body tissues. Fecal excretion was very small - less than 0.03% in 96 hours. The excretion results are summarized in Tables 4 and 5 (539).

Table 4. Cumulative Urinary Excretion of Tritium Activity Following Intravenous Administration of H^3-Pyridoxine · HCl in Rats.

Time after inj. (hr)	Rat No. & cumulative excretion (%)				
	D	E	F	G	Avg
1.5	64.3	54.5	58.0	57.2	58.5
2.5	69.3	62.4	65.1	62.3	64.8
3.5	—	68.1	—	67.5	67.8
4.5	—	—	68.4	—	—
5.5	71.9	—	—	—	—
12	73.3	73.5	69.7	69.1	71.4
24	75.6	75.7	71.5	70.5	73.3
31	76.3	76.2	71.7	70.8	73.7
96	79.8	78.1	78.3	73.8	77.5
122	80.2	78.1	78.4	73.9	77.7
144	80.4	78.2	78.4	75.0	78.0

Table 5. Cumulative Fecal Excretion of Tritium Activity Following Intravenous Administration of H^3-Pyridoxine · HCl in Rats.

Time after inj. (hr)	Rat No. & cumulative excretion (%)				
	H	I	J	K	Avg
12	<.001	<.001	—	<.001	<.001
24	.011	.020	.018	.008	.014
48	.016	.022	.023	.019	.020
72	.019	.027	.025	.022	.024
96	.028	.027	.026	.024	.026

Johansson et al. administered pyridoxine uniformly labeled with tritium to 3 men, aged 37 to 50, who had normal blood chemistry and kidney functions (although 2 of the subjects had passed calcium oxalate kidney stones). Two of the men were given 400 mcg pyridoxine in saline i.v.; the third was given 250 mcg pyridoxine in water orally. The excretion of pyridoxine was studied using urine collected in 4 to 8 hour samples the first day and in 24 hour samples for the other 24 days. Previous experiments had shown that pyridoxine is not excreted in the feces to any great degree. In all 3 subjects, 15 to 20% of the tritium administered was excreted in the urine the first day. The half-life in the body varied from 18 to 38 days. The authors computed the fractional turnover rate of the total amount of vitamin B_6 in the body to be 2.2 to 4.4% per day (1.7 to 3.6 mg per day) from the experimental data, which is

relatively slow rate. The main excretory product of pyridoxine after its administration to humans, is 4-pyridoxic acid, which accounts for 20 to 40% of the vitamin B_6 excreted per day. The effects of dietary intake of pyridoxine were not ascertained, since the subjects were not on diets containing constant amounts of the vitamin. The total amount of vitamin B6 in the body was estimated to be 40 to 150 mg. When one of the subjects was given a diet containing an additional 80 mg pyridoxine per day, the excretion of tritium significantly increased. The authors suggested that the body pool of vitamin B_6 was mobilized by the increased dietary intake of pyridoxine (1232).

Contractor and Shane identified another metabolite of pyridoxine, which could explain the poor recoveries of labelled pyridoxine by other investigators (Rabinowitz and Snell, 1949; Johansson et al., 1966). Both H^3- and C^{14}-labeled pyridoxine were injected i.p. into male Wistar rats. Twenty-four hour urines were chromatographed on DEAE cellulose columns. Examination of the elution patterns showed the following metabolites: 4-pyridoxic acid, pyridoxal phosphate, pyridoxine phosphate, and an unidentified metabolite. This metabolite which was up to 20% of the total radioactivity given, was identified to be 4-pyridoxic acid 5'-phosphate by TLC, electrophoresis, and fluorescence. The authors explained that 2 pathways probably exist in animal tissues for 4-pyridoxic acid formation. The accepted pathway is from pyridoxal phosphate to pyridoxal (by hydrolysis) and then to 4-pyridoxic acid by an aldehyde oxidase. The predominant pathway, according to the authors, may be the oxidation of pyridoxal phosphate to 4-pyridoxic acid 5'-phosphate which is subsequently hydrolysed to 4-pyridoxic acid (513).

Contractor and Shane administered 50 mg pyridoxine HCl orally or i.v. (in tablets or in saline) to men and to pregnant and nonpregnant women. Control levels in blood and urine of pyridoxine, its derivatives and metabolites are shown in Tables 6 and 7. Pyridoxine (pyridoxol) was not detected in the blood of the subjects, and no pyridoxal-5'-phosphate or pyridoxamine-5'-phosphate were detected in the urine of the subjects. Blood pyridoxal 5'-phosphate levels were significantly lower in pregnant women (last trimester) indicating a vitamin B_6 deficiency in pregnancy. This difference was not observed in urinary output, indicating the increased B_6 requirement of the fetus. High levels of pyridoxal-5'-phosphate and pyridoxamine-5'-phosphate were observed in the fetus (from cord blood levels). Pyridoxal 5'-phosphate levels in the blood reached a peak 2 hours after oral pyridoxine loading, which is the time needed for intestinal absorption (see Figure 19). This indicates the rapid phosphorylation or oxidation of pyridoxine. The intravenous administration of pyridoxine caused the same peak in 10 minutes. Similar results were found with pyridoxal and 4-pyridoxic acid levels; however, no effects were found on pyridoxamine or pyridoxamine 5'-phosphate levels. Table 8 shows that a slight decrease in the excretion of pyridoxine metabolites occurred in pregnancy after pyridoxine loading. Within the first 6 hours after the oral loading, 60% of the 24 hour urine 4-pyridoxic acid and 95% of the 24 hour urine pyridoxal were excreted in pregnant women. The absorption of pyridoxine (pyridoxol) was not found to be affected by pregnancy, since the urinary recovery of pyridoxine and metabolites was similar for men, pregnant women, and nonpregnant women (512).

Table 6. Levels of B_6 compounds in blood

Subject	Pyridoxal 5'-phosphate (mμg/ml. ± S.D.)	Pyridoxamine 5'-phosphate (mμg/ml. ± S.D.)	Pyridoxal (mμg/ml. ± S.D.)	Pyridoxamine (mμg/ml. ± S.D.)	4-Pyridoxic acid (mμg/ml. ± S.D.)
Man	13.5 ± 2.9(5)	20 ± 5.5(4)	1.3	24.7 ± 19.5(3)	31.9 ± 6.4(4)
Nonpregnant woman	12.1 ± 2.3(23)	9.3 ± 7.4(21)	0.8 ± 0.2(2)	18.0 ± 3.9(21)	31.8 ± 10.1(23)
Pregnant woman	7.7 ± 3.7(5)	9.5 ± 6.4(4)	1.1 ± 0.7(5)	7.6 ± 1.2(4)	30.4 ± 9.2(4)
Fetus	27.5 ± 8.1(5)	27.4 ± 2.6(4)	2.2 ± 0.9(5)	6.6 ± 3.0(4)	25.2 ± 9.8(4)

Table 11. Levels of B_6 compounds in urine

Subject	Pyridoxal (μg/24 hr. ± S.D.)	Pyridoxol (μg/24 hr. ± S.D.)	Pyridoxamine (μg/24 hr. ± S.D.)	4-Pyridoxic acid (μg/24 hr. ± S.D.)
Man	45.4 ± 11.2(2)	77.5 ± 31.7(2)	208 ± 66(2)	1324 ± 780(3)
Nonpregnant woman	24.5 ± 12.4(19)	94.1 ± 35.1(19)	121 ± 48(19)	1213 ± 848(26)
Pregnant woman	31.5 ± 8.7(2)	71.9 ± 20.1(2)	132 ± 18(2)	1454 ± 474(10)

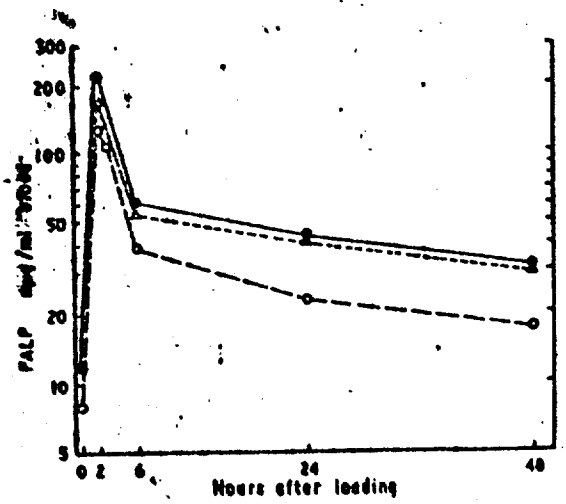


Fig. 17 Levels of pyridoxal 5'-phosphate in blood of different subjects after oral dose of pyridoxol HCl (50 mg.) ●——●, nonpregnant woman; △ - - - - △, man; ○— —○, maternal.

Table 8. Urinary recovery of B_6 metabolites in different subjects after administration of 50 mg. pyridoxol HCl (PAM values are not included as they were not affected by the B_6 loading)

Subject	Method of administration	Metabolite (mg./24 hr. ± S.D.)			Recovery (per cent of dose ± S.D.)
		Pyridoxal	Pyridoxol	4-Pyridoxic acid	
Man	Oral	0.452	0.620	13.2	33
Nonpregnant woman	Oral	0.745 ± 0.107(2)	1.06 ± 0.25(2)	11.7 ± 0.7(2)	31 ± 3
	Intravenous	0.794 ± 0.134(3)	1.77 ± 0.32(3)	14.5 ± 1.2(3)	39 ± 4
Pregnant woman	Oral	0.754 ± 0.353(3)	1.16 ± 0.51(3)	9.74 ± 1.56(5)	27 ± 5
	Intravenous	0.545	3.78	14.2	42

Flexner and Chassin studied the effect of age on the urinary excretion of pyridoxine in 3 groups of patients - with normal renal function, with Parkinson's disease, and with renal insufficiency. A total of 84 patients were studied, all of whom were on standard hospital diets. After obtaining a control urine sample, the patients were given 50 mg pyridoxine i.v. and 400 ml water orally. After 1 hour, another urine sample was obtained. The pyridoxine levels in the urine were calculated according to Scudi. In 10 patients (with normal renal function), 5 to 15 years old, the excretion of pyridoxine after its administration was an average of 21.3% of the dose given. In 13 patients who were 16 to 50 years old the pyridoxine excretion was an average of 8.4% after its administration. Two other patients in this age range excreted only 1.6 and 3.8% pyridoxine. The average output of pyridoxine in 45 patients over 50 years old was 7.2% (see Table 9). Both the 7 patients with Parkinson's disease and the 14 patients with renal insufficiency had below normal pyridoxine outputs, with age not being an important factor. The amount of pyridoxine excreted tends to decrease with increasing age. The authors did note that the 50 mg dose was probably excessive in proportion to body weight in the 5 to 15 year old group, which explained the high pyridoxine output of 21.3% (761).

TABLE 9

Influence of age on pyridoxine output

Group	Number of patients	Urinary output average	Deviation Standard	Deviation Maximum
		per cent	per cent	
5-15	10	21.3	±3.68	6.0
16-50	13	8.4	±2.04	3.1
	2	2.7		
Over 50	32	7.2	±1.87	3.8
	13	2.3		

Sakuragi reviewed the role of pyridoxine in the metabolism of fats. Both McHenry and Gavin (1941) and Sherman (1950) have reported that pyridoxine is involved in the conversion of carbohydrates and proteins to fats. Various researchers (Witten and Holman (1952); Mead and Howton (1957) have stated that pyridoxine is necessary for the conversion of linoleic acid to arachidonic acid, and Champorigny and Breton (1947) reported that pyridoxine may be a cofactor for the conversions of palmitic acid to palmitoleic acid and of stearic to oleic acid. Basnayake and Sinclair (1956) have shown various ways in which pyridoxine is related to essential fatty acids (EFA). Symptoms of vitamin B_6 deficiency and of EFA deficiency are similar - both produce skin lesions in rats. The removal of pyridoxine from the diet produces the symptoms of EFA deficiency, and vice versa; moreover, the deficiency of one of them can be partially caused by giving more of the other. Pyridoxine has been considered by

Hove and Harris (1946) to be a physiological antioxidant, such as in the stabilization of vitamin A in vivo (2213).

Failey investigated the serum cholesterol-lowering effect of pyridoxine in 21 hospitalized patients from 41 to 80 years old, 5 of whom were diabetics. The 400 mg dose of pyridoxine was administered orally every day for 5 to 36 days. The nondiabetic subjects were male, and the diabetic subjects were female. Serum cholesterol levels were determined before, during, and after the pyridoxine administration. There was a slight but significant fall in serum cholesterol levels, which was greater in the diabetic subjects. The diabetic subjects and 10 of the nondiabetic subjects were given pyridoxine for 7 days. A group of 10 nondiabetic patients did not have a significantly lowered serum cholesterol level. The remaining 6 nondiabetic subjects, who were given pyridoxine for 36 days, did show a significantly lower cholesterol level. A delayed increase in cholesterol was observed after the pyridoxine was discontinued, suggesting a long cumulative effect (728).

Lupien studied the effect of pyridoxine on blood, liver, and heart cholesterol levels in 100 rabbits (2 kg) divided into the following 6 groups:

Group N. Twenty animals received the control pelleted diet*.

Group N + B_6. Sixteen animals received the control diet and vitamin B_6 subcutaneously (6 mg/kg body weight per day).

Group N + 1%. Sixteen animals received the control diet supplemented with 1% cholesterol.

Group N + 1% + B_6. Sixteen rabbits received the cholesterol diet and vitamin B_6 subcutaneously (6 mg/kg body weight per day).

Group D. Sixteen animals received a pyridoxine deficient diet**.

Group D + 1%. Sixteen animals of this last group received the pyridoxine deficient diet with the 1% dietary cholesterol supplement.

* The control diet had the following percent composition: vitamin free casein 18%, sucrose 68%, vegetable oil 10%, U.S.P. Salt mixture 2.4% and was supplemented with all the necessary vitamins. The diet was offered to the animals in pelleted form.

** The pyridoxine deficient diet was the same as the control diet except that it did not contain any pyridoxine.

Certain animals were sacrificed at 0, 3, 5, and 8 weeks. The rabbits were injected i.p. with C14 acetate-0.4 ml/kg, 5.7 mcmoles, 6.2 x 10 7 cpm - 3-1/2 hours before they were sacrificed. Daily s.c. pyridoxine injections produced no sustained significant changes in the serum, liver, or heart cholesterol levels of normal rabbits (group N + B_6), except at 3 weeks. At this time, there was a sharp increase in cholesterol levels (Table 1)

and a sharp decrease in the C14 acetate incorporation rate in the serum and liver cholesterol (Table 10) and a significant decrease in the liver incorporation rate (Table 11). The changes returned to normal limits by the 5th week. The other 4 groups had elevated serum and liver cholesterol levels compared to the control group (N). The serum and liver incorporation rates (cholesterol synthesis) in these groups decreased compared to the control group. The author concluded that the administration of pyridoxine to normal animals does not lower or significantly change the serum, liver, or heart cholesterol levels as other investigators have reported. In this study, pyridoxine given to rabbits on high cholesterol diets further increased the cholesterol levels. Pyridoxine deficiency had a cholesterol-elevating effect also (1585).

TABLE 10

MEAN SERUM, LIVER AND HEART CHOLESTEROL LEVELS FOR ALL EXPERIMENTAL GROUPS

Number of animals in each group given in parentheses.

Groups	*0 Weeks*			*3 Weeks*			*5 Weeks*			*8 Weeks*		
	serum (mg/100 ml)	*liver (mg/g)*	*heart (mg/g)*	*serum (mg/100 ml)*	*liver (mg/g)*	*heart (mg/g)*	*serum (mg/100 ml)*	*liver (mg/g)*	*heart (mg/g)*	*serum (mg/100 ml)*	*liver (mg/g)*	*heart (mg/g)*
N	42±4.7	1.8±0.30 (6)	0.54±0.06	38± 5.4	2.2±0.18 (5)	0.54±0.05	37± 3.9	1.8± 0.15 (6)	0.61±0.05	52±10	2.2±0.11 (6)	0.56±0.06
N + 1%				546±188	7.2±1.0 (5)	1.0 ±0.28	906±40	10 ± 3.5 (5)	1.1 ±0.06	—	—	—
N + B_6				87± 10	2.2±0.21 (6)	0.63±0.04	36± 5	1.8± 0.09 (6)	0.67±0.04	41± 6	1.9±1.0 (6)	0.42±0.03
N + B_6 + 1%				560±146	8 ±2.0 (6)	1.2 ±0.07	1195±84	18 ± 2.3 (5)	1.6 ±0.02	—	—	—
D				327± 69	3.7±0.87 (6)	0.85±0.09	498±97	4.2±0.48 (4)	0.71±0.07	383±82	5.9±0.88 (4)	0.65±0.06
D + 1%				617±138	7.9±1.9 (5)	0.82±0.16	693±50	7.1±1.5 (4)	1.2 ±0.5	—	—	—

TABLE 11.

SERUM AND LIVER CHOLESTEROL SPECIFIC ACTIVITIES (COUNTS/MIN PER MG CHOLESTEROL) FOR ALL EXPERIMENTAL GROUPS

Number of animals in each group given in parentheses.

Groups	*0 Weeks*		*3 Weeks*		*5 Weeks*		*8 Weeks*	
	serum (S.A.)[a]	*liver (S.A.)*	*serum (S.A.)*	*liver (S.A.)*	*serum (S.A.)*	*liver (S.A.)*	*serum (S.A.)*	*liver (S.A.)*
N	1100 ± 200 (6)	1775 ±219	1084 ± 214 (5)	2372 ± 422	907 ± 180 (6)	1569 ± 188	805 ± 161 (6)	1221 ± 143
N + 1%			21 ± 6 (5)	36 ± 10	25 ± 4 (5)	65 ± 10	—	—
N + B_6			260 ± 80 (6)	1349 ± 118	1034 ± 258 (5)	1721 ± 283	687 ± 105 (6)	984 ± 35
N + B_6 + 1%			43 ± 14 (6)	63 ± 18	13 ± 4 (5)	34 ± 8	—	—
D			61 ± 22 (6)	954 ± 116	65 ± 45 (4)	474 ± 45	45 ± 13 (4)	102 ± 38
D + 1%			42 ± 20 (5)	66 ± 16	58 ± 16 (4)	80 ± 22	—	—

[a] S.A. = specific activity (counts/min per mg cholesterol).

Kawanabe injected 2 rabbits (each weighing 2 kg) with 10 mg/kg pyridoxine (20 mg/ml) i.v. and 4 rabbits with 20 mg/kg i.v. Control rabbits were given saline in doses of 0.1, 1, 2, 3, or 4 ml/kg i.v. The rabbits had been starved for 20 hours before the experiment. Blood samples were drawn and the glucose level determined right before the pyridoxine was given and every 15 to 30 minutes for 3 hours after the injection. The group which received 10 mg/kg pyridoxine showed a slight decrease in blood sugar right after the injection, followed by an increase, then another decrease 1 hour later. The second group showed a rise in blood sugar followed by a gradual decrease. Compared to the control groups, the pyridoxine appeared to inhibit the elevation of the blood sugar level near the end of the 3 hour test period. The 10 mg/kg dose produced a greater effect than the 20 mg/kg group.

Various combinations of vitamins were then given i.v. to groups of 3 to 5 rabbits - 0.25 ml B_2 and 20 mg/ml B_6; 50 mg/ml nicotinamide and 20 mg/ml B_6; 50 mg/ml nicotinamide, 0.25 mg/ml B_2 and 20 mg/ml B_6. All 3 combinations produced elevated blood sugar levels with the 3 vitamins given together having the greatest effect and the B_2 + B_6 combination having the smallest effect (1295).

Kato reported that feeding excess levels of vitamin B_6 to 40 day old male Wistar white rats, weighing 50 g, temporarily suppressed the biosynthesis of protein-bound lipoic acid within 2 weeks. The rats were fed standard solid feed containing 0.2 mg% pyridoxine (10 to 20 g of feed were consumed daily) for over 4 weeks. A control group was given only the standard feed for the same period. The lipoic acid synthesis was at the control level in the fourth week. An additional treatment was done, in which 20 mg/kg lipoic acid - ^{35}S was given to the excess pyridoxine group during the second week to study the incorporation of lipoic acid in the liver mitochondrial protein. The lipoic acid incorporation was 70% lower than in the control group, but this was also a temporary effect. The author stated that excess vitamin B_6 affects endocrine systems in the pancreas and adrenals, which leads to adverse changes in the synthesis of lipoic acid. Since control levels were reached during continued administration of excess pyridoxine, the author stated that a combating mechanism or adaptable enzyme system must be present (1288).

Hamamoto et al. found that the oral or intraperitoneal administration of 500 mg pyridoxine HCl per kg to male, Wistar rats (150 to 200 g) caused a 2 to 3-fold increase in hepatic tyrosine activity transaminase (a pyridoxal enzyme). Enzyme induction also occurred 2 to 4 days after bilateral adrenalectomy. Seven days after adrenalectomy, no enzyme induction by pyridoxine was observed. The authors feel that the induction of tyrosine transaminase by pyridoxine is mediated by adrenal glucocorticoids, since pretreatment with hydrocortisome 150 mg/kg) restored the inducibility 7 days after adrenalectomy (1001).

Susten and Kicksey administered 1 g pyridoxine HCl per kg body weight, i.p., to pregnant and non-pregnant female Sprague-Dawley rats weighing 200 to 250 g. Half of the rats were given bilateral adrenalectomies 6 days prior to the injection. The pyridoxine was given on the 21st day of gestation in a volume of 2.0 ml saline. Control injections of saline were given to one group. Five hours after the injections,

the rats were sacrificed and the tyrosine transaminase activities of the adult and fetal livers assayed. Daily nutritional pyridoxine intake was calculated to be 124 mcg for nonpregnant and 168 mcg for pregnant rats, which was sufficient for holoenzyme formation in the livers of the pregnant rats. Pyridoxine administration elevated the activity of both the holotyrosine transaminase activity (HTA) and the total tyrosine transaminase activity (TTA) in intact rats compared to the controls. In adrenalectomized rats, the HTA was not increased and the TTA was slightly depressed. The stimulation of the enzyme activity in fetal liver from maternal treatment was not significant, but the mechanism of stimulation was thought to be operative. The pyridoxine treatment did not affect reproduction, weight gain, or food consumption (2502).

Driskell et al. (1971) studied the relationship between dietary pyridoxine and alanine aminotransferase activity in female Sprague-Dawley rats weighing 250 g and in weanling females. Various levels of pyridoxine were included in a basal diet for 28 to 100 days in two studies. In each group, 3 untreated animals were used as controls, 6 to 7 were mated, and 3 were not mated. The experimental diets were begun 1 week before the animals were mated in the 250 g rats and until 80 to 110 days in the weanlings. The alanine aminotransferase activity in the maternal erythrocytes and in the maternal and fetal livers increased with higher levels of pyridoxine up to 10 to 30 mcg. Levels higher than these did not produce increased enzyme activity (662).

Vitamin B_6 (50 mcg) was found by Lecoq et al. to antagonize the effects of adrenaline (augmentation of nerve chronaxia) in the rat when the 2 compounds were injected simultaneously (1495).

Cheldelin and King reviewed some of the following antagonists to pyridoxine: 4-desoxypyridoxine, 5-desoxypyridoxine, 5-desoxypyridoxal, and 5-desoxypyridoxamine. These compounds induce the symptoms of vitamin B_6 deficiency, such as dermatitis and a decrease in body weight (457).

Pyridoxine was found to significantly potentiate the serum cholesterol-lowering effect of niacin in the studies by Goldner and Vallan in 53 patients with varying degrees of hypercholesterolemia. Two groups of patients whose ages ranged from 30 to 96 years, were given oral doses totaling 3 g niacin and 3 mg pyridoxine every day for 10 weeks. The capsules, 2 taken 3 times per day, contained the following ingredients (those other than niacin and pyridoxine were not involved in cholesterol levels):

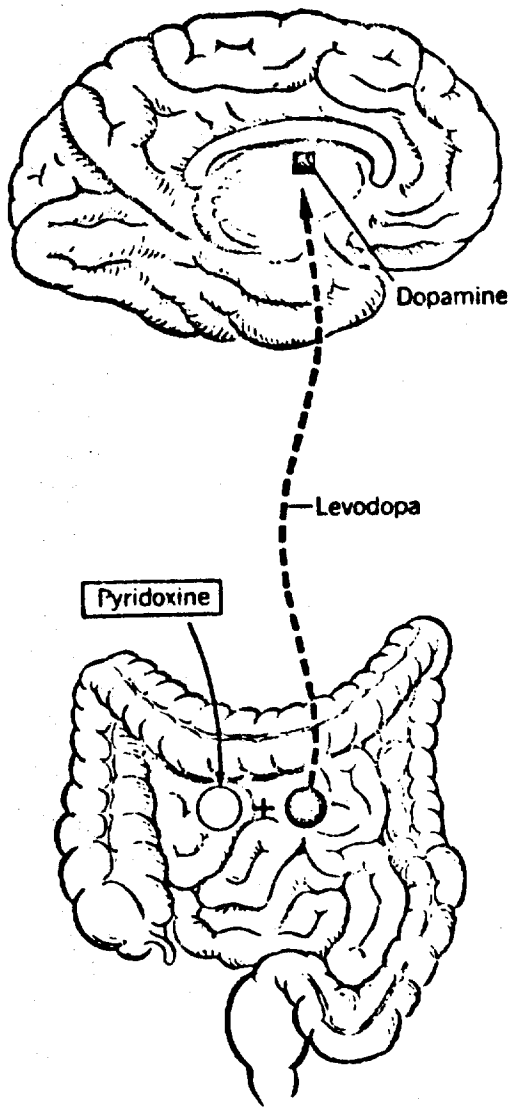
Niacin	330.0	mg
Pyridoxine HCl	0.5	mg
Thiamine mononitrate	1.0	mg
Riboflavin	2.0	mg
Ascorbic acid	20.0	mg
Cyanocobalamine (B_{12} activ.)	.001	mg
Reserpine	.025	mg
Pentylenetetrazole	20.0	mg

In one group of 20 patients, the average cholesterol level went from 338 mg to 203 mg per 100 ml; in the other group, the level decreased from 270 mg to at least 250 mg per 100 ml in 13 of the patients. Both decreases were statistically significant. The effects of niacin and pyridoxine in lowering the cholesterol levels were found even without a low cholesterol diet. The pyridoxine supplements lowered the effective dose of niacin. When the niacin/pyridoxine capsules were discontinued, the hypercholesterolemia reappeared (909).

Teijeira and Martinez-Lage studied the antagonism between pyridoxine and ethyl alcohol in rats, guinea pigs, and cats. Some of the authors' early experiments showed that pyridoxine reduced the incidence of mortality in rats under acute alcoholic intoxication. The authors then did a series of experiments using the EEG and ECG as measurements of the amount of improvement after pyridoxine treatment. The guinea pigs were anesthetized during the ethyl alcohol administration with urethane (10 ml/kg) in a few cases, but with sodium thiopental or thiobarbitol (10 mg/kg) most of the time. The alcohol was given either i.p. in a single dose or by slow injection i.v. in a few cases. Both guinea pigs and cats were given either 20 to 30 ml of 20% alcohol per kg. Cats were given 10, 20 or 40 ml alcohol per kg. The toxic doses of alcohol caused irregular EEG patterns - lentification, paroxistic activity with hypersynchronic spikes - followed by depressed electroencephalographic activity and then death. The main effect of alcohol intoxication on the ECG was the reduction of the cardiac frequency. The authors stated that pyridoxine pretreatment may possibly have a protective effect on alcohol intoxication, although the results were inconclusive. High doses of pyridoxine, 50 mg/kg i.v. given 5 minutes before the alcohol dosage, slowed the appearance of the adverse effects of alcohol and resulted in the total EEG recovery of 52% of the guinea pigs (10 guinea pigs in the group). This was compared to a 30% recovery in control animals. The pyridoxine treatment was not very effective in restoring normal EEG's in cats, but a slight, temporary recovery of the cardiac frequency was noted in cats given 50 to 300 mg/kg pyridoxine i.v. after the induction of alcohol intoxication. Pyridoxine treatment (75 mg/kg) was also effective in lessening the change in cardiac frequency after alcohol intoxication in a group of 5 rats injected with 30 ml/kg of 30% alcohol i.p. The results were compared to 5 untreated controls. The authors believed that the protective effects of pyridoxine in acute alcoholic intoxication may be due to its effect on cerebral metabolism (2562).

Pyridoxine has been found to antagonize the effects of levodopa, the drug used to treat the symptoms of parkinsonism in humans. Leon et al. administered an oral daily dose of 50 mg pyridoxine to 4 patients with parkinsonism, who were also receiving 3.5 to 6.0 g. levodopa daily. The pyridoxal phosphate form of pyridoxine is the coenzyme for the decarboxylation of dopa to dopamine, hence the attempt to use pyridoxine to increase the beneficial effects of levodopa. The present theory is that parkinsonism results from reduced levels of dopamine in the basal ganglia and substantia nigra of the brain. In this study, 3 of the 4 patients reverted to more severe stages of parkinsonism, indicating that pyridoxine interferes with the positive effects of levodopa. Laboratory tests showed that plasma dopa levels and the urinary excretion of dopa and dopamine were decreased by 60%. The fourth patient's condition did not

worsen and 2 of levodopa's side effects, nausea and involuntary movements, diminished. The authors feel that pyridoxine antagonizes levodopa by increasing its metabolism in the GI tract, so that less levodopa is available to the brain (1514).



Levodopa enters the brain and is then presumably decarboxylated to dopamine

(Dopamine is depleted in the basal ganglia and substantia nigra of patients with parkinsonism)

(Dopamine does not cross the blood brain barrier)

EFFECTS OF PYRIDOXINE:

Reduces amount of intact levodopa available to enter the brain

Does not interfere with absorption of levodopa

May accelerate peripheral decarboxylation trans-amination, and/or o-methylation of dopa

Accelerates metabolism of levodopa to dopac and HVA proximal to central circulation in the gastrointestinal tract during absorption and/or during initial passage through the liver via the portal circulation

Hypothesis of possible mechanisms by which pyridoxine interferes with effectiveness of levodopa in treatment of parkinsonism.

Pfeiffer and Ebadi published a paper on the antagonism of L-DOPA (Levodopa) the year after that of Leon et al. Male Sprague-Dawley rats, weighing 180 to 200 g, were given one of these 3 treatments: L-DOPA alone, 100 mg/kg/day i.p. for 4 days; pyridoxine, 100 mg/kg/day i.v. for up to 36 hours; both of the first 12 treatments including the L-DOPA for 4 days and then the pyridoxine. Another group of rats were pretreated with RO 4-4602 (an L-aromatic amino acid decarboxylase inhibitor) and L-DOPA and then some rats were given pyridoxine. Both treatment with L-DOPA alone and pyridoxine alone increased the level of dopamine in the basal ganglia of the rat brains. When both drugs were given, the L-DOPA-induced increase in dopamine was reversed (see Table 12) after an initial increase. When L-DOPA and RO 4-4602 were given together, the dopamine level increased to almost twice as much. Pyridoxine caused this level to decrease (see Table 13) after 6 hours. The authors suggested that pyridoxine antagonizes L-DOPA by the formation of a condensation product between L-DOPA and pyridoxal phosphate, which has not yet been identified (1994).

TABLE 12—THE EFFECTS OF L-DOPA AND OF PYRIDOXINE (GIVEN SINGLY OR CONCOMITANTLY) ON THE LEVEL OF DOPAMINE IN THE BASAL GANGLIA OF RATS

Experimental conditions		Time after drug treatment to decapitation (h)			
Groups	No. of rats	After L-DOPA	After pyridoxine	Dopamine μg/g of fresh brain tissue	*P* value
A. Control	19	—	—	6·66 ± 0·34	
B. L-DOPA—100 mg/kg i.p. per day for 4 days	16	96	—	8·61 ± 0·50	$P < 0·05$
C. Pyridoxine—100 mg/kg iv.	7	—	2	6·28 ± 0·55	
	8	—	6	9·74 ± 0·63	$P < 0·05$
	8	—	12	8·09 ± 0·47	$P < 0·05$
	8	—	24	7·48 ± 0·36	$P < 0·05$
	8	—	36	8·73 ± 0·55	$P < 0·05$
D. Pyridoxine—100 mg/kg iv. given to rats which had received a 100 mg/kg dose of L-DOPA/day for 4 days	6	96	1	9·66 ± 0·97	
	7	96	2	7·74 ± 1·06	$P < 0·05$
	6	96	24	6·00 ± 0·55	$P < 0·05$

The procedure for determination of dopamine has been described in the section on materials and methods. Values represent means ±S.E.M. for the number of animals indicated. Abbreviations: i.p. = intraperitoneally; i.v. = intravenously.

TABLE 13—THE EFFECT OF L-DOPA (75 mg/kg i.p.) GIVEN ALONE; L-DOPA (75 mg/kg i.p.) AND Ro 4-4602 (50 mg/kg i.p.); OR L-DOPA (75 mg/kg i.p.), Ro 4-4602 (50 mg/kg i.p.) AND PYRIDOXINE (250 mg/kg i.v.) GIVEN CONCOMITANTLY ON THE LEVEL OF DOPAMINE IN THE BASAL GANGLIA OF RATS

Experimental conditions	No. of rats	Time after drug treatment to decapitation (h)	Dopamine μg/g wet wt. of brain tissue	*P* value
Control	19	—	6·66 ± 0·34	
L-DOPA	5	1·5	8·67 ± 0·50	$P < 0·05$
L-DOPA and Ro 4-4602	5	1·5	15·66 ± 1·61	$P < 0·05$
L-DOPA, Ro 4-4602 and pyridoxine	5	1·5	16·97 ± 2·22	$P < 0·05$
L-DOPA and Ro 4-4602	5	6	10·62 ± 0·74	$P < 0·05$
L-DOPA, Ro 4-4602 and pyridoxine	5	6	8·79 ± 0·87	

The schedule for drug administration and the procedure for determination of dopamine have been described in the section on Materials and Methods. Values represent means ±S.E.M. for the number of animals indicated. Abbreviations: i.p. = intraperitoneally; i.v. = intravenously.

NEURO 19/9—L

Chertkova and Udilova studied the beneficial interaction between isoniazid (INH) and pyridoxine in white mice. Pyridoxine has been used clinically to eliminate the toxic side effects of INH, which is used in the chemotherapy of tuberculosis in humans. Doses of 3, 5, and 10 mg of pyridoxine prevented the death of mice given 3 mg INH, a lethal dose. The protective effect of pyridoxine was observed when the 2 drugs were given simultaneously either per os or s.c. The authors then tested the drug combination in mice experimentally infected with tuberculosis mycobacteria. The best therapeutic results were observed when 18 mice were given 0.6 mg INH and 0.2 mg pyridoxine daily for 1 month, beginning 24 hours after the mice were infected. The drug combination decreased the degree of necrotic changes in the liver, spleen, and lungs and inhibited the tuberculotic process (464).

Goldman and Braman discussed the use of pyridoxine for treating the adverse effects of isoniazid (INH), used in tuberculosis therapy. Pyridoxine, in doses of 10 mg per 100 mg INH, is effective in preventing peripheral neuropathy in patients receiving high doses of INH and in alcohol or malnourished patients (the most susceptible groups). Peripheral neuropathy begins as numbness in the feet and eventually spreads to the knees, hands, and fingers. Other symptoms include muscle aching, weakness, abnormal achilles and patellar reflexes, muscle atrophy and disturbances in pain, touch, and temperature senses. The syndrome appears within 3 to 5 weeks with high INH dosage and in 6 months with the normal low dosage of 3 mg/kg (a frequency of about 2% in normal patients). Pyridoxine has also been used to treat INH-induced optic neuritis, but no success was observed in treating psychosis, ataxia, dizziness, or encephalopathy. The authors stated that the interactions between pyridoxine and INH are not fully understood (907).

McCoy and Colombini administered red oil of marijuana in a single dose of 500 mcg/kg i.p. or 300 mcg/kg i.p. daily for 8 days to Swiss-Webster mice. The dosage contained 20% delta-9-tetrahydrocannabinol. The mice were then injected with C^{14}-pyridoxine, and thirty minutes later, the mice were sacrificed. Chronic marijuana administration increased the C^{14} retention in the brain and altered the distribution of C^{14} in the forms of pyridoxine when compared to the control animals. The pyridoxal phosphate and pyridoxal radioactivity decreased and the pyridoxine phosphate and pyridoxamine phosphate activity increased in the marijuana-treated mouse brains. The authors concluded that chronic administration of marijuana inhibited the oxidation of pyridoxine by pyridoxine oxidase in the brain (1678).

VI. Consumer Exposure

Pyridoxine is used as a nutritive agent in foods and also occurs naturally. Consumer usage levels, annual poundage, and intake levels are included in NAS/NRC tables 14, 15, 16, 17, and 18 at the end of this section (1846). Also included from a textbook on vitamins (2294) are Tables 19 through 25 on the content of vitamin B_6 in various foods. the last 2 tables tabulate the pyridoxine requirements of domestic and laboratory animals and of humans for NAS/NRC data.

Gyorgy (1971) advocates that the vitamin B_6 requirements for adult humans be increased from 2.5 mg/day to 25 mg/day. This statement may have been based on the higher requirements for B_6 with increasing age and in the presence of arteriosclerosis and other geriatric diseases (978).

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TABLE 14 -- USAGE LEVELS REPORTED FOR NAS APPENDIX A SUBSTANCES (GROUP I) USED IN REGULAR FOODS(R)

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO.	NAME	# FIRMS REPORTING	*** USUAL USE *** WTD MEAN, %	*** MAXIMUM USE *** WTD MEAN, %
PYRIDOXINE HYDROCHLORIDE NAS 0167	01	BAKED GOODS(R)	4	.00099	.00155
	02	BREAK CERLS(R)	8	.00286	.00482
	05	MILK PRODS(R)	5	.00277	.00360
	10	MEAT PRODS(R)	4	.00082	.00146
	22	SNACK FOODS(R)	4	.00029	.00029
	23	BEV TYPE I(R)	4	.00000	.00000
	26	RECONST VEG(R)	4	.00040	.00040
	28	IMIT DAIRY(R)	4	.00200	.00200

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TABLE 15 -- USAGE LEVELS REPORTED FOR NAS APPENDIX A SUBSTANCES (GROUP II) USED IN INFANT FORMULA PRODUCTS & BABY FOODS(B)

NAS SURVEY NO.	SUBSTANCE NAME	FOOD CATEGORY NO.	NAME	# FIRMS REPORTING	*** USUAL USE *** WTD MEAN, %	*** MAXIMUM USE *** WTD MEAN, %
0167	PYRIDOXINE HYDROCHLORIDE	81	BAKED GOODS(B)	4	.00500	.00580
		82	CEREALS(B)	4	.00018	.00055
		83	FORMULAS(B)	7	.00007	.00479

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TABLE 16, PART A -- ANNUAL POUNDAGE DATA FOR NAS APPENDIX A SUBSTANCES (GROUPS I & II)

SUBSTANCE NAME (SURVEY NO.)	# REPORTS TO NAS 1960/1970	POUNDAGE REPORTED TO NAS (MATCHING REPORTS FOR BOTH YEARS) 1960	1970	TOTAL 1970 POUNDAGE REPORTED TO NAS	# REPORTS TO FEMA	POUNDAGE REPORTED TO FEMA-- 1970 ONLY	TOTAL 1970 POUNDAGE NAS + FEMA
PYRIDOXINE HYDROCHLORIDE NAS 0167	19/ 20	2,400	16,834	17,314			17,314

TABLE 17, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBIT SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO.	NAME	NO OF FIRMS	(AGE)	POSSIBLE DAILY INTAKE, MG. AVERAGE	HIGH A	HIGH B
PYRIDOXINE HYDROCHLORIDE NAS 0167	01	BAKED GOODS(R)	4	0-5 MO.	.033660	.044550	.052700
				6-11 MO.	.251460	.512820	.393700
				12-23 MO.	.539550	.889020	.844750
				2-65+ YR.	1.358280	2.017620	2.126600
PYRIDOXINE HYDROCHLORIDE NAS 0167	02	BREAK CERLS(R)	8	0-5 MO.	.017160	.048620	.028920
				6-11 MO.	.537780	1.710280	1.074860
				12-23 MO.	.746460	1.455740	1.258020
				2-65+ YR.	.572000	1.481480	.964000
PYRIDOXINE HYDROCHLORIDE NAS 0167	05	MILK PRODS(R)	5	0-5 MO.	.149580	.110800	.194400
				6-11 MO.	1.728480	8.312770	2.246400
				12-23 MO.	1.509650	4.830880	1.962000
				2-65+ YR.	1.094150	3.340620	1.422000
PYRIDOXINE HYDROCHLORIDE NAS 0167	10	MEAT PRODS(R)	*	0-5 MO.	.009020	.023780	.016060
				6-11 MO.	.169740	.457560	.302220
				12-23 MO.	.247640	.425580	.440920
				2-65+ YR.	.642880	1.066820	1.144640
PYRIDOXINE HYDROCHLORIDE NAS 0167	22	SNACK FOODS(R)	*	0-5 MO.	**********	.000290	**********
				6-11 MO.	.001160	.003190	.001160
				12-23 MO.	.003190	.008990	.003190
				2-65+ YR.	.003770	.010770	.003770
PYRIDOXINE HYDROCHLORIDE NAS 0167	23	BEV TYPE II(R)	*	0-5 MO.	.000000	.000000	.000000
				6-11 MO.	.000000	.000000	.000000
				12-23 MO.	.000000	.000000	.000000
				2-65+ YR.	.000000	.000000	.000000

TABLE 17, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBIT SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO.	NAME	NO OF FIRMS	(AGE)	POSSIBLE DAILY INTAKE, MG. AVERAGE	HIGH A	HIGH B
PYRIDOXINE HYDROCHLORIDE NAS 0167	26	RECONST VEG(R)	*	0-5 MO.	.000000	.000000	.000000
				6-11 MO.	.000000	.000000	.000000
				12-23 MO.	.000000	.000000	.000000
				2-65+ YR.	.000800	.002400	.000800
PYRIDOXINE HYDROCHLORIDE NAS 0167	28	IMIT DAIRY(R)	*	0-5 MO.	.000000	.000000	.000000
				6-11 MO.	.028000	.046000	.028000
				12-23 MO.	.016000	.068000	.016000
				2-65+ YR.	.018000	.030000	.018000
PYRIDOXINE HYDROCHLORIDE NAS 0167	81	BAKED GOODS(B)	*	0-5 MO.	.005000	.015000	.005800
				6-11 MO.	.015000	.015000	.017400
				12-23 MO.	**********	**********	**********
PYRIDOXINE HYDROCHLORIDE NAS 0167	82	CEREALS(B)	*	0-5 MO.	.024300	.041940	.074250
				6-11 MO.	.016560	.043380	.050600
				12-23 MO.	.003600	.016200	.011000
PYRIDOXINE HYDROCHLORIDE NAS 0167	83	FORMULAS(B)	7	0-5 MO.	.234990	.430500	16.080030
				6-11 MO.	.047880	.228130	3.276360
				12-23 MO.	.015400	.004340	1.053800
PYRIDOXINE HYDROCHLORIDE NAS 0167	99	ALL CATEGORIES	22	0-5 MO.	.473710	.715480	16.452160
				6-11 MO.	2.896050	11.329130	7.390700
				12-23 MO.	3.081490	7.638750	5.589680
				2-65+ YR.	3.689880	7.949670	5.679810

TABLE 18, PART A -- POTENTIAL DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUP I & II), PER FOOD CATEGORY, BASED ON FOOD CONSUMPTION BY EATERS ONLY - - - SEE EXPLANATORY NOTES IN EXHIBITS SECTION

FOOD CATEGORY	SUBSTANCE NAME / NO. NAME / AGE)	NO. OF (SURVEY NO.) AVERAGE	********** POTENTIAL DAILY INTAKE, MG., PER CATEGORY ********** FIRMS (VERY HIGH HIGH A		HIGH B		
33 FORMULAS(B)	PYRIDOXINE HYDROCHLORIDE NAS 0167	7	.282030	0-5 MO.	.456960	19.298910	31.269120
			.204470	6-11 MO.	.426790	13.991590	29.204630
			.251090	12-23 MO.	.520590	17.181730	35.623230

TABLE 19

VITAMIN B_6 CONTENT IN MEAT

Meat	mg/100 gm fresh meat
Beef	
Standing rib roast	0.32
Standing rib roast (cooked)	0.28
Standing rib roast	0.59[a]
Standing rib roast (cooked)	0.40[a]
Boston cut	0.38
Boston cut (cooked)	0.25
Boston cut	0.54[a]
Boston cut (cooked)	0.46[a]
Lean	0.5
Beef	0.15–0.33 (c)[b]
Tongue	0.13
Heart	0.29
Liver	0.71
	1
	3.8
	1.42[a]
	0.74
	0.45
	0.38 (c)
Brain	0.13 (c)
Veal	
Leg	0.33
Leg (cooked)	0.29
Leg	0.57[a]
Leg (cooked)	0.44[a]
Leg	0.37
Leg (roasted)	0.20
Shoulder	0.30
Shoulder (roasted)	0.14
Chops (sirloin)	0.41
Chops (braised)	0.11
	0.12
Chops (shoulder)	0.35
Stew meat	0.33
Stew meat (cooked)	0.10
Liver	0.9
	0.55
Pork	
Lean	0.4
	0.20–0.35 (c)
Liver	1.11[a]
	0.51
	0.35 (c)

TABLE 19—*Continued*

Meat	mg/100 gm fresh meat
Wholly fatty tissue	0.05
Kidney	0.30–0.32 (c)
Lamb	0.2
Leg	0.29
Leg (roasted)	0.12
Sirloin chop	0.22
Sirloin chop (broiled)	0.11
Leg	0.25
Leg (cooked)	0.16
Leg	0.52[a]
Leg (cooked)	0.43[a]
Stew meat	0.23
Stew meat (cooked)	0.06
Ham	0.33
Smoked	0.4
Cured	0.19
Frankfurters	0.15
	0.13
	0.15
	0.31[a]
Pork sausage	0.2
Liver sausage	0.45[a]
	0.23
Liver paste	0.2

[a] Rat growth assay.

[b] (c) = chemical assay method.

TABLE 20

VITAMIN B_6 CONTENT IN FISH

Fish	mg/100 gm fresh meat
Cod	0.2
	0.34[a]
	0.51 (c)[b]
Eel	0.28
Eel (smoked)	0.15
Flounder	0.25
Herring	0.45
Herring (pickled fillets)	0.15
Herring (salted)	0.22
Herring (smoked)	0.35
Mackerel	0.7
Mackerel (Atlantic)	0.21
Mackerel (Pacific)	0.27
Mackerel (smoked)	0.5
Sardine (Atlantic)	0.16
Sardine (Pacific)	0.28
Salmon	0.59[a]
Salmon (canned, wet solids)	0.45
Tuna (canned, wet solids)	0.44

[a] Rat growth assay.
[b] (c) = chemical assay method.

TABLE 21

VITAMIN B_6 CONTENT IN EGGS

Egg (chicken)	mg/100 gm fresh material
Whole	0.12
Yolk	0.30
	0.66–0.67 (c)[a]
White	0.02
	0.11 (c)
Whole	0.68
Yolk	1.5

[a] (c) = chemical assay method.

TABLE 22

LOSS OF VITAMIN B_6 BY COOKING MEAT[a]

Meat	Loss (%)
Veal	
By roasting	34
By braising	19
By stewing	18
Lamb	
By roasting	29
By braising	34
By stewing	16
Ham	
By curing	57

[a] J. M. McIntire, B. S. Schweigert, and C. A. Elvehjem, *J. Nutr.* 28, 219 (1944).

TABLE

VITAMIN B_6 IN FRESH MILK

Milk	Vitamin B_6 content	Method
Fresh	0.41 and 0.37 mg/100 gm	SC[a]
	0.1–0.2 mg/100 ml	NS[b]
	0.023 mg/100 ml	NS
	0.041 mg/100 ml	NS
	0.033 mg/100 ml	SC
	0.035 mg/100 ml	NS
	0.050 mg/100 gm	SC
	0.035 mg/100 gm	SC
	0.18–0.23 mg/100 gm	(c)[c]
Shop-bought (average of January–December)	0.036–0.046 mg/100 ml	SC
Average of 40 cows		
1st month of lactation	0.081 mg/100 ml	SC
5th–11th month of lactation	0.035 mg/100 ml	SC
Sample taken under aseptic conditions	0.147 mg/100 ml	NS

[a] *Saccharomyces carlsbergensis.*
[b] *Neurospora sitophila.*
[c] (c) = chemical assay method.

TABLE 23

VITAMIN B_6 IN CHEESE

Cheese	mg/100 gm
Gruyère	0.08
Rind	0.28
Cake	0.08
Swiss	0.09
	0.28 (c)[a]
Emmental	0.3–0.8
Edam	0.08
20% fat	0.08
45% fat	0.09
Camembert	0.25
	0.25
Rind	0.40
Cake	0.20
Brie	
Rind	0.47
Cake	0.15
Roquefort	0.10
Rind	0.19
Cake	0.10
Gorgonzola	
Rind	0.36
Cake	0.11
Processed cheese	0.048
Average of 21 varieties	0.094

[a] (c) = chemical assay method.

TABLE 24

VITAMIN B_6 IN CEREALS, CEREAL PRODUCTS, AND POTATOES

Food	mg/100 gm	Method
Wheat	0.34	SC[a]
	1.46	SC
Wheat (whole meal)	0.40	SC
	0.32	SC
	0.28	*b*
	0.5	*b*
Wheat meal		
85%	0.42	*b*
73%	0.25	*b*
80%	0.12	SC
70%	0.07	SC
	0.085	SC
Barley meal	0.3	SC
Buckwheat groats	0.4	SC
Corn flour	0.05	SC
Rice	0.15	SC
Bread (white)	0.09	SC
Rolls	0.09	SC
Bread (graham)	0.2	SC
Crispbread	0.3	SC
Bread (rye, brown)	0.22	SC
Potatoes	0.14	SC
	0.22–0.45	(c)[c]
Sweet (frozen)	0.125	*b*
Sweet [heat processed (canned)]	0.053	*b*

[a] *Saccharomyces carlsbergensis.*
[b] Rat growth assay.
[c] (c) = chemical assay method.

TABLE 2.5

VITAMIN B_6 CONTENT IN VEGETABLES AND FRUITS

Vegetable or fruit	mg/100 gm	Method
Peas		
Split	0.075	SC[a]
Green	0.16	SC
Green (sweet)	0.15	SC
Green (sweet, frozen)	0.14	SC
Beans		
Green (French)	0.1	SC
Green (frozen)	0.090	b
Green (French style, frozen)	0.081	SC
Lima beans		
Fordhook	0.17	SC
Baby	0.11	SC
Frozen	0.18	b
Heat processed (canned)	0.078	b
Baby green fresh	0.17	SC
Same batch frozen	0.14	SC
Fresh	0.15	SC
Same batch frozen	0.13	SC
Fresh	0.14	SC
Same batch cooked	0.12	SC
Cabbage		
White	0.11	SC
Frozen	0.12	b
Heat processed (canned)	0.11	b
Brussels sprouts	0.28	SC
	0.16	SC
Cauliflower	0.2	SC
	0.18	SC
Carrots (fresh)	0.94	SC
Spinach	0.22	SC
Leaf	0.18	SC
Tomato	0.10	SC
Apple (fresh)	0.045	SC
	0.042	SC
Cherry	0.045	SC
Peach	0.03	SC
	0.02	SC
Strawberry	0.054	SC
	0.06	SC
	0.061	SC
Sliced frozen	0.048	SC
Banana	0.5	SC
	0.4–0.6	(c)[c]
Orange	0.05	SC
California	0.089	SC
Juice (frozen, reconstituted)	0.026	SC
Grapefruit (Florida)	0.030	SC
Avocado	0.24	SC
California (3 varieties)	0.39	SC
	0.61	SC
	0.62	SC

[a] *Saccharomyces carlsbergensis.*
[b] Rat growth assay.
[c] (c) = chemical assay method.

TABLE 26 ANIMAL REQUIREMENTS (per kilo ration) (NAS-NRC)

Animal	Purpose	Py (mg)
Cat (1962)	Growing	2
Dog (1962, revised)	Growing	1
Fox[e] (1968)	Growing	2
Guinea pig (1962)	Growing	4
Hamster (1962)	Growing	6
Mink[f] (1968)	Growing	1.1
Monkey	Growing	1.2
Mouse (1962)	Growing	1
	Pregnancy and lactation	[h]
Rat (1962)	Growing	1.2
	Gestation	0.6
	Lactation	0.6
Poultry		
Chicken (1966)	Growing 0–8 weeks	3
	Growing 8–16 weeks	[h]
	Laying	[h]
	Breeding	4.5
Duck (1966)	Growing	2.6
Pheasant (1966)	Growing	[h]
Quail (1966)	Growing	[h]
Turkey 1966)	Growing 0–8 weeks	3
	Growing 8–16 weeks	[h]
	Breeding	[h]
Swine (1968)	Growing 5–10[d]	1.5
	Growing 10–20	1.5
	Growing 20–35	1.1
	Growing 35–60	[h]
	Growing 60–100	[h]
	Breeding 110–160	[h]
	Lactating 140–200	[h]
	Boars 110–150	[h]

Compiled by J. C. Bauernfeind and E. DeRitter.

[a] AA, Ascorbic acid; Bi, Biotin; Ch, Choline; Cy, Cyanocobalamin; FA, Folic acid; Ni, Niacin; Pa, Pantothenic acid; Py, Pyridoxine; Ri, Riboflavin; Th, Thiamine.

[b] Required but specific needs not established. AA only a dietary essential for monkey and guinea pig.

[c] NAS-NRC publications also exist for the vitamin requirements of large animals.

[d] Kilograms

[e] Not a dietary essential.

[f] From 7–31 weeks of age, male body wt. goes from 690 to 2,070 g with consumption of 27 to 75 g of dry food daily; female body wt. goes from 560 to 1,110 g with consumption of 32 to 60 g of dry food daily.

[g] From 7–31 weeks of age, male body wt. goes from 1.4 to 5.7 kg with consumption of 59 to 160 g of dry food daily; female body wt. goes from 1.3 to 4.7 kg with consumption of 36 to 118 g of dry food daily.

[h] Growing.

FOOD AND NUTRITION BOARD, NATIONAL ACADEMY OF SCIENCES–NATIONAL RESEARCH COUNCIL
RECOMMENDED DAILY DIETARY ALLOWANCES,[a] REVISED 1968

Designed for the maintenance of good nutrition of practically all healthy people in the U.S.A.

	AGE[b] (years)		WEIGHT		HEIGHT			PROTEIN[d]	VITAMIN B_6
	From	Up to	(kg)	(lbs)	cm	(in.)	kcal	(gm)	(mg)
Infants	0	1/6	4	9	55	22	kg × 120	kg × 2.2[e]	0.2
	1/6	1/2	7	15	63	25	kg × 110	kg × 2.0[e]	0.3
	1/2	1	9	20	72	28	kg × 100	kg × 1.8[e]	0.4
Children	1	2	12	26	81	32	1,100	25	0.5
	2	3	14	31	91	36	1,250	25	0.6
	3	4	16	35	100	39	1,400	30	0.7
	4	6	19	42	110	43	1,600	30	0.9
	6	8	23	51	121	48	2,000	35	1.0
	8	10	28	62	131	52	2,200	40	1.2
Males	10	12	35	77	140	55	2,500	45	1.4
	12	14	43	95	151	59	2,700	50	1.6
	14	18	59	130	170	67	3,000	60	1.8
	18	22	67	147	175	69	2,800	60	2.0
	22	35	70	154	175	69	2,800	65	2.0
	35	55	70	154	173	68	2,600	65	2.0
	55	75+	70	154	171	67	2,400	65	2.0
Females	10	12	35	77	142	56	2,250	50	1.4
	12	14	44	97	151	61	2,300	50	1.6
	14	16	52	114	157	62	2,100	55	1.8
	16	18	54	119	160	63	2,300	55	2.0
	18	22	58	128	163	64	2,000	55	2.0
	22	35	58	128	163	64	2,000	55	2.0
	35	55	58	128	160	63	1,850	55	2.0
	55	75+	58	128	157	62	1,700	55	2.0
Pregnancy							+200	65	2.5
Lactation							+1,000	75	2.5

[a] The allowance levels are intended to cover individual variations among most normal persons as they live in the United States under usual environmental stresses. The recommended allowances can be attained with a variety of common foods, providing other nutrients for which human requirements have been less well defined. See text for more-detailed discussion of allowances and of nutrients not tabulated.

[b] Entries on lines for age range 22–35 years represent the reference man and woman at age 22. All other entries represent allowances for the midpoint of the specified age range.

[c] The folacin allowances refer to dietary sources as determined by *Lactobacillus casei* assay. Pure forms of folacin may be effective in doses less than ¼ of the RDA.

[d] Niacin equivalents include dietary sources of the vitamin itself plus 1 mg equivalent for each 60 mg of dietary tryptophan.

[e] Assumes protein equivalent to human milk. For proteins not 100 percent utilized factors should be increased proportionately.

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EFFECT OF PYRIDOXINE HYDROCHLORIDE (VITAMIN B_6) UPON DEPRESSION ASSOCIATED WITH ORAL CONTRACEPTION

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Summary The known association between combined œstrogen-progestagen oral contraceptive (O.C.) administration and abnormalities of tryptophan and vitamin-B_6 metabolism has been investigated in a group of 22 depressed women whose symptoms were judged to be due to the effects of O.C. 11 of these women showed biochemical evidence of an absolute deficiency of vitamin B_6. In a double-blind crossover trial this group of women responded clinically to the administration of pyridoxine hydrochloride. The remaining 11 women showed no such response. Placebo administration was without effect. Possible mechanisms for depression due to O.C. use and its treatment with pyridoxine hydrochloride are discussed.

Introduction

DEPRESSION is a problem in women taking œstrogen-progestagen oral contraceptives (O.C.) [1-3] and it is a common cause of women stopping the pill.[4] The frequency of this depression is difficult to ascertain, but a figure as high as 6·6% has been reported in one British study.[5] The symptoms of pill-induced depression differ from those found in endogenous and reactive depression; pessimism, dissatisfaction, crying, and tension being predominant, whereas sleep disturbance and appetite disorders are not common.[5]

There is evidence that brain-amine metabolism may be disturbed in affective disorders, and many attempts have been made to link various changes in the numerous substances involved with depression. Thus defective synthesis of brain 5-hydroxytryptamine (5-H.T., serotonin) has been implicated [5,6]; low levels of the 5-H.T. metabolite 5-hydroxyindoleacetic acid (5-H.I.A.A.) have been found in the cerebrospinal fluid of depressed patients,[7] there is some direct evidence of low 5-H.T.[8] and 5-H.I.A.A.[9] in the hindbrains of depressive suicides, and the administration of the 5-H.T. precursor L-tryptophan may be effective in the treatment of depression.[10]

5-H.T. is synthesised from L-tryptophan, which, in addition to its involvement in protein synthesis, is also metabolised via the nicotinic-acid ribonucleotide pathway. The steps involved in the 5-H.T. and nicotinic-acid ribonucleotide pathways are summarised in fig. 1. Several enzymatic reactions require pyridoxal-5-phosphate, the coenzyme derived from the various forms of vitamin B_6, as a cofactor. Administration of œstrogen-containing O.C., or œstrogen alone, increases metabolism of tryptophan via the nicotinic-acid ribonucleotide pathway.[11] This is due to induction of the rate-limiting enzyme, hepatic tryptophan oxygenase, by the œstrogen, an effect mediated by way of the adrenal glands.[12] Œstrogen increases cortisol activity in the liver,[13] and hydrocortisone administration greatly increases hepatic tryptophan oxygenase levels.[12,14] Because of the enhanced metabolism of tryptophan via the nicotinic-acid ribonucleotide pathway, spontaneous urinary excretion of 3-hydroxyanthranilic acid (H.A.) is increased,[15] and after an oral load of tryptophan much increased urinary excretion of the tryptophan metabolites kynurenine (K), 3-hydroxykynurenine (HK), 3-hydroxyanthranillic acid (HA), and xanthurenic acid (XA) is found.[11,16,17] The amount of kynurenine excreted in the urine correlates with the level of tryptophan oxygenase in the liver.[14] Because of the increased metabolism of tryptophan down the nicotinic-acid ribonucleotide pathway the requirements for pyridoxal phosphate are increased. The altered excretion of tryptophan metabolites observed in women on O.C. is similar to that found in nutritional vitamin-B_6 deficiency,[18] and is corrected by the administration of vitamin B_6.[11,16,17] Vitamin-B_6 requirements may be further increased by the effect of œstrogen conjugates competitively inhibiting pyridoxal phosphate.[19]

We have shown that about 80% of women taking O.C. have abnormal tryptophan metabolism indicating a relative vitamin-B_6 deficiency, and that about 20% have evidence of absolute deficiency of this vitamin.[20] This was demonstrated by low urinary excretion of 4-pyridoxic acid, which is the major excretory product of vitamin B_6,[21] increased urinary HK/HA ratios,[22] and decreased activity of pyridoxal-phosphate-dependent erythrocyte aspartate and alanine aminotransferase enzymes.[23]

There are three ways in which the changes in tryptophan metabolism may lead to reduced formation of 5-H.T. in the brain and, therefore, depression. First, increased metabolism of tryptophan along the nicotinic-acid ribonucleotide pathway may result in an

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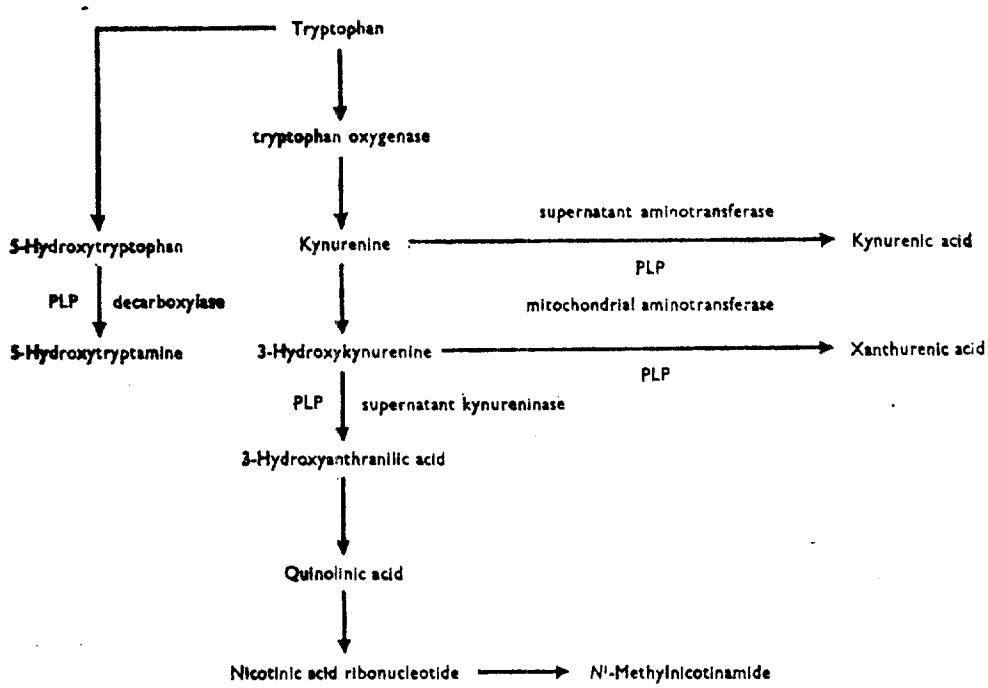

Fig. 1—Major pathways of tryptophan metabolism.
PLP indicates the known pyridoxal-phosphate-dependent enzyme reactions.

inadequate supply of precursor for 5-H.T. synthesis. In this context it is of interest that cerebrospinal fluid-levels of tryptophan are low in depressed patients,[24] and, although concentrations of total plasma tryptophan are unaltered, concentrations of free plasma-tryptophan, which alone can cross the blood/brain barrier, are reduced.[25] Second, there is experimental evidence that the metabolites of tryptophan, K and 3-HK, inhibit the transport of tryptophan across the blood/brain barrier.[26] This mechanism could apply to women on O.C. because of increased formation of these metabolites. Third, increased requirements of vitamin B_6 in women on O.C. due to increased pyridoxal-phosphate-dependent enzyme activity may result in inadequate amounts of this coenzyme for normal 5-hydroxytryptophan decarboxylase activity in the brain. Though decarboxylation is not the rate-limiting step in 5-H.T. synthesis in rats, there is some evidence that it may be in man.[27]

Winston suggested that vitamin-B_6 administration might relieve depression due to O.C. by overcoming deficiency of this vitamin.[28] Subsequently, Baumblatt and Winston [29] reported that administration of pyridoxine to 58 women on O.C. complaining of premenstrual depression relieved the symptoms in 18 and caused an improvement in a further 26. There have, however, been no double-blind placebo controlled crossover trials of the effect of treating depression due to O.C. with pyridoxine and in which the vitamin-B_6 status of the women has been evaluated.

Pyridoxine administration can correct two of the possible mechanisms whereby abnormal tryptophan metabolism may cause depression during O.C. administration. Thus it could prevent the accumulation of tryptophan metabolites which inhibit tryptophan transport into the brain, and it could restore normal activity of 5-hydroxytryptophan decarboxylase. We have done a double-blind crossover study to determine the effect of pyridoxine upon depression due to O.C., and to assess whether absolute deficiency of vitamin B_6 was present in these women, and whether any clinical improvement that might result from the administration of pyridoxine reflected the woman's initial vitamin-B_6 status.

Patients and Methods

Patients

All women taking O.C. attending the Margaret Pyke Centre, London, were asked to complete a Beck self-rating depression questionary [30] which has been validated in England by Metcalfe and Goldman.[31] Those women with a score of 23 or above, indicating moderate to severe depression, were referred to the metabolic unit. There they were interviewed by P. W. A., and those women with a previous history of psychiatric illness, personality disorder, symptoms of a reactive or endogenous depression, or with obvious factors contributing to their depression such as marital disharmony, financial worries, or job dissatisfaction, were excluded from the trial. 32 women judged to have depression due to O.C. were admitted to the trial after informed consent had been obtained with their general practitioners' agreement. 22 women completed the trial, the remaining 10 not completing the study because they moved from the area or failed to reattend for follow-up or because new serious personal or marital problems appeared or because of continuing severe depression during the early stages of the trial necessitating active therapy. These women have been excluded from the report. The clinical details of the women studied and the nature and duration of the O.C. therapy are shown in table I.

Design of Trial

This was carried out on an outpatient basis. On

TABLE I—CLINICAL DETAILS OF THE WOMEN

n	Age (yr.)	Marital status	o.c. preparation	Total duration of o.c. use (mo.)
	22	M	Minilyn	42 (*12*)
	21	S	Minilyn	8
	21	S	Minovlar	12
	22	S	Norlestrin	12 (*4*)
	24	M	Minilyn	7
	25	M	Gynovlar	10
	25	S	Norlestrin	12
	22	S	Orthonovin 1/50	11 (*8*)
	22	S	Norlestrin	13 (*4*)
	23	S	Volidan	34
	25	S	Minovlar	24
	20	M	Minovlar	42 (*10*)
	25	M	Norlestrin	14 (*2*)
	26	M	Norinyl-1	36
	26	M	Minovlar	5
	25	S	Minilyn	22
	25	M	Minovlar	18 (*10*)
	28	M	Minilyn	5
	25	M	Minovlar	60 (*24*)
0	29	M	Norinyl-1	17 (*6*)
1	25	M	Minovlar	28
2	25	M	Gynovlar	60 (*10*)
.	..	..	..	22 (*mean*)

men with absolute vitamin-B_6 deficiency.
res in parentheses relate to the duration of the currently used o.c.
tituents of individual o.c., 'Minilyn' (ethinylœstradiol 50 µg., lynœstrenol 2·5 mg.): 'Minovlar' (ethinylœstradiol 50 µg., norethisterone acetate 1 mg.): 'Norlestrin' (ethinylœstradiol 50 µg., norethisterone acetate 2·5 mg.): 'Gynovlar' (ethinylœstradiol 50 µg., norethisterone acetate 3 mg.): 'Orthonovin' (mestranol 50 µg., norethisterone 1 mg.); 'Volidan' (ethinylœstradiol 50 µg., megestrol acetate 4 mg.); 'Norinyl-1' (mestranol 50 µg., norethisterone 1 mg.).

nission to the study vitamin-B_6 status and tryptophan tabolism were assessed (see below). A 24-hour urine lection was obtained for 4-pyridoxic acid measurement. the following morning the women came fasting to the it's day ward, where a sample of blood was taken for asurement of erythrocyte aminotransferase activities. ter this they were given 2 g. of L-tryptophan suspended milk, and all the urine passed for 24 hours was collected. n completion of these investigations they were given ther pyridoxine hydrochloride 20 mg. twice a day or a lacebo for two months. The nature of the tablets supplied each woman was known only to Hoffmann-La Roche Basle). A two-month treatment schedule was used to avoid any possible early placebo effects. Every fortnight each woman completed and returned a Beck scale sent to her by post.

At the end of the two months the biochemical investigations were repeated and then the alternative tablets were given for a further two months, the Beck scales being completed and returned fortnightly. At the end of this second two months' treatment the biochemical investigations were repeated. Throughout the study the women were given every opportunity to contact the clinical members of the staff for advice.

Although the biochemical measurements indicated during each woman's study the sequence in which she received pyridoxine and placebo treatment, this information was withheld from the staff involved in the clinical management of the trial.

Laboratory Methods

Urine samples were stored at −15°C until analyses were carried out. K, HK, and HA were determined by the ion-exchange column procedures of Brown and Price,[32] as modified by Heeley[34]; XA was determined by thin-layer chromatography[33] and 4-pyridoxic acid (4-P) by ion-exchange chromatography.[31] Erythrocyte alanine aminotransferase (Al-AT) and aspartate aminotransferase (Asp-AT) activities were assayed by the method of Woodring and Storvick,[34] with and without the addition of 100 µg. of pyridoxal phosphate to the 3 ml. assay medium. The assay without added coenzyme is a measure of basal activity of the enzyme in vivo. The assay with added coenzyme enabled calculation of the percentage stimulation of the enzyme in vitro.

Analysis of Results

The mean levels of K, HK, HA, HK/HA ratio, 4-P, Al-AT, and Asp-AT activities and percentage stimulation in vitro in the depressed women were compared with the results in 31 non-depressed women on o.c., and in a control group of women not on o.c. Absolute vitamin-B_6 deficiency in the depressed women, as opposed to functional insufficiency of this vitamin, was considered to be present if they fulfilled two or more of the following criteria derived from data from the control group of women not taking o.c.: HK/HA ratio greater than 2 S.D. above the mean ratio of the control group (>2·25); urinary 4-P 2 S.D. below the mean level of the control group (<2·5 µg. per 24 hours); and Al-AT and Asp-AT activities 2 S.D. below the mean

ABLE II—ENTRY URINARY TRYPTOPHAN METABOLITES (µmole/24 hr.), URINARY 4-PYRIDOXIC ACID (µmole/24 hr.), AND ERYTHROCYTE AMINOTRANSFERASE ACTIVITIES (µg. PYRUVATE OR OXALOACETATE/hr./mg. Hb) AND % STIMULATION IN VITRO.

Woman	XA	K	HK	HA	HK/HA	4-P	AL-AT activity	AL-AT % stim.	Asp-AT activity	Asp-AT % stim.
* 1	110	29	18	13	1·39	3·28	*0·43*	*60·0*	21·9	*101·0*
* 2	154	115	113	40	*2·82*	3·28	*0·69*	14·0	20·8	78·0
* 3	902	456	666	241	*2·77*	*1·21*	*0·43*	*57·0*	*16·0*	94·0
* 4	562	106	198	52	*3·81*	4·63	*0·40*	*90·0*	*14·1*	*114·0*
* 5	269	110	112	49	*2·30*	3·30	*0·45*	*74·0*	*19·8*	87·0
* 6	144	60	113	26	*4·50*	3·10	*0·74*	*46·0*	22·8	81·0
7	547	45	38	20	1·91	*1·95*	0·92	15·0	22·9	79·0
* 8	226	82	99	35	*2·8*	*2·2*	0·78	33·0	23·2	36·0
* 9	..	14	22	9	1·10	*2·3*	*0·75*	23·4	*18·3*	55·0
10	..	12	7	9	0·77	3·4	1·03	*97·0*	25·8	49·0
*11	..	50	76	42	1·80	6·30	*0·64*	*53·1*	22·0	69·9
*12	151	26	45	33	1·37	7·49	*0·59*	26·0	*19·4*	73·0
13	480	48	45	13	*3·46*	3·64	2·50	26·0	27·9	58·0
14	..	233	271	209	1·30	2·90	0·77	34·3	20·9	67·9
*15	432	231	207	68	*3·04*	2·73	1·01	29·0	*19·5*	58·0
16	130	21	34	22	1·55	8·80	1·12	16·0	26·5	57·0
17	451	45	54	26	2·08	*1·70*	1·70	27·0	26·4	65·0
18	494	110	135	65	2·08	4·62	0·95	24·0	26·7	80·0
19	86	26	27	26	1·04	5·40	1·29	17·0	25·3	52·0
20	331	74	85	45	1·89	4·96	1·62	38·0	24·1	54·0
21	394	36	77	45	1·70	5·70	1·23	12·0	24·7	63·0
22	446	34	72	49	1·47	2·90	1·01	16·0	37·2	50·0
Mean ± S.D.	339 ± 225	89 ± 102	114 ± 140	52 ± 59	2·13 ± 0·96	3·89 ± 1·91	0·86 ± 0·16	37·6 ± 24·6	22·7 ± 0·8	69·2 ± 18·8

* Indicates women with absolute vitamin-B_6 deficiency.

activities of the control group (<0·75 μg. of sodium pyruvate per hour per mg. Hb and <19·5 μg. oxaloacetate per hour per mg. Hb, respectively). The erythrocyte aminotransferase activity data were analysed in logarithmic form because the distribution of results was skewed. The criteria for percentage stimulation of Al-AT and Asp-AT were that they should exceed the mean plus 2 S.D. of the percentage stimulation in vitro in the control group (>40% and >98%, respectively). Using the above criteria 11 of the depressed women had absolute vitamin-B_6 deficiency.

For assessment of changes in Beck scores during treatment, results for women receiving placebo or pyridoxine first were analysed separately to avoid any error that might arise should there be a carry-over effect of pyridoxine on Beck scores. The changes in Beck scores were analysed in the groups as a whole, and also in the subgroups of women with and without evidence of absolute vitamin-B_6 deficiency. The Beck score on entry into the trial was compared with the score at the end of the first treatment period, and the latter score was compared with the score at the end of the second treatment period. A decrease in score of 5 or more was taken to indicate improvement in depression, an increase of 5 or more a deterioration, and changes of less than 5 indicated no change in depression. Standard statistical calculations were employed, including Student's *t* test, and χ^2 test.

Results

Clinical

Table I shows the clinical details of the 22 women, those women subsequently judged to be vitamin-B_6 deficient being indicated by an asterisk. There were no differences between the vitamin-B_6 deficient and non-deficient women, of age, duration of O.C. use, or nature of O.C. preparation. All O.C.s contained 50 μg. of œstrogen, and approximately equal numbers were of high or low progestagen content. Five of the vitamin-B_6-deficient women were married (46%), compared to 8 of the non-deficient women (73%). The symptoms (with the number of women in parentheses) most commonly indicated in the entry Beck forms were those of pessimism (19), dissatisfaction (18), lethargy (17), loss of libido (16), and irritability (8). None complained of alteration of appetite or of sleep disturbance.

Urinary Tryptophan Metabolites, Urinary 4-P, and Erythrocyte Aminotransferase Activities and Per Cent Stimulation in Vitro (tables II–IV)

Table II shows the results obtained in the individual depressed women. All but 4 (nos. 1, 10, 16, and 19) had abnormal urinary tryptophan metabolite excretion compared to the mean results in women not taking O.C. (table III). Results indicating absolute vitamin-B_6 deficiency are in italic type, and (see Methods) 11 out of the 22 depressed women fulfilled the criteria of absolute vitamin-B_6 deficiency. A raised HK/HA ratio was more common than a low 4-P excretion (7 out of 11 and 3 out of 11 women, respectively). Indications of absolute vitamin-B_6 deficiency were most frequently found in the erythrocyte aminotransferase results, and of these a low Al-AT activity was found in 9 out of 11 women and a low Asp-AT activity in 6 out of 11. An increased Al-AT per cent stimulation in vitro was found in 6 out of 11 women.

Table III shows the mean results (±1 S.D.) in a group of women not taking O.C., a group of women taking O.C. but not complaining of depression, the whole group of 22 depressed women on O.C., and a subdivision of this last group consisting of those women with absolute vitamin-B_6 deficiency. The urinary tryptophan metabolite data of the 22 depressed women did not differ from that of the non-depressed women on O.C. Their results, however, differed significantly from the women not on O.C. Mean urinary 4-P levels were not significantly different in any of the groups. Asp-AT activity was significantly increased in the non-depressed women on O.C. compared to the women not on O.C., and Al-AT per cent stimulation in vitro tended to be increased in the former group. In the 22 women with depression on O.C., Al-AT and Asp-AT activities were lower ($P<0·01$ and $P<0·005$)

TABLE III—MEAN (±S.D.) URINARY TRYPTOPHAN METABOLITES (μmole/24 hr.), URINARY 4-PYRIDOXIC ACID (μmole/24 hr.), AND ERYTHROCYTE AMINOTRANSFERASE ACTIVITIES (μg. PYRUVATE OR OXALOACETATE/hr./mg. Hb) AND % STIMULATION IN VITRO.

Group		XA	K	HK	HA	HK/HA	4-P	Al-AT activity	Al-AT % stim.	Asp-AT activity	Asp-AT % stim.
Controls	Mean ±S.D.	63±40	36±20	33±17	28±15	1·25±0·5	3·9±0·7	1·05±0·15	18·7±10·5	21·9±1·2	68·8±15
	N	22	22	22	22	22	28	26	26	18	18
Non-depressed women on O.C.	Mean ±S.D.	385±304	123±151	158±207	96±141	2·2±1·2	3·4±1·3	1·17±0·15	22·0±15·0	26·4±1·1	58±14
	N	31	31	31	31	31	31	31	31	28	28
	P*	<0·001	<0·01	<0·01	<0·025	<0·01	N.S.	N.S.	N.S.	<0·001	N.S.
Depressed women (whole group) on entry	Mean ±S.D.	339±225	89±102	114±140	52±59	2·13±0·96	3·69±1·91	0·86±0·16	37·6±24·6	22·7±0·8	69·2±18·8
	N	18	22	22	22	22	22	22	22	22	22
	P†	<0·001	<0·02	<0·01	N.S.	<0·001	N.S.	N.S.	<0·005	N.S.	N.S.
Depressed women (B_6 deficient) on entry	Mean ±S.D.	328±263	116±128	152±181	55±64	2·5±1·1	3·6±1·8	0·60±0·13	45·9±23·4	19·9±0·5	77·1±22·1
	N	9	11	11	11	11	11	11	11	11	11
	P‡	<0·001	<0·01	<0·01	N.S.	<0·025	N.S.	<0·001	<0·001	N.S.	N.S.
	P§	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	<0·001	<0·001	<0·001	N.S.
Depressed women after B_6 administration	Mean ±S.D.	82·3±80·4	22·1±16·4	27·3±10·7	18·1±5·6	1·6±0·9	127±67	2·7±1·2	9·1±8·5	42·6±10·1	19·4±9·2
	N	12	18	18	18	18	21	20	20	20	20

* = Statistical significance between control and non-depressed women on O.C.
† = " " " " " " whole depression group.
‡ = " " " B_6-deficient O.C. patients and control group.
§ = " " " " " " " " non-depressed women on O.C.
N = No. of women studied.
N.S. = Not significant.

TABLE IV—URINARY TRYPTOPHAN METABOLITE, URINARY 4-PYRIDOXIC ACID, AND ERYTHROCYTE AMINOTRANSFERASE DATA IN WOMEN 8 WK. AFTER STOPPING PYRIDOXINE ADMINISTRATION: UNITS AS IN EARLIER TABLES

Woman	XA	K	HK	HA	HK/HA	4-P	Al-AT activity	Al-AT % stim.	Asp-AT activity	Asp-AT % stim.
3	710	523	329	111	*2·96*	4·10	*0·53*	19	*17·9*	77
5	..	40	48	40	1·2	6·70	*0·24*	*103·1*	20·9	90·8
11	..	42	62	34	1·8	..	*0·6*	*89*	21·5	83
15	523	98	122	78	1·57	8·10	1·36	19	28	43
16	182	19	32	20	1·60	6·20	1·75	27	36·1	35
17	..	68	67	47	1·01	..	2·2	23·6	34·4	22
18	470	115	158	84	1·88	3·14	1·19	12	42·2	47
20	163	48	90	33	*2·73*	7·60	2·90	4	36·1	35
Mean ± S.D.	409 ± 234	119 ± 166	113 ± 96	56 ± 32	1·8 ± 0·7	5·97 ± 1·96	1·34 ± 0·9	37 ± 37	30 ± 9	54 ± 26

TABLE V—MEAN (± 1 S.D.) FORTNIGHTLY BECK SCORES DURING THE TRIAL

Group	Entry Score	Pyridoxine				Placebo			
		Wk. 2	Wk. 4	Wk. 6	Wk. 8	Wk. 10	Wk. 12	Wk. 14	Wk. 16
Combined (N = 11)	30 ± 7	20 ± 12	25 ± 12	17 ± 11	18 ± 12	17 ± 10	16 ± 9	15 ± 12	13 ± 9
*P		N.S.	N.S.	< 0·01	< 0·05				
†P						N.S.	N.S.	N.S.	N.S.
Not B_6-deficient women (N = 5)	29 ± 5	32 ± 12	27 ± 11	25 ± 10	28 ± 12	25 ± 7	20 ± 3	21 ± 10	17 ± 6
*P		N.S.	N.S.	N.S.	N.S.				
†P						N.S.	N.S.	N.S.	N.S.
B_6-deficient women (N = 6)	31 ± 9	25 ± 12	24 ± 15	11 ± 8	12 ± 6	12 ± 9	13 ± 11	9 ± 12	9 ± 12
*P		N.S.	N.S.	< 0·01	< 0·005				
†P						N.S.	N.S.	N.S.	N.S.
Group		**Placebo**				**Pyridoxine**			
Combined (N = 11)	33 ± 8	31 ± 7	22 ± 10	31 ± 14	32 ± 15	25 ± 11	23 ± 11	22 ± 11	21 ± 11
*P		N.S.	N.S.	N.S.	N.S.				
†P						N.S.	< 0·05	< 0·05	< 0·05
Not B_6-deficient women (N = 6)	34 ± 7	31 ± 6	24 ± 13	28 ± 11	33 ± 13	26 ± 13	27 ± 11	25 ± 11	28 ± 6
*P		N.S.	N.S.	N.S.	N.S.				
†P						N.S.	N.S.	N.S.	N.S.
B_6-deficient women (N = 5)	31 ± 9	32 ± 8	21 ± 4	35 ± 17	32 ± 20	24 ± 11	17 ± 7	18 ± 10	12 ± 8
*P		N.S.	N.S.	N.S.	N.S.				
†P						N.S.	N.S.	N.S.	< 0·05

*P = Significance of change of Beck score between entry and subsequent weeks during initial treatment period.
†P = Significance of change of Beck score between score at 8 weeks and subsequent weeks during second treatment period.
N = No. of women studied.

and Al-AT per cent stimulation in vitro higher ($P < 0·01$) than in the non-depressed women on O.C., so that in the former group enzyme results were similar to those of women not taking O.C., except for a significantly raised Al-AT per cent stimulation in vitro. In the subgroup of depressed women on O.C. with absolute vitamin-B_6 deficiency these changes were greater, so that Al-AT and Asp-AT activities were lower than in the control group, and the Al-AT per cent stimulation in vitro was still higher. The administration of pyridoxine 20 mg. twice a day for eight weeks restored to normal the abnormal tryptophan metabolite excretion. With pyridoxine treatment the erythrocyte aminotransferase activities were greatly increased and percentage stimulation in vitro decreased.

Table IV shows the individual and mean (± 1 S.D.) results in 8 of the 11 women two months after discontinuing pyridoxine treatment while continuing O.C. and placebo. 3 women were unable to collaborate with this aspect of the study. The data show that at this time the mean urinary tryptophan metabolites were moderately elevated, though the HK/HA ratio was still low and the 4-P excretion raised. The erythrocyte aminotransferase activities were still high, but Al-AT per cent stimulation in vitro was elevated. Thus, in the group as a whole, there was biochemical evidence of a continuing effect of the preceding pyridoxine administration. However, inspection of the individual results shows that in patients 3, 5, and 11 data indicating absolute vitamin-B_6 deficiency were present.

Urinary tryptophan metabolite, 4-P levels, and erythrocyte aminotransferase enzyme results (un-

TABLE VI—ANALYSIS OF CLINICAL RESPONSE TO ADMINISTRATION OF PYRIDOXINE OR PLACEBO*

Treatment	Improved	Unchanged	Deteriorated
All women:			
Pyridoxine	15	5	2
Placebo	9	6	7
Vitamin-B_6-deficient group:			
Pyridoxine	11	0	0
Placebo	4	3	4
Not vitamin-B_6-deficient:			
Pyridoxine	4	5	2
Placebo	5	3	3

* To carry out the χ^2 test the number of women unchanged or deteriorated were combined. For the "all" group $\chi^2 = 3·30$; for the vitamin-B_6-deficient women $\chi^2 = 10·26$ ($P < 0·01$); and for the non-deficient group $\chi^2 = 0·18$ (N.S.).

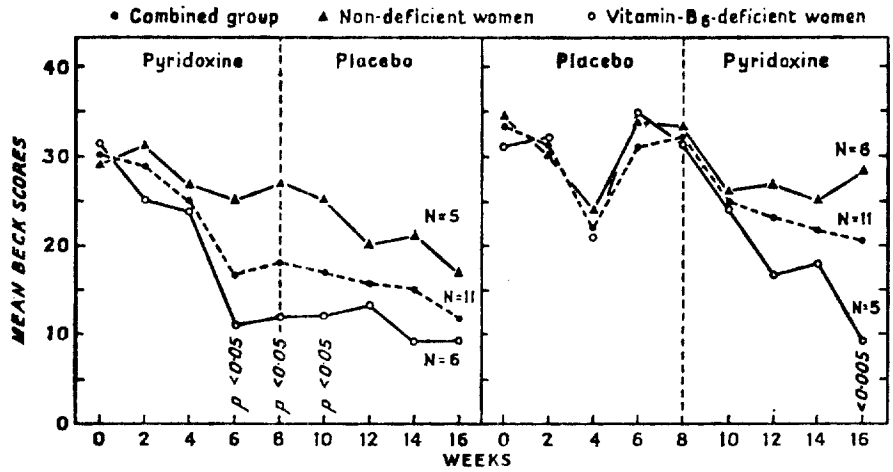

Fig. 2—Mean fortnightly Beck scores during the two treatment schedules in 22 depressed women on O.C.
P shows the statistical significance between the mean Beck scores of the vitamin-B_6-deficient and non-deficient women.

published) in the women given placebo for the first two months were substantially unchanged from those obtained from the analyses performed on entry into the trial, though the trend was towards greater evidence of vitamin-B_6 deficiency.

Changes in Beck Scores with Pyridoxine and Placebo (tables V and VI)

Mean (± 1 S.D.) Beck scores during the trial are shown in table V and in fig. 2, divided according to the sequence of pyridoxine and placebo treatment. In both treatment schedules the results in the vitamin-B_6 deficient and non-deficient women have been analysed separately and as a combined group. In the combined group there was a significant fall in Beck scores during pyridoxine treatment in both treatment schedules. Further analysis, however, showed that it was only the vitamin-B_6-deficient women who improved with pyridoxine. The improvement in Beck scores when pyridoxine was given first was maintained during the subsequent administration of placebo. When placebo was given first there was a fall (not statistically significant) in Beck scores at four weeks in both vitamin-B_6-deficient and non-deficient women. Unlike the effect of pyridoxine, the placebo effect was not maintained. Table VI shows that by the χ^2 test significant improvement in Beck scores during pyridoxine treatment occurred only in those women with absolute vitamin-B_6 deficiency, their Beck scores falling to levels found in non-depressed subjects.

Discussion

In women taking O.C. it was possible to identify a characteristic form of depression in which pessimism, anxiety, dissatisfaction, lethargy, and loss of libido were prominent, confirming the findings of Herzberg et al.[3] Although almost every woman complained during the first interview of greater lability of emotions with increased weepiness and irritability, these two symptoms were not prominent in the Beck scores, perhaps because it contains only two questions relating to such symptoms. Since disturbances of sleep pattern characteristic of endogenous or reactive depression [35] were used to exclude patients from this study at the initial interview, none of the women had such an abnormality.

The investigation was not designed to determine the frequency of O.C.-induced depression because, though most of the women were detected by means of a routine questionary at a family-planning clinic, a minority were referred directly by their general practitioners. The women were strictly selected because this study was planned to establish whether depression, appearing de novo during O.C. therapy without evident predisposing factors, was due to absolute vitamin-B_6 deficiency. Such a deficiency was demonstrated in half the women, and their depression responded to the administration of pyridoxine. In the remaining women pyridoxine was without apparent effect.

Several biochemical criteria were used to identify absolute vitamin-B_6 deficiency. Urinary levels of tryptophan metabolites were abnormal in most of the women studied, and could not be used to identify an absolute deficiency of vitamin B_6 because the changes are similar to those due to tryptophan-oxygenase induction and relative vitamin-B_6 deficiency. A raised HK/HA ratio, however, which is considered to be a reliable indication of vitamin-B_6 deficiency in man,[22,36] was significantly raised in 7 out of the 11 women judged to have absolute vitamin-B_6 deficiency. Urinary 4-P excretion, on the other hand, was a poor discriminant, because it was significantly low in only 3. Erythrocyte aminotransferase activities and stimulation in vitro provide a reliable measure of vitamin-B_6 status in nutritional vitamin-B_6 deficiency, activities falling and stimulation by pyridoxal phosphate in vitro rising, with increasing vitamin depletion.[20,23,24] O.C. administration, however, renders the interpretation of erythrocyte aminotransferase data difficult, as there may be induction of enzyme activity in the red blood-cells similar to that shown in the liver transaminases.[12] Thus we have previously reported that Asp-AT activity was raised in non-depressed women on O.C.,[37] confirming the findings of Aly et al.[38] In view of these findings it has been suggested that measurement of Asp-AT activity would not be able to identify women with absolute vitamin-B_6 deficiency while

taking O.C.[37] However, 6 of the 11 women judged to have absolute deficiency of this vitamin had Asp-AT activities which were significantly lower than those observed even in women not taking O.C. Al-AT activity may remain unchanged [37] or fall in women on O.C.,[38] though in the latter report there was considerable overlapping between basal activities of the 11 control and 13 O.C. users. In our study, in the 11 vitamin-B_6-deficient women the mean Al-AT activity was lower than that of women not taking O.C. Al-AT per cent stimulation in vitro, which is increased during O.C. administration,[37,39] increased further with absolute vitamin-B_6 deficiency.

There are two possible mechanisms whereby pyridoxine can correct impaired brain 5-H.T. synthesis. First, large doses of pyridoxine could overcome competitive inhibition of pyridoxal phosphate by œstrogen conjugates, thereby restoring normal enzyme function in the tryptophan/nicotinic-acid-ribonucleotide pathway. Accumulation of the metabolites K and HK may, therefore, be prevented so that the transport of tryptophan into the brain is no longer impeded. If this mechanism alone were responsible for increasing the rate of brain 5-H.T. synthesis, most of the women in our series should have improved with pyridoxine treatment, since 18 out of 22 had urinary tryptophan metabolite abnormalities. Since the clinical improvement was confined to the group with absolute viramin-B_6 deficiency it is possible that the second mechanism was more important—namely, that brain 5-hydroxytryptophan decarboxylase activity was increased by pyridoxine therapy. There is some evidence that this enzyme, as opposed to tryptophan hydroxylase, may be rate-limiting for the synthesis of brain 5-H.T. in man.[37] In addition, there is evidence that coenzymes may not only activate pre-existing latent apoenzymes but may also influence the synthesis of the apoenzyme itself. Thus excess pyridoxine has been shown to increase the level of rat liver tyrosine aminotransferase,[40] and we have reported [37] that erythrocyte aminotransferase activities were progressively increased in women on O.C. the longer the duration of pyridoxine administration. Conversely, lack of coenzyme is associated with reduced enzyme activity and decreased amount of latent apoenzyme, as shown by low enzyme levels after correction of coenzyme deficiency in short-term experiments. Thus 5-hydroxytryptophan decarboxylase activities in vitamin-B_6-deficient rats were increased with pyridoxine, but to lower levels than those before the induction of vitamin-B_6 deficiency.[41] These changes were very striking in the liver and kidney but were less so in the brain. It is possible, therefore, that in the vitamin-B_6-deficient women of our study brain 5-hydroxytryptophan decarboxylase apoenzyme synthesis was reduced and that the relief of depression was due to induction of synthesis of this apoenzyme in addition to activation of latent apoenzyme.

Duration of administration of O.C. did not appear to correlate with the development of absolute vitamin-B_6 deficiency. The progestagen content of the O.C. also seemed to be unimportant in causing depression, confirming the findings of Herzberg et al.,[3] and contrary to the report of Grant and Mears,[42] who implicated a high progestagen content. Our findings suggest that it is the œstrogen component which is most important, and, furthermore, there is evidence that progestagens alone do not affect tryptophan metabolism.[17]

It is of interest to note that as judged by Beck scores the beneficial clinical effect of pyridoxine treatment when given first in the trial was maintained for two months during the placebo treatment. Mean tryptophan metabolite and erythrocyte aminotransferase results indicated that the biochemical effects of pyridoxine were still evident eight weeks after discontinuing therapy, because 4-P excretion and Al-AT and Asp-AT activities were raised. XA excretion, however, was raised, which may be explained by the observation of Ogasawara et al. that pyridoxal phosphate is less tightly bound to supernatant kynureninase than to mitochondrial kynurenine aminotransferase.[43] Thus œstrogen conjugates may displace pyridoxal phosphate more readily from the former, so that HK is preferentially metabolised to XA. Three women did, however, show evidence of absolute vitamin-B_6 deficiency two months after pyridoxine treatment was discontinued. No explanation can be offered for this observation, their diet not differing from the remaining 5 women with still replete vitamin-B_6 stores.

It has been suggested that pyridoxine supplements should be given to all women on O.C.[44-47] In view of the clinical response to pyridoxine of the depression in the women in the present study with absolute vitamin-B_6 deficiency, it is tempting to agree with these views. However, administration of large doses of pyridoxine may not be without complications. Alanine and tyrosine aminotransferase activities are increased in rat liver with œstrogen treatment[12]; plasma-tyrosine levels [47] and total plasma-aminoacid levels fall in women on O.C.[48] These effects may be due to œstrogen-mediated increased glucocorticoid activity inducing transaminases leading to increased aminoacid catabolism. Furthermore, as has been discussed above, increased apoenzyme synthesis due to coenzyme induction may occur. Thus, if pyridoxine supplements were given to all women on O.C. it is possible that coenzyme induction of apoenzyme could enhance the plasma-aminoacid-lowering action of œstrogen, which may be undesirable, especially in areas of the world where protein malnutrition exists.

In the group of women whose depression was thought on clinical grounds to be due to O.C. administration, only half were found to have absolute vitamin-B_6 deficiency, and only this group responded to the administration of the vitamin. Other causes must therefore be sought to explain the depression in those who did not respond. It is possible that substrate deficiency (tryptophan lack) due to tryptophan oxygenase induction could account for some of these cases. This and other aspects of the problem are being investigated.

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EXPERIMENTAL STUDY OF THE EFFECTS PRODUCED BY LARGE DOSES OF VITAMIN B_6*

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The recent introduction of vitamin B_6 in clinical medicine renders essential the understanding of its possible toxic effects. This study was undertaken in order to throw some light on the problem. The relative amounts of vitamin B_6 used in these experiments were far in excess of quantities employed in human therapy: the massive doses were used so that the pathological changes could be studied in their fullest phases of development.

Material and Methods. Fifteen adult male rats and 4 adult male and female dogs were used in these experiments (chart). Vitamin B_6** was administered orally or subcutaneously as the base or hydrochloride. Three rats and 2 dogs receiving adequate stock laboratory diets were used as controls. Four other rats which were on a vitamin B_6 deficient diet and in which no abnormalities of the central nervous system were found on microscopic examination, served as further controls. All animals receiving vitamin B_6 in the large doses showed essentially the same disability. The clinical features associated with this hypervitaminosis have been reported by Unna (1).

Dog 1 is representative of those which received the larger doses. This dog, of 10 kg. body weight, received 0.5 gm./kg. of vitamin B_6 HCl by gavage at the following times:

Aug. 4 - 9 a.m. and 5 p.m.;
Aug. 5 - 9 a.m. and 5 p.m., and
Aug. 6 - 9 a.m.

Altogether it received 2.5 gm./kg. On August 4, the dog vomited at every dose and a considerable portion of the drug therefore might have been lost. For two days the animal appeared normal, after that time a gradually increasing ataxia became evident. Five days after the administration of vitamin B_6, the animal showed ataxia and frequently fell. When examined the next day, the dog was unable to stand, and when an attempt was made to place the dog on his feet his limbs would spread apart and he would fall to the floor. He would respond to a pin prick by moving the stimulated limb. Deep reflexes of all limbs were absent. He lay quietly moving his limbs only in response to painful stimuli. Tone was diminished generally and deep reflexes did not return. The dog continued in this condition until he was found dead on the morning of the 14th day after he was given the first dose of vitamin B_6.

In other dogs the effects were essentially the same as in the dog. In about 24 hours after receiving a dose of 3 gm. or more per kg., there was incoordination and the movements were awkward, slow and jerky. The forelimbs were often flexed under the body with the paws close to the chest and limbs spread apart and directed forward. There was marked hypotonia of all joints, although periodic outstretching of the limbs, especially the hind limbs, occurred during which there was transient increase of tone. In animals receiving the larger doses the symptoms usually persisted up to the time of sacrifice. With smaller doses, recovery seemed to occur frequently. No convulsions were observed in any of the animals.

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** Pyridoxine or Pyridoxine hydrochloride were employed.

All dogs, excepting dog 4 who died, were killed during the stage of severe ataxia or following recovery from the ataxia by the injection of air into the saphenous vein. The rats were killed by decapitation. Complete autopsies were performed.

Care was taken to avoid injury to the brain and spinal cord during their removal. Pieces of the brain, spinal cord, ganglia and nerves, taken at various levels, were placed in the fol-

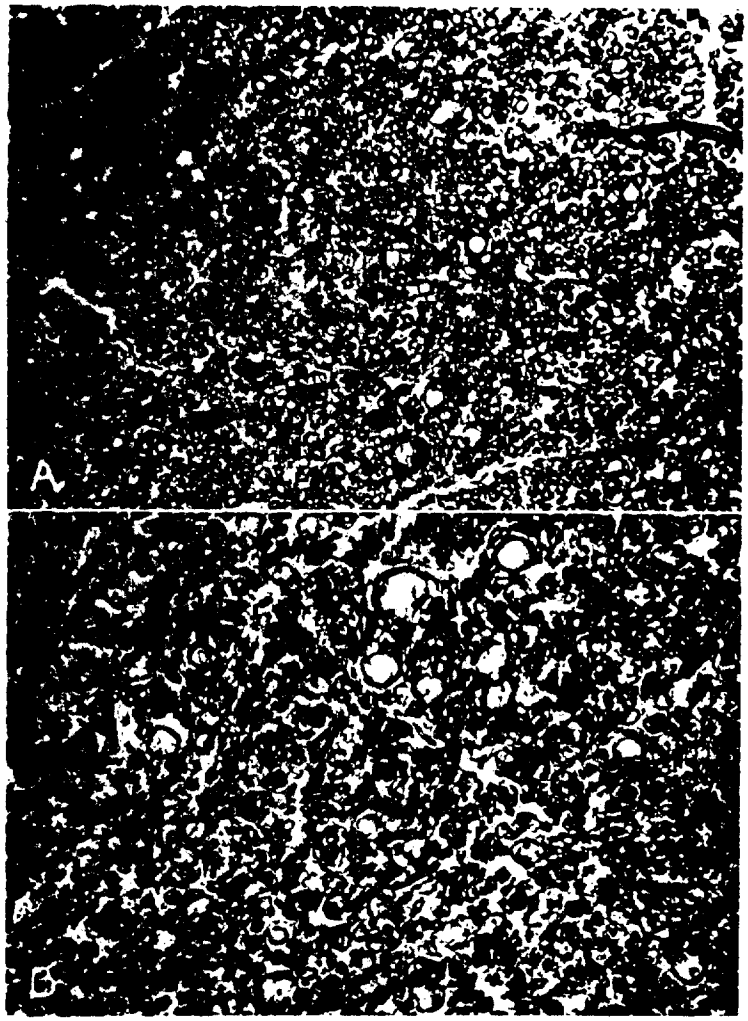

FIG. 1. Weigert-Pal stains showing A, normal myelin sheaths of the posterior columns in the control dog and B, swelling and other degenerative changes of myelin sheaths in the posterior columns in the treated dog (× 200).

lowing fixatives: 10% formalin for hematoxylin and eosin, Weigert-Pal, Scharlach-R, and Gros-Bielschowsky stains; 95% alcohol for 1 day, then followed by absolute alcohol for celloidin embedding and toluidine blue staining of nerve cells; formalin urea potassium iodide for oligodendroglia (2), and formalin ammonium bromide for microglia and astrocytes. In the case of dog 4, additional pieces of the nervous system were fixed in Muller's fluid for Marchi preparations.

Anatomical observations. No macroscopic abnormalities were encountered. Microscopic examination of the spinal cord, posterior roots, posterior root ganglia and peripheral nerves showed well marked changes. The posterior columns showed a honeycombed appearance in hematoxylin and eosin preparations. Weigert-Pal stains revealed rarefaction and swelling of myelin sheaths (fig. 1). Fragmentation and loss of axones was prominent in

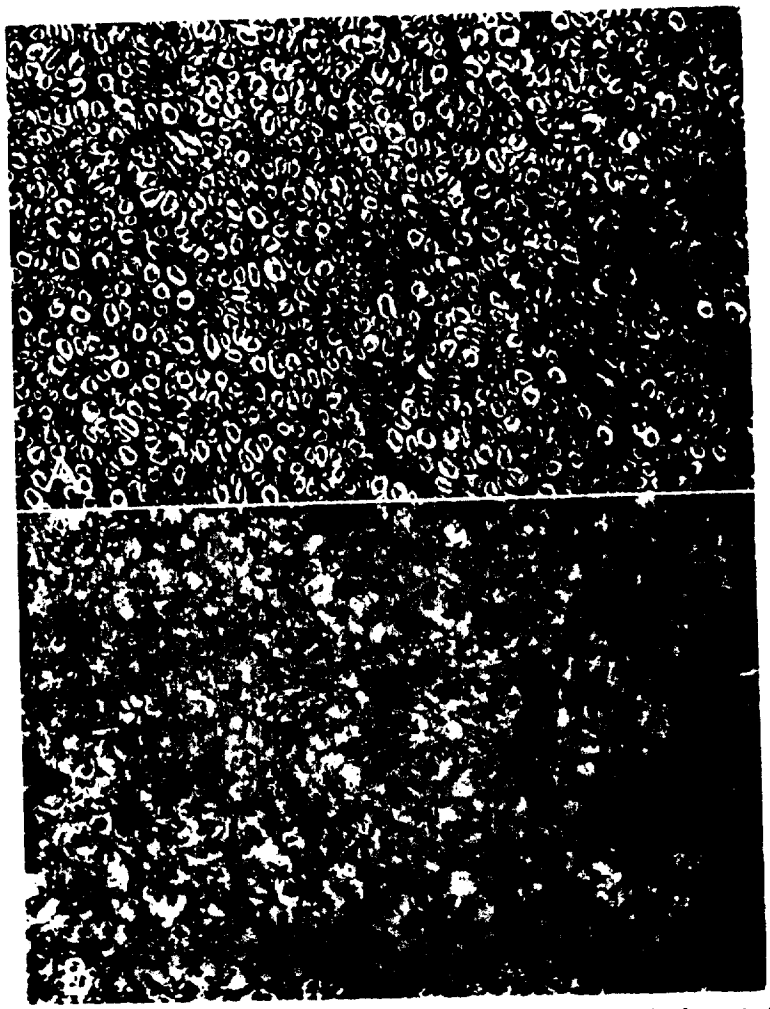

FIG. 2. Gros-Bielschowsky preparations showing A, intact axis cylinders of the posterior columns in the control dog and B, degenerated axis cylinders of the posterior columns in the treated dog (× 200)

Gros-Bielschowsky preparations (fig. 2). Degeneration of axones was less marked than the degenerative myelin sheath changes. Swelling of oligodendroglia and the occurrence of rod cells and compound granular corpuscles were seen in sections impregnated with silver carbonate. Sections impregnated with gold chloride sublimate showed hypertrophy of astrocytes with fragmentation of processes and the formation of numerous "filling bodies."

Large amounts of fat were seen in Scharlach R stains. All of the above changes were confined to the posterior columns of the spinal cord and were absent in the medulla, higher levels of the brain stem, cerebrum and cerebellum. They occurred at all levels of the spinal cord, occasionally more marked at some segments than others, but without consistent sparing of any segments. In the rats the degenerative changes spared the most central portion of the posterior columns in which region the pyramidal tract is situated (4).

In those cases in which the posterior column changes were most marked, the posterior roots, and to a less extent, the peripheral nerves showed vacuolization, poor staining of

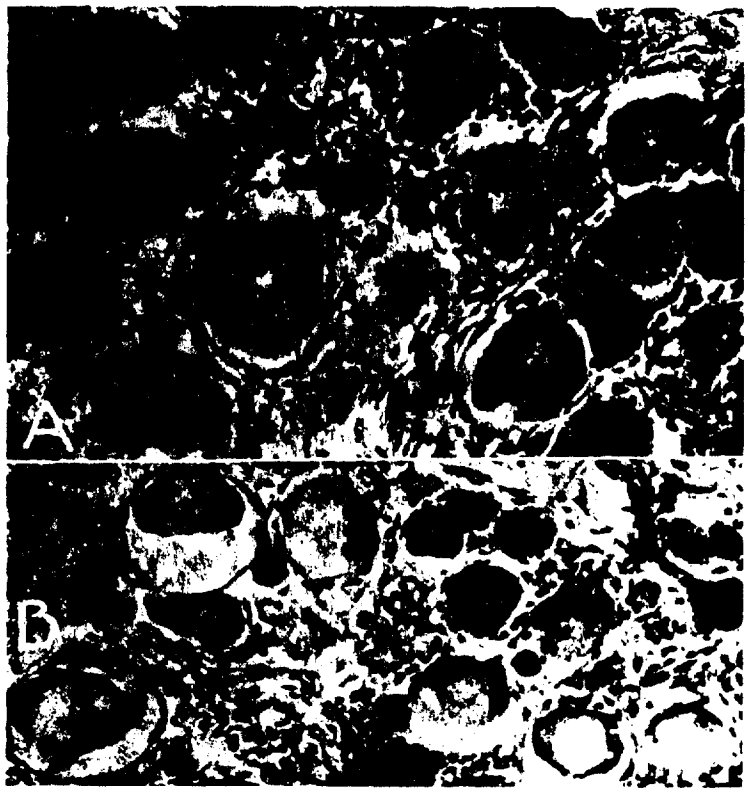

FIG. 3. Toluidine blue stains showing A, normal nerve cells of the posterior root ganglion in the control dog and B, markedly chromatolytic nerve cells of the posterior root ganglion in the treated dog (× 290).

myelin sheaths with swelling and fragmentation of the axis cylinders. No such abnormalities were observed in the anterior roots. Furthermore in those cases in which marked degenerative changes occurred in the posterior columns, Nissl preparations showed marked chromatolysis of the nerve cells of the posterior root ganglia (fig. 3). The chromatolysis began in the perinuclear region. Some of the cells showed a mere peripheral ring of ill defined chromatin granules with an eccentric nucleus. Other cells were devoid of nucleus and chromatin and presented a cell membrane only. Still other cells showed rupture of cell membranes, leaving empty spaces surrounded by capsular cells. It is of interest that in no case did the sympathetic ganglia show evidence of chromatolysis or other changes. In dog 4, Marchi preparations verified the marked degenerative changes of the posterior

column (fig. 4), and to a less extent, of the posterior roots. Few degenerated fibers appeared in the anterior and lateral columns.

None of the above-recorded changes occurred in either the normal control or B_6 deficient animals. The changes in the animals receiving large doses of vitamin B_6 were noninflammatory in nature although in rat 4, a focus of round cell accumulation with chromatolysis was observed in one anterior grey horn at a single level; in rat 7, a similar inflammatory focus occurred at the posterior root entry zone at one level. In 2 rats (8 and 9) which received vitamin B_6, no abnormal changes were found.

FIG. 4. Marchi stain showing degeneration of the posterior columns of the spinal cord of the dog treated with vitamin B_6 (× 14) and B, higher magnification view of the posterior columns shown in A. (× 270).

In all of the dogs and in all but two of the rats, the changes in the posterior column were cured [illegible] possible to distinguish quite readily, with the above two exceptions, from animals which received vitamin B_6 from the control animals by the state of the posterior columns. These changes were much more constant and much more marked than any of the other abnormalities which were described. The posterior column changes in all animals ability preceded the less constant changes in the posterior roots, posterior ganglions or peripheral nerves. The degeneration of the axones was less marked than the degeneration of the myelin sheaths. The fact that time favored clinical improvement and the disappearance of many of the degenerative cord changes is a further indication that the axis cylinders were less affected than the myelin sheaths.

CHART

Summary of pathologic changes produced by excessive amounts of vitamin B_6

ANIMAL	DOSAGE	ROUTE OF ADMINISTRATION	SURVIVAL PERIOD	HISTOLOGIC CHANGES
Rat 1	3 gm./kg.*	Oral	4 days?	Marked rarefaction of posterior columns
Rat 2	3 gm./kg.*	Oral	4 days?	Slight rarefaction of posterior columns
Rat 3	3 gm./kg.*	Oral	4 days?	Marked rarefaction of posterior columns
Rat 4	3 gm./kg.*	Subcutaneous	25 days	1. Moderate rarefaction of posterior columns. 2. Vacuolization of posterior roots 3. Focus of necrosis in anterior grey horn consisting of chromatolysis of ganglion cells with infiltration by lymphocytes
Rat 5	5 gm./kg.*	Oral	10 days	Slight rarefaction of posterior columns
Rat 6	5 gm./kg.*	Oral	10 days	Slight rarefaction of posterior columns
Rat 7	3 gm./kg.*	Oral	20 days	1. Slight rarefaction of posterior columns 2. Focus of necrosis of same type as in Rat 4 at posterior root entry zone
Rat 8	5 gm./kg.†	Oral	4 days	No abnormality
Rat 9	5 gm./kg.†	Oral	4 days	No abnormality
Rat 10	6 gm./kg.*	Oral	2 days	Very slight rarefaction of posterior columns
Rat 11	6 gm./kg.*	Oral	2 days	1. Marked rarefaction of posterior columns 2. Moderate chromatolysis of posterior root ganglion cells
Rat 12	7 gm./kg.*	Oral	16 days	1. Moderate rarefaction of posterior columns 2. Vacuolization of posterior roots
Rat 13	6 gm./kg.*	Oral	4 days	1. Marked rarefaction of posterior columns 2. Marked chromatolysis of posterior root ganglion cells
Rat 14	6 gm./kg.*	Oral	37 days	1. Moderate rarefaction of posterior columns 2. Slight chromatolysis of posterior root ganglion cells
Rat 15	5 gm./kg.*	Oral		Moderate rarefaction of posterior columns
Dog 1	2 gm./kg.† in 4 doses over period of 36 hours	Oral	8 days	1. Moderate degeneration of axis cylinders and myelin sheaths of posterior columns 2. Marked chromatolysis of posterior root ganglion cells
Dog 2	2 gm./kg.† in 4 doses over period of 36 hours	Oral	13 days	Slight rarefaction of posterior columns
Dog 3	1 gm./kg.†	Oral	14 days	Slight rarefaction of posterior columns
Dog 4	2.5 gm./kg.† in 5 doses over period of 48 hours	Oral	14 days	1. Marked degeneration of myelin sheaths and, to a less extent, of axis cylinders of posterior columns and posterior roots: few degenerated fibers seen in Marchi preparations in anterior and lateral columns. 2. Occasional chromatolysis in posterior root ganglion cells

* Vitamin B_6 base.

† Vitamin B_6 HCl.

The vitamin was administered in one dose unless otherwise stated.

No striking changes were found in other viscera except for considerable enlargement, marked congestion and occasional hemorrhage in the adrenals of the rat.

SUMMARY

The administration of excessively large doses of vitamin B_6 to rats and dogs results in a few days in pronounced ataxia and weakness. The abnormalities of the nervous system on microscopic examination consist of degeneration of the posterior columns of the spinal cord; in some cases, there was well-marked degeneration of the posterior roots, posterior ganglia and peripheral nerves.

We are grateful to Doctors H. Molitor and K. Unna of the Merck Institute of Therapeutic Research for supplying some of the dogs and rats used in these studies, and to Doctors J. M. Carlisle and D. F. Robertson of the Medical Department of Merck & Co. for the vitamin B_6.

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GASTROENTEROLOGY

Sites of absorption in the small intestine

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UNTIL RECENTLY LITTLE WAS KNOWN of the functions of individual segments of the small intestine. There are no obvious differences in mucosal cell structure in the proximal or distal parts of the small intestine and this makes the concept of differential function difficult for many physiologists to accept. As long ago as 1887, however, Tappeiner (37) showed in experimental animals that the bile salts are not absorbed in the duodenum, but that absorption is maximal in the ileum. More recently, the discovery that vitamin B_{12} is specifically absorbed in the ileum has stimulated greater interest in sites of absorption. A great deal of work has been carried out in experimental animals but very much less attention has been paid to sites of absorption in man.

This paper first describes some of the methods which have been used to study sites of absorption in man, and indicates the principal factors that determine the site of absorption of a substance. The sites of absorption of the different dietary constituents are then considered separately.

METHODS

Modern techniques usually involve some method of intestinal intubation. The double lumen tube was introduced in 1934 by Miller and Abbott (26); it was passed by mouth and a test substance was introduced into the intestine through one lumen of the tube. The unabsorbed material was then aspirated through the other lumen from the distal site. For accuracy this technique required complete recovery of the unabsorbed material from the distal end of the segment under study and this is virtually impossible. Recently, the introduction of nonabsorbable markers has greatly improved the value of intubation techniques of this sort. Using such markers, substances can be perfused through a limited segment of either the proximal or the distal small intestine, and absorption is determined by measuring the ratio of the concentration of the test substance to that of the unabsorbed marker (34). This is a considerable advance because it is not necessary to recover all of the unabsorbed material. These methods indicate the capacity of a given segment of the gut to absorb and are best used for kinetic studies. They do not necessarily demonstrate the normal site of absorption. A more physiological method was suggested by Blankenhorn, Hirsch and Ahrens (2) and this has been used particularly by Borgström and others (8). A thin polyvinyl tube is swallowed and allowed to pass through the entire gastrointestinal tract. A meal, mixed with a nonabsorbable marker, is given and the intestinal contents are sampled through the tube at various levels. From analysis of the ratio of the various food substances to the concentration of the unabsorbed marker, absorption at different levels can be calculated. These methods, however, cannot be used for fat-soluble substances, since water-soluble markers are unsatisfactory for the study of fat-soluble material (39).

One of the most widely quoted methods of assessing sites of absorption is to measure absorption after resection of various segments of the small intestine. Such observations must be interpreted with considerable caution [illegible] compensatory changes may occur in the [illegible] after extensive resection. Other methods have included [illegible] ment of the active uptake of a test substance by [illegible] intestinal mucosa freshly taken from different areas and in the case of B_{12}, assessment of the distribution of radioactivity at laparotomy after oral administration of a labeled test dose.

When sites of absorption in man are considered, it will be seen that for many substances there is relatively little evidence as to the site of absorption, the observations often being limited to the results of only one method at most.

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FACTORS INFLUENCING SITES OF ABSORPTION

The factors which influence the site of absorption are first the relationship between the rate of absorption and the rate of transit through the small intestine. Since the jejunum is a highly motile organ, only substances which are absorbed fairly rapidly will be completely absorbed there. The slower the absorption rate, the more the substance will be propelled on into the distal intestine before absorption is complete. Many absorption mechanisms are in fact less active in the ileum than in the jejunum, but the motility of the ileum is sluggish and transit slow, so that even with relatively inactive transport systems in the ileum effective absorption can occur there.

The second factor is the mechanism of absorption, whether by passive diffusion or by an active process, and in the case of absorption by an active transport process, the distribution of this mechanism in the intestinal mucosa is important. Finally, the form in which the substance exists in food and the rate at which it is released from complex or bound forms may determine its site of absorption.

If a substance is absorbed by passive diffusion, whether through the water or the lipid phase of the intestinal cell membrane, it will clearly be absorbed by the proximal part of the intestine after oral feeding, and how far it travels down the gut during absorption will depend on its rate of diffusion. Furthermore, since the rate of diffusion is directly related to the concentration of the substance, the site of absorption will remain the same regardless of the amount of the test substance given. If, however, absorption occurs by active transport, absorption will again occur in the proximal intestine if the transport mechanism is present there; but as the oral load is increased the active mechanism may become saturated; the site of absorption may then become more distal, the distal intestine absorbing what has overflowed from the proximal. Under these circumstances the ileum acts as a functional intestinal reserve. But that is not the only function of the ileum, because if a substance is absorbed by an active transport mechanism which is only present in the ileum, absorption can only occur there and will not take place proximally under physiological circumstances.

CARBOHYDRATES

There is good evidence that sugars are absorbed from the proximal part of the small intestine. Borgström and his colleagues (8), using the Blankenhorn tube technique, studied the site of absorption of glucose from a mixed meal of corn oil, lactose, and protein and demonstrated that glucose was virtually entirely absorbed by the time the meal had passed through the duodenum and proximal 100 cm of jejunum.

On theoretical grounds, sugars which are not actively transported should be absorbed from both proximal and distal small intestine, but after oral feeding the proximal part of the intestine should be the main site of absorption. In man, indirect evidence has suggested that rha[illegible] arabinose, and xylose are absorbed in the jejunum and more recently direct studies by Fordtran, S[illegible] and Ingelfinger (16) have indicated that xylose is [illegible]mally absorbed from the proximal small intestine; [illegible] ileum is also capable of absorbing xylose but only [illegible] one-sixth as effectively as the jejunum.

Much of the dietary carbohydrate is absorbed [illegible] disaccharide, and the final stage of digestion, hydr[illegible] of the disaccharide, takes place in the intestinal m[illegible] cells through the activity of the small intestinal [illegible]charidases. The sites of absorption of the disacch[illegible] are, therefore, likely to be related to the sites in the [illegible] intestine of maximal disaccharidase activity. In [illegible] there are low levels of sucrase, isomaltase, and lact[illegible] the first part of the duodenum, but all the disaccharidases are present in the jejunum as well as in the ileum (1). The site of absorption of sucrose, maltose, and lactose was studied directly in man by Dahlqvist and Borgström (12) using an intubation technique and they found that although lactose and maltose were absorbed proximally, sucrose appeared to be absorbed in the ileum, a surprising finding since sucrase is present in the jejunal mucosa. This was reinvestigated by Gray and Ingelfinger (17), however, and these workers showed that sucrose absorption can occur from the jejunum.

PROTEIN

The most widely accepted view on the site of protein absorption in man is derived from the study of Borgström et al. (8) already referred to. In addition to studying glucose, these workers measured protein absorption at different levels in the small intestine and absorption appeared to occur from the proximal part of the gut. In these experiments, however, it was not protein that was measured but the absorption of a marker of ^{131}I labeled human serum albumin. This is unsatisfactory, since the ^{131}I label may become detached from the protein during absorption and this may give erroneous results (20). The site of absorption of dietary protein must therefore remain sub judice. Studies of nitrogen balance and of the absorption of tritium-labeled albumin in patients with extensive gut resections suggest a proximal absorption site for protein (21); but this is not yet proven and the extent to which the ileum participates in protein absorption is unknown.

FAT

There has been considerable controversy about the site of fat absorption in recent years. Some workers have claimed that the ileum is the main site of fat absorption, whereas others have suggested the jejunum. In experimental animals there is evidence that the jejunum is the major site of fat absorption. In man, histological and electron-microscopic studies have shown fat in the jejunal epithelial cells during absorption and these observations would suggest a proximal absorption site. Other evidence has been derived from intestinal intubation techniques

and from studies of patients with small intestinal resections. The intestinal intubation evidence, however, is of uncertain significance since it depends on the use of water-soluble markers. As already indicated, the results of intubation studies which have relied on water-soluble markers are not satisfactory for when a water-soluble marker is mixed with fat, there is separation of the two phases (39).

The evidence provided by studies of absorption in patients subjected to intestinal resections must therefore be considered, but the interpretation of such data in patients with gut resections is difficult. If the absorption of fat by patients with jejunal or ileal remnants is compared, it appears superficially that the ileum may be important in fat absorption, for ileal resection certainly can cause steatorrhea. But although frequently there is steatorrhea when the distal gut is resected, much of the dietary fat can still be absorbed even when there is only a foot of jejunum remaining—and some absorption occurs when there is as little as 8 inches (4). These findings support the idea of a proximal absorption site for fat in man and have suggested that the ileum is normally only necessary for completing the last stages of fat absorption, mopping up what the jejunum has missed as it were, and acting as a reserve when dietary intakes are high. The ileum can absorb fat well after the main absorbing area in the proximal intestine has been resected. A resection of 8 feet of the jejunum, for example, causes no steatorrhea, and even though there is some steatorrhea after resection of all the small gut but for the distal 18 inches of the ileum, much of the dietary fat can still be absorbed (4). These data have been used to indicate the sites of fat absorption but there are a number of snags. When there is ileal resection, for instance, there may be a reduction of bile salt reabsorption in the small intestine, and the steatorrhea in distal resection patients may therefore be more than can be explained by the resection alone (18, 19). Furthermore, compensatory changes may occur after resection.

RESPONSE OF RESIDUAL INTESTINE AFTER PARTIAL RESECTION

It was said many years ago that the intestinal villi become longer after gut resection (15), but a recent paper has failed to confirm this (30). After distal resection, however, the villi in man can show hypertrophy. The mean villus height in jejunal biopsy samples obtained by Dr. R. H. Dowling from a group of patients with distal gut resection is compared with that of control subjects in Table 1. There is a significant increase in villus height in the resection patients. This structural change is accompanied by an increase in function. Figure 1 shows the amount of glucose absorbed by a 25-cm segment of jejunum in control subjects and in patients with distal gut resection (13). The observations were made using a double-lumen tube perfusion technique. There is a significant but not great increase in glucose absorption after distal gut resection.

TABLE 1. *Mean villus height in control subjects compared with distal intestinal resection*

	No. of Subjects	Villus Height, μ*	Statistical Significance
Control subjects	11	382 ± 20	$P < 0.05$
Patients with intestinal resection	12	440 ± 16	

* Mean ± SEM

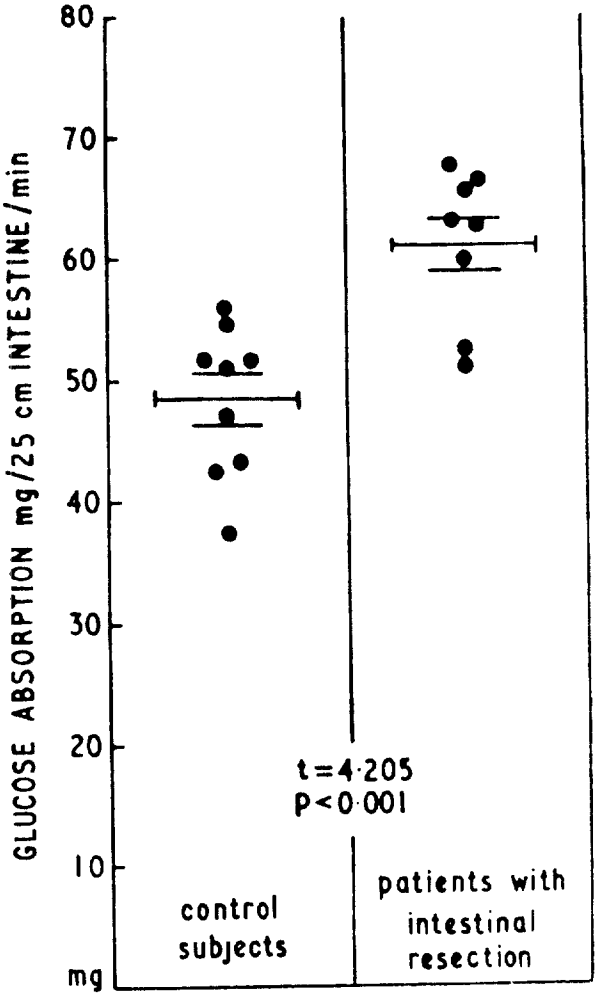

FIG. 1. Results of glucose absorption in milligrams (mean ± SEM) using the segmental-perfusion technique.

It is possible, however, that the functional changes may be much more striking in the ileum after a proximal resection, for studies in experimental animals have shown a difference in the functional response of the jejunum and ileum after resection. Groups of rats were subjected to resection of two-thirds of either the proximal or the distal small intestine (14). Table 2 shows the villus heights in control rat jejunum and ileum, in the jejunum after distal resection, and in the ileum after proximal resection. As in man, there is a slight but significant increase in jejunal villus height after distal resection.

But the changes in the ileal villi (which are normally shorter than those in the jejunum) are very much more striking. This anatomical change is accompanied by an increase in function. Figure 2 shows the amount of glucose absorbed by rat jejunum or ileum; the results were obtained using a recirculation perfusion technique. In the jejunum there was a significant increase in the absorption of glucose after distal resection, but (as in man) this increase was not gross. In the ileum, on the other hand, the increase was much more marked, the absorption of glucose from the ileum becoming virtually as effective as in the jejunum after the proximal intestine was resected. It is, therefore, clear that absorption sites may change under the unphysiological conditions of intestinal resection. It is not yet known whether the same change in absorptive capacity occurs in the ileum in man after a proximal resection, but the clinical observation that absorption is so much better after a proximal resection than after a distal resection may well be due to a similar mechanism, and data obtained after gut resection, particularly proximal resection, do not necessarily provide a valid method for determining sites of absorption. Since these are virtually the only data available on the site of fat absorption in man, with the exception of Borgström's observations using the Blankenhorn tube, it is clear that further information is required on the sites of fat absorption, and this necessitates the development of a fat-soluble, nonabsorbable marker.

FAT-SOLUBLE VITAMINS

There is even less information on the sites of absorption of the fat-soluble vitamins for there are no direct data available. But supposing these vitamins are absorbed by passive diffusion through the lipid phase of the intestinal cell membrane, it would be expected that they would be absorbed proximally in the gut. Direct observations on the site of absorption of vitamin A in animals suggest that vitamin A is absorbed in the jejunum (29). As far as vitamin D is concerned, Norman and de Luca (28) and Kodicek (22) have favored the unlikely idea that the ileum is the site of vitamin D absorption. On the other hand, Schachter, Finkelstein and Kowarski (33), using tied intestinal loops in the rat, have suggested that the jejunum is the main site of absorption of vitamin D. In man, the only data available are derived from absorption studies after gut resection. Figure 3 shows the plasma radioactivity in a patient with only 4 feet of jejunum remaining after being given an oral dose of 1 mg of ^{3}H-labeled vitamin D_3. Although the plasma radioactivity here was slightly subnormal, 60% was absorbed from this dose, which is normal (38). Subject to the criticism that this is a resection situation, it seems likely that vitamin D can be absorbed in the proximal small intestine in man.

TABLE 2. *Villus height after resection of two-thirds of either proximal or distal small intestine compared with villus height in control rats**

	No. of Rats	Mean Villus Height†	Statistical Significance
Control jejunum	24	323 ± 11	$t = 3.18$
Jejunum after distal resection	23	389 ± 9	$P < 0.01$
Control ileum	27	232 ± 7	$t = 3.58$
Ileum after proximal resection	28	354 ± 12	$P < 0.001$

* From Dowling and Booth (14). † Mean ± SEM

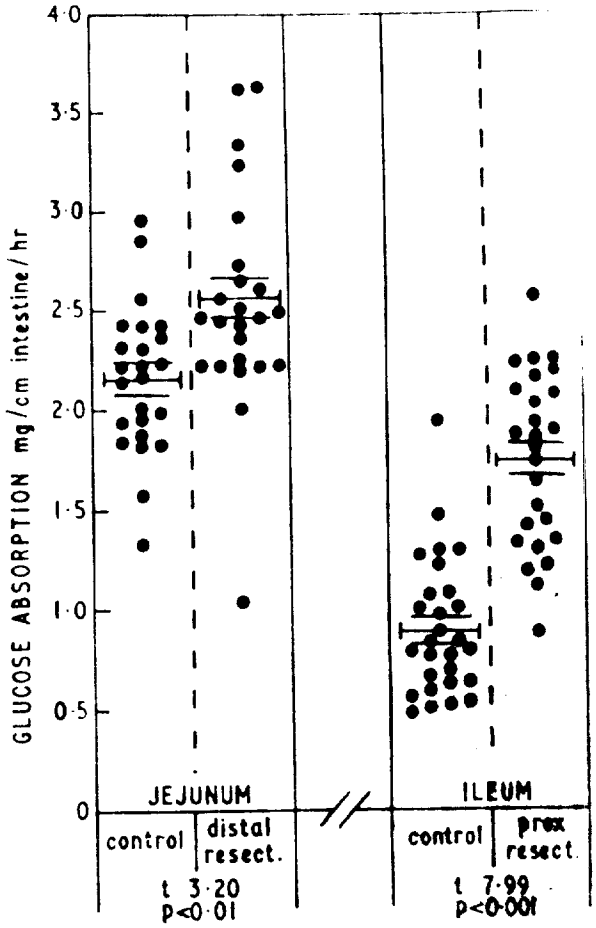

FIG. 2. Mean glucose absorption (± SEM) irrespective of time after operation, from the jejunal and ileal remnants after resection, compared with absorption from corresponding lengths of control intestine in the rat. Concentration of glucose in the infused fluid was 380 mg/100 ml. Absorption is expressed as milligrams per centimeter per hour (14).

WATER-SOLUBLE VITAMINS

As regards the water-soluble vitamins, there is again relatively little direct evidence on sites of absorption in man. No active transport mechanisms have yet been defined for ascorbic acid or for most of the B group vitamins. If their absorption is passive, as has been claimed for riboflavin (36), vitamin C (35), and pyridoxine (10), then absorption should occur proximally. Direct studies were carried out for vitamin C by Nichol-

[illegible] and Chornock (27) using the Miller Abbott tube, and these showed rapid absorption of vitamin C from the jejunum. The mechanism of absorption of folic acid is not yet known, but it may be that some form of facilitated diffusion is involved. Intubation studies demonstrate that the jejunum can absorb folic acid and, as with xylose, the ileum absorbs less (Hepner and Hoffbrand, unpublished data). There are many clinical observations in resection patients which suggest that folic acid is absorbed in the jejunum (11), and similar data are also available for pyridoxine (9), so that the available evidence suggests a proximal absorption site for vitamin [illegible] and many of the B group vitamins.

But vitamin B_{12}, as is well known, behaves quite differently. Vitamin B_{12} is unique in requiring an intrinsic factor secreted by the stomach to ensure its absorption; it is also unique in that there is a curious delay in the transport of B_{12} across the intestinal mucosa during absorption (5, 6); and it has a specific absorption site in the ileum (7). This was first suggested by clinical studies of B_{12} absorption in resection patients by McIntyre et al. (25), and their observation that B_{12} cannot be absorbed in the absence of the ileum has been repeatedly con-

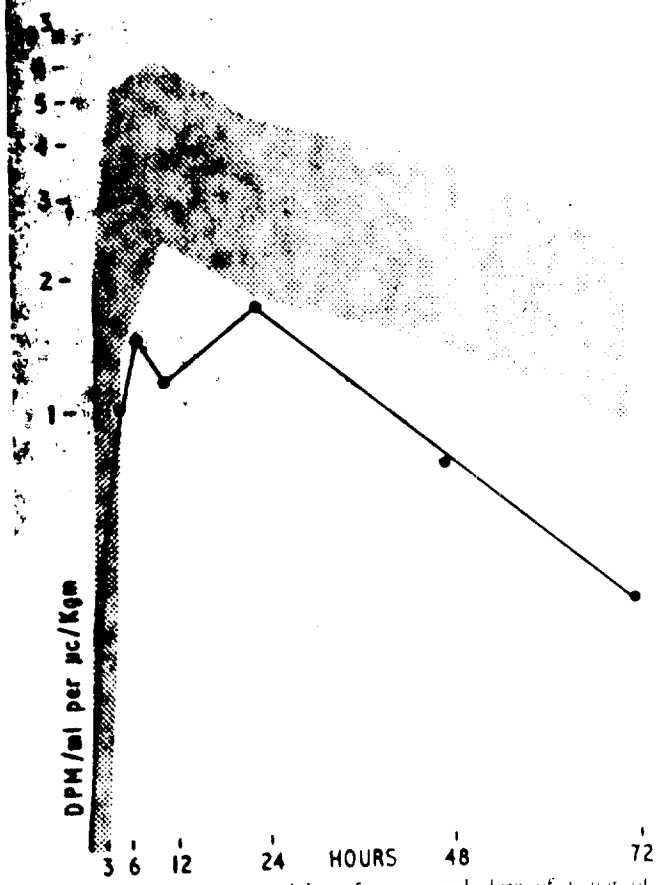

FIG. 3. Plasma radioactivity after an oral dose of 1 mg of H-labeled vitamin D_3 in a patient who had undergone resection of all the small intestine but for the proximal 4 feet of the jejunum. Hatched area indicates the normal range (38).

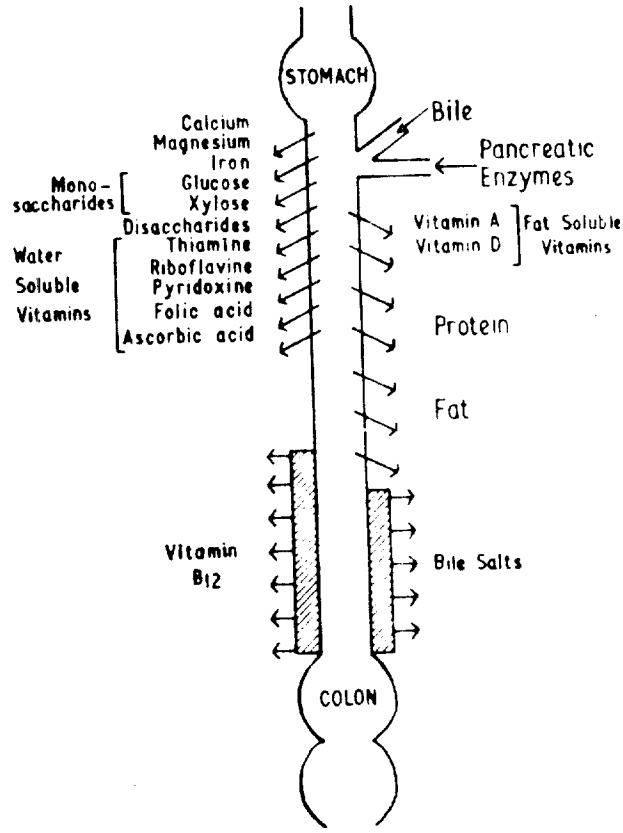

FIG. 4. Sites of absorption in the small intestine [illegible]

firmed. In man, the first direct data on absorption in the ileum were obtained by studying the distribution of radioactivity in the gut at laparotomy, after oral administration of labeled B_{12} (7). Since then ileal absorption of vitamin B_{12} has been demonstrated in man using the double-lumen tube technique by Rennov, Jessen and Hansen (31). In addition, Rosenberg, Lau and Herbert (32) have shown that homogenates of intestinal mucosa from human jejunum cannot take up B_{12} bound to intrinsic factor, whereas the vitamin can be effectively taken up by homogenates of the ileum. Data from at least four different methods of approach therefore indicate that the ileum is the site of absorption of B_{12} [illegible]. A recent paper, however, has suggested that the [illegible] can absorb B_{12} (24). This observation was made by a surgeon who studied B_{12} absorption from [illegible] of intestine isolated between clamps at laparotomy and [illegible] was apparently no difference between [illegible] ileum. Whether such studies have physiological significance, however, is uncertain for McC[illegible] found that intrinsic factor did not seem to be necessary for the B_{12} absorption that was demonstrated.

MINERALS

Finally, the sites of absorption of minerals should be considered. The first point is that although in [illegible]

studies in animals suggest that iron is specifically absorbed in the duodenum, this is certainly not true in vivo. In man, absorption can occur from the ileum if iron is put there through a tube. Similarly with calcium and magnesium, although absorption may take place proximally, distal absorption can also occur and it is not yet known how far the different regions of the gut normally contribute to the absorption of these divalent cations.

CONCLUSION

Figure 4 summarizes diagrammatically what seems to be true about sites of absorption in the small intestine (3). The diagram represents the small intestine as a simple tube, and shows that the proximal part of small intestine is the major area of absorption of dietary constituents. However, the ileum can also ab virtually all the substances normally absorbed proxim and it is therefore a reserve area for the absorptio substances that have escaped absorption proximally when a proximal transport mechanism has been rated, or after resection of the proximal small gut. A although absorption is in general less active in the ile its sluggish motility favors absorption of those substances that reach this region. The ileum, therefore, not only absorbs B_{12} and the bile salts, but also acts as an adaptable functional reserve.

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THE ABSORPTION OF TRITIUM-LABELLED PYRIDOXINE HYDROCHLORIDE IN THE RAT

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Until recently it was only possible to study th eabsorption of pyridoxine indirectly by measuring the urinary excretion of pyridoxine and its metabolites following relatively large oral doses. The initial studies of Scudi, Unna & Antopol (1940) and Scudi, Koones & Keresztesy (1940) in man, the dog and the rat, were made using a colorimetic method for estimating pyridoxine and its metabolites. Although this method fails to estimate 4-pyridoxic acid, now known to be the principal urinary metabolite of pyridoxine in man (Reddy, Reynolds & Price, 1958), these workers did show that pyridoxine was excreted in the urine very soon after it was ingested, and they therefore concluded that absorption must be exceedingly rapid.

Recently pyridoxine hydrochloride labelled with tritium has become available, and it is now possible to measure small amounts of this substance with accuracy. In this paper we report the results of studies of absorption of tritiated pyridoxine in the rat. Observations were made on the rates and sites of absorption of varying doses; the capacity of the small intestine of the rat to absorb increasing amounts of pyridoxine was also studied.

METHODS

Experimental animals. Black and white rats weighing between 200 and 250 g were used throughout the experiments. Most of the observations were made on intact animals. A few studies were also made in a group of five animals in which between 50 and 70 % of the distal small intestine had been removed. These animals were prepared by the operative technique described by Booth, Evans, Menzies & Street (1959), and the experiments were carried out one month later when the animals had fully recovered and were in good health.

[^{3}H]pyridoxine hydrochloride. Commercial pyridoxine HCl prepared by British Drug Houses was labelled with tritium by the Radiochemical Centre, Amersham. The resulting material had a specific activity of 1·6 mc/mg and was 97 % pure by isotope dilution assay.

Radioactive test doses, containing between 0·05 and 5·0 mg of pyridoxine HCl, were fed to the animals. These doses were prepared by dilution of the labelled material and the addition of suitable amounts of unlabelled pyridoxine. Doses were usually adjusted so that 1 ml. contained the required amount of pyridoxine and 1 μc of radioactivity.

Electrophoresis of pyridoxine HCl and 4-pyridoxic acid. Pyridoxine HCl was subjected to paper electrophoresis at 120 V for 6–8 hr, with an acetate buffer (pH 5·1) as described by Siliprandi, Siliprandi & Lis (1954). When electrophoresis of a mixture of 4-pyridoxic acid and pyridoxine HCl was carried out, a carbonate buffer at pH 11·1 was used. Electrophoretic strips were first examined under ultra-violet light, and the pyridoxine band was identified by its fluorescence. They were then stained with diazotized *p*-aminoacetophenone. The bands were identified by comparison with a parallel run of each compound.

Measurement of radioactivity. All radioactive samples were counted in a Packard Tricarb liquid scintillation counter. The liquid scintillator used consisted of 2-5,diphenyloxazole 2 g/l. and 1,4-bis(2,5-diphenyloxazolyl)benzene 0·025 g/l. dissolved in a mixture of equal volumes of toluene and ethanol. Fluid specimens were assayed by mixing 0·25 ml. samples with 8 ml. of liquid scintillator. Internal standards were used throughout and were prepared from 0·25 ml. samples of 1 in 20 dilution of the test dose in the specimen to be assayed. To determine the distribution of radioactivity in electrophoretic strips the paper was cut into 0·5 cm. lengths, each of which was counted directly in 8 ml. of the liquid scintillator.

Experimental procedure

Urinary excretion of radioactivity after 'oral' doses of [^{3}H]pyridoxine HCl. Labelled pyridoxine was given through a fine polythene tube passed into the oesophagus of the unanaesthetized animal. The rats were starved for 24 hr before the test. After the oral doses had been given each rat was placed in a separate metabolism cage (Hanna & Allcock, 1961) and the urine passed during the ensuing 12 hr was collected. Specimens which were contaminated by faeces were discarded. The radioactivity in the urine samples was then measured according to the methods already described.

Six rats were given oral test doses of 0·05 mg of [^{3}H]pyridoxine, first before and then after previous 'saturation' with daily intraperitoneal injections of 10 mg unlabelled pyridoxine for the preceding 3 days. Since relatively little radioactivity was excreted when test doses were given in this way, these rats were then given similar oral test doses of [^{3}H]pyridoxine and at the same time parenteral injections of increasing amounts of non-radioactive pyridoxine were given intraperitoneally, a technique which was found greatly to increase the percentage of the dose excreted in the urine.

In order to determine the absorption of larger amounts of pyridoxine, six other rats were given increasing oral test doses of [^{3}H]pyridoxine (from 0·05 to 5·0 mg) together with a constant intraperitoneal injection of unlabelled pyridoxine, and the urinary excretion of radioactivity was measured.

Nature of urinary radioactivity. The amount of radioactivity in the urine was relatively low when test doses labelled with 1 μc of tritium were used. Four rats were therefore given 0·05 mg of pyridoxine labelled with 40 μc of tritium, in order to obtain highly radioactive urine samples. Two of these rats received this dose only; the other two animals were given a simultaneous injection of 10 mg of unlabelled pyridoxine to increase the urinary excretion of radioactivity. The urine was collected for 12 hr as before. Carrier pyridoxine HCl at a concentration of 5 mg/ml. and 4-pyridoxic acid at 1 mg/ml. were then added to the urine samples and 10 μl. volumes were subjected to electrophoresis in carbonate buffer at pH 11·1.

Rate and site of absorption of [^{3}H]pyridoxine. Two groups of twelve animals were used. Test doses of 0·05 mg [^{3}H]pyridoxine were given to the first group of animals; the second group received doses of 5·0 μg [^{3}H]pyridoxine. Three animals from each group were then killed by ether anaesthesia at each of the following times after the oral test doses: 7½, 15, 30 and 60 min. After death, the gastro-oesophagal junction, pylorus and terminal ileum were clamped and the stomach and entire small intestine were removed. The small intestine was divided into four segments of equal length as follows: (1) duodenum and upper jejunum; (2) lower jejunum; (3) upper ileum; (4) lower ileum. The contents of the stomach and each segment of the small intestine were then washed out with 10 ml. of normal saline and the amount of radioactivity in the washings was measured.

Effect of resection of the distal small intestine. Absorption tests were also carried out in the five animals in which between 50 and 70 % of the distal small intestine had been resected. These animals were given successive oral doses of between 0·05 and 5·0 mg of [^{3}H]pyridoxine together with an intraperitoneal injection of 10 mg of unlabelled pyridoxine. The urinary excretion of radioactivity was measured as in normal rats.

Absorption from the stomach was studied in three rats in which the gastro-oesophagal junction and pylorus had been ligated under ether anaesthesia, and in which 0·05 mg of [^{3}H]pyridoxine (contained in 0·5 ml. volume) was injected into the stomach. The animals were allowed to recover and were killed half an hour later. The stomachs were washed out with normal saline and the radioactivity in the washings was measured.

Comparison of absorption from jejunum, ileum and colon. Three groups of five rats were used. Under ether anaesthesia 0·05 mg of [^{3}H]pyridoxine contained in a volume of 0·25 ml. was injected from a graduated Mantoux syringe directly into the upper jejunum, upper ileum or caecum. At the same time 10 mg of unlabelled pyridoxine was given intraperitoneally, to ensure maximal excretion of radioactivity. The animals were then placed in separate metabolism cages and allowed to recover. Urinary radioactivity was measured as already described.

RESULTS

Electrophoresis of [^{3}H]pyridoxine HCl

[^{3}H]pyridoxine, to which carrier pyridoxine at a concentration of 5 mg/ml. had been added, was subjected to electrophoresis in acetate buffer at pH 5·1. The ultra-violet fluorescence due to pyridoxine and the distribution of radioactivity in the electrophoretic strip are shown in Fig. 1.

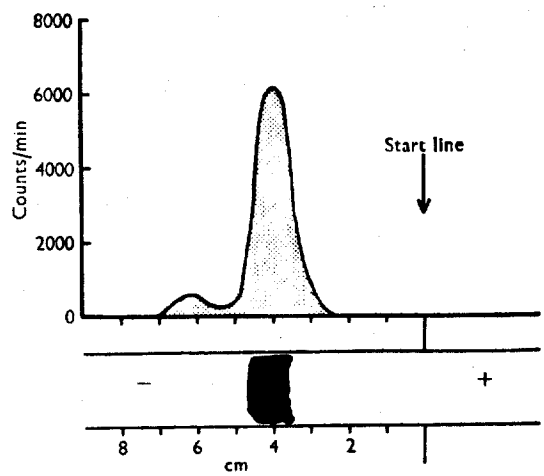

Fig. 1. Electrophoresis of [^{3}H]pyridoxine HCl in acetate buffer at pH 5·1. In Figs. 1, 2 and 4; above, distribution of radioactivity; below, ultra-violet fluorescence.

More than 50 % of the radioactivity corresponded to the position of pyridoxine HCl (Fig. 1). A small fraction (less than 5 %) was represented by a faster-moving component. This radioactivity did not correspond with any

known pyridoxine derivative and the nature of the impurity remains uncertain.

TABLE 1. Urinary radioactivity during 12 hr after oral doses of 0·05 mg [^{3}H]pyridoxine

Rat no.	Percentage of dose excreted	
	Before saturation	After saturation*
1	34·1	30·5
2	40·0	31·6
3	34·8	15·3
4	35·0	18·2
5	43·7	38·7
6	16·6	23·0
Mean ± S.D.	34·0 ± 8·9	26·2 ± 8·2

* Animals were 'saturated' by intraperitoneal injection of 10 mg pyridoxine HCl daily for 3 days.

Urinary excretion of radioactivity after oral doses

Effect of 'saturation' with pyridoxine HCl

Six rats (Nos. 1–6) were given test doses of 0·05 mg of [^{3}H]pyridoxine. They were then given the same test dose after previous 'saturation' with daily intraperitoneal injections of 10 mg unlabelled pyridoxine HCl for 3 days. The percentage of these doses excreted in the urine is given in Table 1. These animals excreted between 16·6 and 43·7 % (mean 34·0 %) of the first test dose, and there was no significant difference when the doses were given after the animals had been 'saturated' (Table 1).

Nature of urinary radioactivity. The results of electrophoresis of the urine collected after an oral dose of 0·05 mg of pyridoxine HCl labelled with 40 μc of tritium are shown in Fig. 2. Unlabelled pyridoxine HCl and 4-pyridoxic acid were added to the urine as markers. Approximately 60 % of the excreted radioactivity corresponded to the position of pyridoxine HCl. The remainder moved to a position between that of pyridoxine and 4-pyridoxic acid (Fig. 2).

Effect of simultaneous parenteral injection of increasing amounts of unlabelled pyridoxine HCl

The six rats (Nos. 1–6) were next given successive test doses of 0·05 mg of [^{3}H]pyridoxine and at the same time injections of unlabelled pyridoxine was given intraperitoneally, the parenteral injection being increased from 0·5 to 8·0 mg. The amounts of radioactivity excreted are illustrated in Fig. 3.

The amount of radioactivity excreted when the dose was given alone ranged from 18·2 to 38·3 % (Fig. 3). When a parenteral injection of non-labelled pyridoxine was given at the same time as the test dose was fed, larger amounts of radioactivity were excreted. The mean excretion after a parenteral dose of 0·5 mg was 54·8 % (range 31·2–70·2). When larger

parenteral injections were given the urinary excretion of radioactivity increased, the mean excretion reaching a maximum of 83·6% (range 80·0–94·0) when 8·0 mg was injected (Fig. 3).

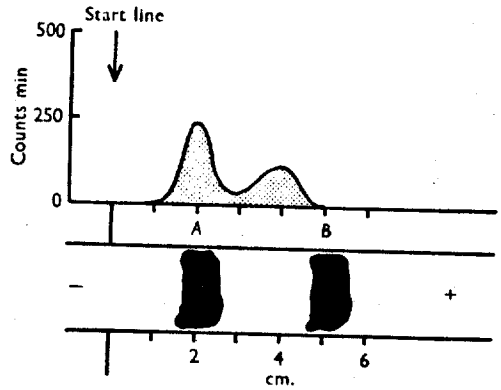

Fig. 2. Electrophoresis of urine after oral dose of [^{3}H]pyridoxine HCl given alone. Pyridoxine HCl (*A*) and 4-pyridoxic acid (*B*) have been added as markers. Carbonate buffer at pH 11·1.

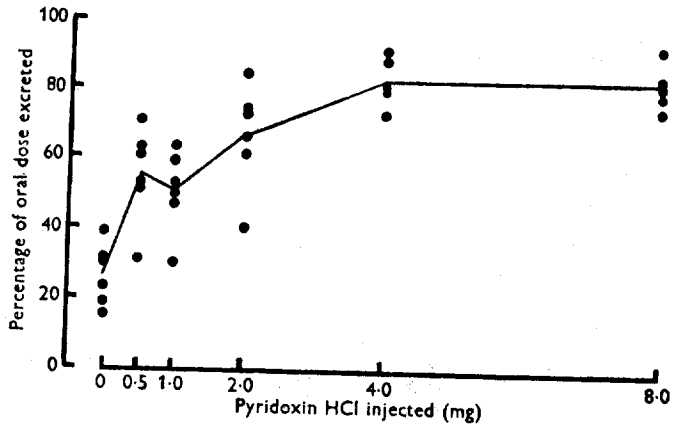

Fig. 3. Effect of simultaneous parenteral injections of non-radioactive pyridoxine HCl (from 0·5 to 8 mg) on the urinary excretion of radioactivity in 24 hr following an oral dose of 0·05 mg [^{3}H]pyridoxine HCl.

Nature of urinary radioactivity. Electrophoresis of the urine collected after an oral dose of 0·05 mg of pyridoxine HCl labelled with 40 μc of tritium, accompanied by a parenteral injection of 8 mg pyridoxine, was carried out as already described. The results are shown in Fig. 4. Most of the urinary radioactivity corresponded to pyridoxine HCl. There was also a small amount of the faster moving component already noted (Fig. 2)

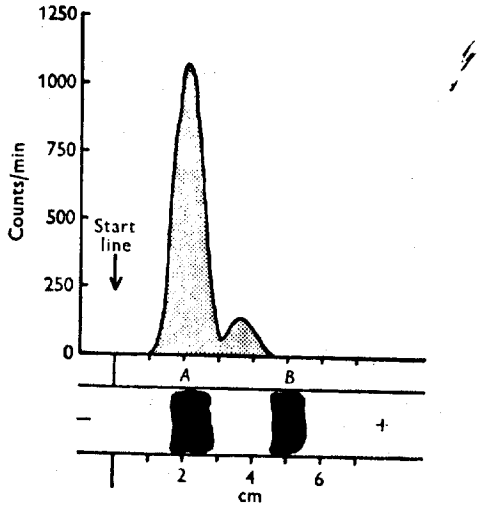

Fig. 4. Electrophoresis of urine after oral dose of [^{3}H]pyridoxine HCl given with simultaneous parenteral injection of 10 mg non-radioactive pyridoxine. Markers and buffer as in Fig. 2.

TABLE 2. Percentage of [^{3}H]pyridoxine excreted in urine by normal rats during 12 hr after oral doses of 0·05–5 mg*

Rat no.	[^{3}H]pyridoxine fed (mg)				
	0·05	0·1	0·5	1·0	5·0
7	86·0	83·9	73·0	79·0	63·5
8	59·8	72·2	53·0	73·5	56·0
9	74·0	72·3	52·1	76·2	78·5
10	60·0	77·0	53·5	59·0	73·5
11	89·0	80·2	56·8	62·7	74·5
12	—	73·0	—	—	63·5
Mean ± S.D.	73·7 ± 12·4	76·4 ± 4·4	57·7 ± 7·8	70·1 ± 7·8	68·2 ± 7·8

* 10 mg of unlabelled pyridoxine HCl was given intraperitoneally at the same time as the oral doses were fed.

Increasing oral doses of [^{3}H]pyridoxine HCl

Six other rats (Nos. 7–12) were given increasing oral test doses of [^{3}H]-pyridoxine (from 0·05 to 5·0 mg) together with an intraperitoneal injection of 10 mg of unlabelled pyridoxine to ensure maximal excretion of radioactivity. The percentages of these doses excreted in the urine are given in Table 2 and the actual amounts of radioactivity excreted, expressed as mg of [^{3}H]pyridoxine, are illustrated in Fig. 5. As Table 2 shows, there was little difference in the percentage of the dose excreted in the urine, irrespective of the size of the oral test dose. The mean excretion after a

dose of 0·05 mg was 73·7 % (range 59·8–89·0) and the percentage excretion was not significantly different after a 5·0 mg dose (range 56·0–78·5, mean 68·2 %). In terms of the actual amount of pyridoxine excreted, however, there was a progressive and linear increase in the excretion with increase in the amount of pyridoxine fed (Fig. 5).

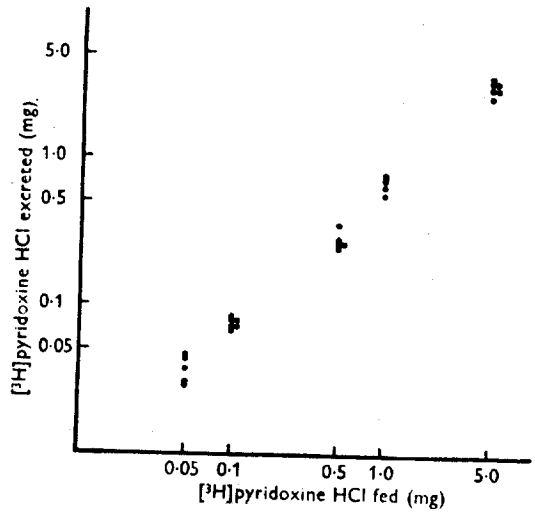

Fig. 5. Amounts of [^{3}H]pyridoxine HCl excreted in the urine of normal rats during 24 hr after increasing oral doses of [^{3}H]pyridoxine (from 0·05 to 5·0 mg). A 'flushing' injection of 10 mg was given simultaneously.

Relationship between rate and site of absorption

Rate of absorption

The absorption by the animals killed at intervals of 7½–60 min after being given either 0·05 or 5·0 mg of [^{3}H]pyridoxine was calculated by assuming that the radioactive material not recovered had been absorbed. The percentage of the dose absorbed by each rat is illustrated in Fig. 6; the mean absorption at the varying time intervals after feeding is also shown. The initial rate of absorption of pyridoxine was remarkably rapid and both doses were absorbed at a similar rate (Fig. 6). By 15 min the mean absorption was 74·7 % after the 0·05 mg dose and 69·2 % after the dose of 5·0 mg. After this time absorption continued more slowly, so that by 60 min more than 90 % had been absorbed from either dose (Fig. 6).

Distribution of radioactivity in the gastro-intestinal tract

The mean percentage of the administered radioactivity found in the stomach and the four different segments of the small intestine of the three

rats killed at each time are shown in Fig. 7. The range of results in each group of animals is also shown.

At 7½ min after the doses were fed most radioactivity was present in the stomach (Fig. 7). The gastric radioactivity had fallen considerably by 15 and 30 min, and at 60 min after either dose less than 2 % of the administered pyridoxine remained.

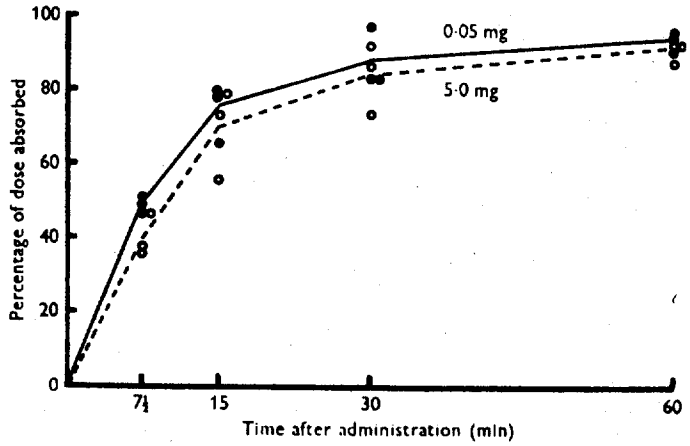

Fig. 6. Percentage absorption of [^{3}H]pyridoxine HCl by rats killed at varying intervals of time after receiving oral test doses of 0·05 (●) or 5·0 mg (○). Mean absorption at each time is indicated by the two lines.

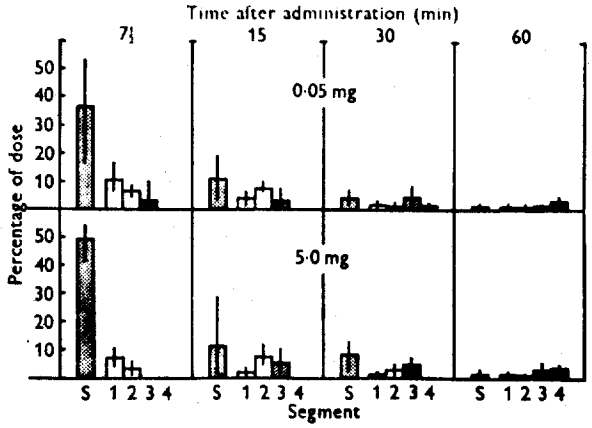

Fig. 7. The columns represent the mean amount of radioactivity in stomach ▤ and four equal segments of the small intestine of three rats killed at intervals of 7½–60 min after receiving 0·05 or 5·0 mg of [^{3}H]pyridoxine HCl orally. The intestinal segments were numbered as follows: (1) Duodenum and upper jejunum □; (2) lower jejunum ▢; (3) upper ileum. ▨; (4) lower ileum ▨ The range of results is indicated by the vertical line.

Despite the rapid gastric emptying, the amounts of either dose recovered from segments 1 and 2 were rarely as high as 10 % (Fig. 7). The third segment contained virtually no radioactivity after either dose at 7½ min, except in a single animal. At 15 min the mean amount of radioactivity in this segment had risen to 3·1 % after the 0·05 mg dose and to 5·9 % after the larger dose. Less than 5 % was present in segment 3 in any of the animals at 30 or 60 min.

Insignificant amounts of radioactivity were found in the fourth segment at 7½ or 15 min after either test dose. After half an hour a small percentage was recovered from this segment in two of the animals given the smaller dose, but there was no radioactivity in the fourth segment at this time following the 5·0 mg dose; by 60 min the mean radioactivity in segment 4 had risen to 3·2 % after the 0·05 mg dose and to 3·3 % after the larger dose (Fig. 7).

Effect of resection of the distal small intestine

The animals in which between 50 and 70 % of the distal small intestine had been resected were given test doses of between 0·05 and 5·0 mg of [^{3}H]pyridoxine. They also received an intraperitoneal injection of 10 mg unlabelled pyridoxine to ensure maximal excretion of radioactivity. The percentage of the oral dose excreted in the urine is given in Table 3; the actual amount of labelled pyridoxine excreted is shown in Fig. 8. The amounts of [^{3}H]pyridoxine excreted in the urine by these animals (Fig. 8) were similar to those in the normal animals in which no resections had been carried out (Fig. 5).

TABLE 3. Percentage of [^{3}H]pyridoxine excreted in urine during 12 hr after oral doses of 0·05–5·0 mg* by rats subjected to resection of distal small intestine

Rat no.	[^{3}H]pyridoxine fed (mg)				
	0·05	0·1	0·5	1·0	5·0
37	77·1	78·0	62·6	60·8	75·0
38	66·0	77·0	71·5	79·0	78·0
39	50·5	84·1	87·5	—†	—†
40	67·0	80·5	85·1	67·7	84·0
41	—†	77·0	76·9	—†	—†
Mean	65·1	79·3	80·6	69·2	78·2

* 10 mg of unlabelled pyridoxine HCl was given intraperitoneally at the same time as the oral doses were fed. † Urine contaminated with faeces during experiment.

Comparison of absorption from stomach, jejunum, ileum and colon

Stomach. The percentages of the dose recovered from the stomachs of the three rats given 0·05 mg of labelled pyridoxine intragastrically, with the pylorus and oesophagus ligated, were 99, 101, and 98 %, respectively, indicating that no significant absorption of pyridoxine occurs from the stomach.

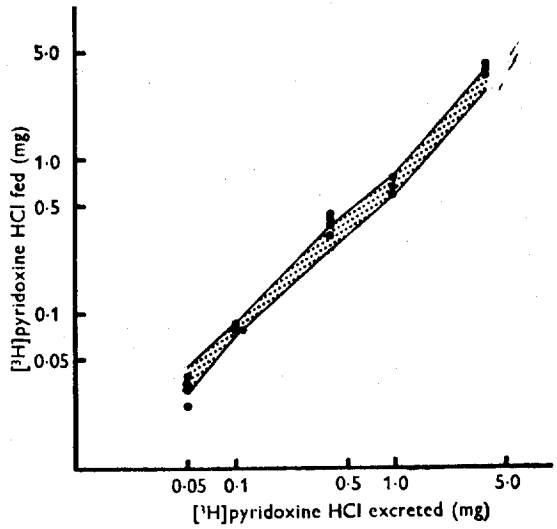

Fig. 8. Amount of [^{3}H]pyridoxine HCl excreted in the urine of rats subjected to resection of the distal small intestine during 24 hr after increasing oral doses of [^{3}H]pyridoxine HCl from 0·05 to 5·0 mg. A 'flushing' intraperitoneal injection of 10 mg was given simultaneously. The range of results in normal animals is indicated by the hatched area. Each point represents the amount of [^{3}H]pyridoxine excreted by a single resected animal.

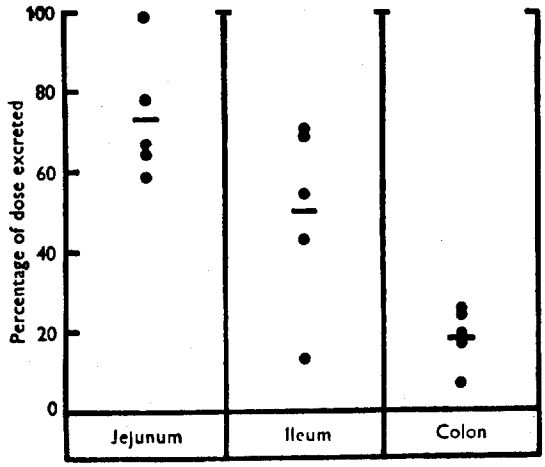

Fig. 9. Comparison of urinary excretion of radioactivity after 0·05 mg of [^{3}H]-pyridoxine was introduced directly into jejunum, ileum or colon.

Jejunum, ileum or colon. The amounts of radioactivity excreted in the urine by each group of five rats given 0·05 mg [^{3}H]pyridoxine directly into the jejunum, ileum or caecum are shown in Fig. 9; an intraperitoneal injection of 10 mg unlabelled pyridoxine was given at the same time to ensure maximal excretion of radioactivity. From 59 to 99 % (mean 71 %) was excreted when the dose was given into the upper jejunum (Fig. 9). The amount excreted after injection into the ileum of the test dose ranged from 13 to 70 % (mean 50 %). Very much less radioactivity was excreted when the dose was given into the caecum (range 7–27 %, mean 18 %) (Fig. 9).

DISCUSSION

The results given in this paper illustrate that [^{3}H]pyridoxine HCl may be used for absorption studies. When the radioactive vitamin is given to rats orally, relatively little radioactivity is excreted in the urine (Table 1). However, if the oral test dose is accompanied by a large parenteral injection of non-radioactive pyridoxine HCl very much larger amounts of radioactivity are excreted (Fig. 3, Table 2). With this technique it is possible to study absorption by measuring the urinary excretion of radioactivity after giving an oral test dose of [^{3}H]pyridoxine HCl.

The results indicate that the intestinal tract of the rat has a remarkable capacity to absorb pyridoxine. When increasing doses of labelled pyridoxine (from 0·05 to 5·0 mg) are given, increasing amounts are excreted in the urine, indicating an increasing absorption (Fig. 5). The daily pyridoxine requirement of a 200 g rat has been estimated to be approximately 10 μg (Brown & Sturtevant, 1949). Yet the rat is clearly able to absorb as much as 500 times its daily requirement with ease (Fig. 5). This extraordinary capacity for pyridoxine absorption in the rat appears to be a function of the jejunum. As is shown by the results in Fig. 6, absorption of either a small or a large dose of pyridoxine occurs very rapidly and this absorption takes place in the jejunum regardless of the size of the dose, relatively little radioactivity ever reaching the ileum when either 0·05 or 5·0 mg of pyridoxine HCl was fed (Fig. 7). The results in the rats subjected to resection of the distal small intestine confirm the importance of the jejunum in the absorption of pyridoxine, for even when the largest doses were given to these animals, absorption was as complete as in normal rats (Fig. 8).

This response of the jejunum to increasing oral doses of pyridoxine suggests that absorption occurs by diffusion. For if absorption of a substance occurs through an active transport mechanism, it may be possible to saturate the absorptive capacity of this system. In the case of a substance normally absorbed proximally by such a mechanism, the site of absorption becomes progressively more distal with increase in the dietary

load. Such a response of the small intestine has been demonstrated for fat (Booth, Read & Jones, 1961; Booth, Aldis & Read, 1961), a substance which is generally considered to be absorbed by active mechanisms. The failure of even 5 mg of pyridoxine to saturate any more of the jejunum than the dose of 0·05 mg suggests that there may be no transport mechanism and that absorption may be occurring by diffusion. This conclusion is supported by unpublished observations of J. S. Stewart and C. C. Booth on everted sacs of rat intestine, which have failed to demonstrate any active transport system for pyridoxine absorption *in vitro*. Although the jejunum appears to be the major site of absorption, the ileum is also capable of absorbing this vitamin and some absorption may occur from the colon when pyridoxine is directly introduced into this organ. These observations are in keeping with a diffusion mechanism of absorption. Evidence suggests that other water-soluble vitamins of the B group may be absorbed in the same way (Morrison & Campbell, 1960; Spencer & Zamcheck, 1961; Turner & Hughes, 1962).

The labelled pyridoxine used in this study appears to be stable and was not broken down by absorption or by metabolism in the body, for most of the radioactivity excreted in the urine was shown to be due to labelled pyridoxine (Figs. 2 and 4). Furthermore, the great increase in urinary excretion when intraperitoneal injections of non-radioactive material are used to 'flush' radioactivity from the body suggests that the labelled pyridoxine behaves in the same way as the unlabelled vitamin.

The urine also contained small amounts of radioactivity which did not appear to correspond electrophoretically to any known derivative of pyridoxine but which was present in the original labelled material (Fig. 1). The nature of this radioactivity remains to be determined.

SUMMARY

1. The absorption of oral doses of tritium-labelled pyridoxine HCl (from 0·05 to 5·0 mg) was studied in the rat. The results indicate that pyridoxine is absorbed rapidly from the upper intestine regardless of the size of the dose given. Absorption may also occur from the ileum and to a small extent from the colon.

2. There is a linear relationship between oral dose and the amount absorbed in normal animals and in those in which the distal small intestine has been resected. These observations suggest the possibility that pyridoxine may be absorbed by diffusion.

3. The tritium-labelled material used in this study was more than 95 % pure. The label did not appear to become detached during absorption or passage through the body. Urinary excretion experiments with a simul-

taneous parenteral injection of unlabelled pyridoxine suggested that the radioactive vitamin behaved in a similar way to the non-radioactive pyridoxine.

We wish to thank Dr J. H. Belcher for advice on the assay of tritium in tissues and body fluids and Miss Janet Heath for her assistance.

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WATER-SOLUBLE VITAMINS, PART II[1,2]

(PANTOTHENIC ACID, THIAMINE, LIPOIC ACID, RIBOFLAVIN, VITAMIN B_6, NIACIN, ASCORBIC ACID, AND MISCELLANEOUS FACTORS)

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INTRODUCTION

The year 1953 witnessed the further acceleration of a trend that has been in progress in vitamin research for several years, away from observations on whole animals and toward an examination of vitamin action at the cellular or subcellular level. The latter approach originally furnished information concerning individual enzyme systems, which has been continually expanded into an integrated picture of larger areas of metabolism and physiologic behavior.

The fields of vitaminology and enzymology become identified with each other at the molecular level, and the extension of our knowledge of the finer reaction mechanisms that include these vitamins has enabled biochemists to explain an increasing number of aspects of metabolism in terms of simple chemical reactions. Especially valuable, for example, have been the identification of "active acetate" as acetyl coenzyme A, and the discovery of the importance of other acyl coenzyme A compounds in fat metabolism; the studies on interaction between coenzyme A and thiamine and lipoic acid containing systems are clarifying to a considerable extent the important details of the metabolism of two-carbon compounds. The finding that metals occur as integral units of flavoproteins, and new information on the behavior of ascorbic acid, add to the assurance that the current emphasis on enzymology in vitamin research will be maintained.

[1] This review deals with the vitamins connected with C_2 metabolism (pantothenic acid, thiamine, and lipoic acid); with the electron transport vitamins, riboflavin, and niacin; the B_6 group; ascorbic acid; and new growth factors. Inositol is omitted because of the relatively few papers that were devoted to it during the past year.

The literature survey was concluded with journals available to the authors to December, 1953.

[2] The following abbreviations are used: Acetyl LBF for acetyl pentetheine; ADP for adenosinediphosphate; AMP for adenosine-5'-phosphate; ATP for adenosinetriphosphate; CoA for coenzyme A; DPN for diphosphopyridine nucleotide; DPNH for reduced diphosphopyridine nucleotide; TPP for thiamine pyrophosphate (diphosphothiamine); FAD for flavin-adenine-dinucleotide; FMN for flavin mononucleotide; GAP for glyceraldehyde-3-phosphate; LBF for *Lactobacillus bulgaricus* factor = pantetheine; LBF-γ-phosphate for pantetheine-4'-phosphate; LTPP for lipothiamide pyrophosphate; NMN for N'-methyl nicotinamide; PA for pantothenic acid; PAB for p-aminobenzoic acid; PAC for pantothenic acid conjugate; P-P for inorganic pyrophosphate.

This emphasis, which will also be felt in the present review, should not be interpreted to detract from the significance of many nutrition type experiments. These continue to be necessary, not only for themselves but also to provide a background against which subcellular studies may be evaluated. They have been included in this discussion wherever possible, within the space limitations imposed.

Pantothenic Acid

Information has accumulated during the past year which consolidates the position of coenzyme A (CoA[2]) in the formation of "active acetate," and also gives further evidence of the versatility of this coenzyme as a biocatalyst. Since several of its functions are considered in detail in the chapter on carbohydrate metabolism, it will suffice here to record that CoA has been shown in the laboratories of Green, Lynen, Ochoa, and Stadtman to be a key component in systems for fatty acid breakdown, through the formation of acyl and β-ketoacyl CoA derivatives (1 to 5), probably at each step in the "cycle" (3) whereby the fatty acids are oxidized into C_2 units (acetyl CoA) (5). Moreover, the reactions appear to be reversible in purified enzyme preparations (5, 6). The latter studies thus confirm at the molecular level the observations (7, 8) that CoA catalyzes the formation of longer chain fatty acids (as well as sterols) from acetate and open to view the extended series of degradations that occur during the burning of fatty acid molecules. Related observations (9, 10) indicate that α-glycerol phosphate is esterified with fatty acid CoA derivatives, and thus the biosynthesis of phospholipids and neutral fats may also be foreshadowed through the use of this coenzyme. Other reactions that appear to be CoA-dependent are the enzymatic decarboxylation of malonate (11) and succinate (12, 13) in microorganisms, the furoylation of glycine in liver and kidney preparations (14), and probably the acylation of glycine in *Clostridium kluyveri* (14a). Since neither of the latter decarboxylations also involves a carbonyl group in the substrate (in contrast to pyruvate, α-ketoglutarate, or β-keto acids), this may be viewed as a new type of CoA-dependent cleavage (possibly through malonyl or succinyl phosphate), to yield acetate and propionate, respectively. The co-dependence of succinate decarboxylation upon thiamine pyrophosphate (TPP[2]) and the simultaneous formation of CO_2 in both reactions, may suggest an interrelationship between the CoA-systems and those involved in production of the active aldehyde fragment.

The importance of acyl CoA derivatives has prompted several workers to investigate methods for their preparation in highly purified form. Numerous enzymatic syntheses have been described (1, 15, 16). Among the nonenzymatic methods, thioacetic acid (17), S-acetylthiophenol (18), thiopalmitic acid (10), and succinic (2), crotonic (19), and benzoic (20) anhydrides have been used as acylating agents. Acetyl pantetheine (Acetyl LBF[2]) has been made from LBF[2] with either acetylthiophenol (18) or acetyl chloride (21) and from pantothenic ethyl imide with thioacetic acid (22). High yields to 90%

...at) purity preparations of CoA for these reactions can be readily isolated ...y adsorption and elution from charcoal (23), followed by chromatography ...o charcoal (23, 24) and Dowex 50 or Dowex 1, with or without the interme-...iate co-precipitation of CoA with glutathione as a copper salt.

Although the structure of CoA has not yet been completely confirmed by synthesis, it seems reasonably clear that the configuration given in a previous review (25) is essentially correct. Hydrolysis of the third phosphate group (a monophosphate) indicates (26) that the linkage corresponds to that in adenylic acid *b*; this may suggest attachment at carbon 3′ of the adenylic residue. Other data relating to degradation of CoA and enzymatic resynthesis, which generally confirm the structure of the coenzyme, have been effectively reviewed elsewhere (27 to 30). The conversion of LBF to CoA in liver tissue was pictured as proceeding via (*a*) a phosphorylation (with ATP[2]) to produce pantetheine-4′-phosphate (LBF-γ-phosphate[2]) (30, 31); (*b*) condensation of the latter with ATP to give "dephospho CoA"; (*c*) phosphorylation of this product with ATP to yield CoA (32). These reactions were catalyzed by soluble, nonparticulate enzyme fractions. Yields of 80 to 90 per cent were reported for the conversion of LBF-γ-phosphate to CoA (29).

The isolation of LBF of about 50 per cent purity has been described, from culture filtrates of *Ashbya gossypii* (33), and methods for the synthesis of this compound in pure form have been developed in several laboratories. Four of these procedures (34 to 37) involved the condensation of the sulfur-containing moiety with β-alanine or a derivative to form "β-aletheine" (35), followed by coupling with pantolactone. A fifth method (22) employed the formation of pantothenic ethylene imide, followed by S-acylation with thio- or phenylthioacetic acid, and hydrolysis of the mercaptoester. Good yields were generally claimed.

The question naturally arises concerning the possible biological activity *per se* of the various moieties and sub-units of CoA. Several of them are active presumably as precursors of CoA. An organism that responds especially well to these forms is *Acetobacter suboxydans*, which was early shown to be more responsive to CoA and PAC[2] (38, 39, 40) than to free pantothenic acid (PA[2]). Likewise, the pyrophosphatase-split product from CoA was active, and was called the "*A. suboxydans* stimulatory factor" (41). It was first regarded as a pantothenic acid phosphate but was later judged to be LBF-γ-phosphate (42). More recently, pantothenyl cysteine was found (43) to strongly stimulate this organism, and was considered to be a precursor of LBF and CoA in *A. suboxydans*. An inventory was therefore made of the activity of various PA derivatives for the bacterium, and the following order of effectiveness was established (44, 45): LBF-γ-phosphate = CoA > pantothenyl cysteine = LBF (approx.) > PA = pantoic acid > LBF diphosphate ≧ PA-α- or γ-phosphate (→0), with CoA and LBF-γ-phosphate about 10 to 20 times as active as CoA. It is evident from these results that growth stimulation in *A. suboxydans* is not the property of a single compound in the PA-CoA family. Of interest was the fact that the sulfur-containing structures were active princi-

pally, if not exclusively, in the reduced state. Apparently this organism, despite its facility for converting pantoic acid through several intermediates into CoA, lacks the ability to reduce the S—S bond in these compounds once they are formed.

The foregoing series of studies do not support the idea that PA derivatives other than CoA may possess intrinsic enzymatic activity. The latter view might be held, from analogy with flavin- and pyridinenucleotides, and from the observation (46) that rat growth assay values for PA were higher than microbiological values in certain materials, under conditions where CoA and other known PA conjugates were believed to be completely converted to PA for the microbial tests. The higher rat assay values may have a partial explanation in the finding (43) that avian liver enzyme preparations which cleave CoA to PA are only feebly active upon pantothenylcystine. If the latter compound were active for the rat, its presence in natural materials could contribute to the total apparent PA content, and indeed the microbial values would then be spuriously low on account of incomplete liberation of PA by the enzyme treatment. The distribution of pantothenyl cyst(e)ine in natural materials requires additional study, and a further inventory of PA derivatives is needed, against the possibility that minor, yet possibly important relatives of CoA may have thus far been obscured from view.

The biosynthesis of PA has also been examined. It is reportedly increased in streptomycin-resistant strains of *Escherichia coli* (47). The reason is unknown but the experiments recall the report (48) that an alternate scheme may exist in some organisms for the condensation of pyruvate and oxalacetate, which is affected by streptomycin. Possibly the CoA requirements of such a condensation (and the succeeding degradations) are different from those encountered in the traditional Krebs cycle. The coupling of β-alanine to pantoate in *E. coli* has been shown to be mediated by ATP, with cleavage of the latter to form AMP[2] and P-P[2] (49). ADP[2] could not substitute for ATP as an energy source. Preliminary to this coupling, pantoate was thought on the basis of studies with *E. coli* mutants (50) to be formed from "dihydroxyvaline" or "ketovaline," which had also been viewed as precursors of valine (51). The proposed reaction sequence is shown, as follows:

$$\underset{\text{"dihydroxyvaline"}}{(CH_3)_2C(OH)\cdot CHOH\cdot COOH} \rightarrow \underset{\text{"ketovaline"}}{(CH_3)_2CH\cdot CO\cdot COOH} \rightarrow \text{valine}$$

$$\underset{\text{"ketopantoic acid"}}{CH_2OH\cdot CH(CH_3)_2\cdot CO\cdot COOH} \rightarrow \text{pantoic acid}$$

This sequence differs from earlier suggestions regarding pantoate synthesis in that neither valine (52) nor the α-amino analogue of pantoic acid [pantonine (53)] is regarded as a direct precursor. However, "ketopantoic acid," in contrast to pantonine, has been shown in two laboratories (51, 54) to be an effective growth promoter in a pantoicless mutant and also to be a potent reversing agent of salicylate inhibition. Evidence for the steps preceding

-mation of ketopantoic acid was obtained in the manner common to mu-
-t studies: accumulation of intermediates in blocked mutants and growth
f the latter with supposed products of these intermediates. Although such
-eriments when taken alone may not reveal the major normal pathways of
-etabolism, nevertheless the scheme offered appears to fit the available data
-d furnishes a pattern for further examination of these interconversions.

The formation of ketopantoate in the foregoing outline involves a hydrox-
-ethylation; this may suggest the participation of the folic acid-citrovorum
-ctor group. Of possible special interest, then, are the continuing studies on
-terium linens (55, 56), in which the biosynthesis of PA was mediated by
-aminobenzoic acid (PAB[2]) (although only slightly by folic or folinic acids).
-ese studies may thus also point to a possible function for PAB apart from
-contribution to the folic acid molecule. In addition, the unique feature
-s presented that PA and PAB were alternately required for growth; in the
-ersence of either vitamin, the other was synthesized. Growth inhibition by
-lfanilamide could be reversed not only by PAB but also by PA, pantoic
-d, or biotin. The interrelationships that exist here are unclear, but they
-all related observations in rats (57) and *E. coli* (58). It seems evident that
-AB functions in the synthesis of PA, and possible that CoA or biotin or
-th may be involved in the formation of PAB or other aromatic compounds.
-periments with appropriate cell-free systems should aid materially in ex-
-ining these interesting relationships.

The interactions between PA and other vitamins or hormones are even
-s understood. Much attention has been paid to the influence of PA, as
-as ascorbic acid,[3] on adrenal function, and progress in this field has been
-ewed elsewhere (30, 59, 60). In passing, it should be emphasized that a
-e relationship exists between PA (presumably as CoA) content of the
-es and adrenal cortex function. Although many of the changes are com-
-, perhaps as a result of participation of the pituitary, it would appear
-t most of the adrenal dependence upon PA has its basis in the catalysis of
-id synthesis, particularly cholesterol, by CoA. Some current articles in
-field (61, 62) reporting the response of normal and PA-deficient rats to
-s, fit reasonably into this pattern, although in two studies (62, 63) it was
-ested that some cortisone-like hormone or hormones may still be pro-
-d in the PA-deficient rat. The complex character of this deficiency is ex-
-ted by the fact that, despite many evidences of improvement in adrenal
-tion by administration of adrenal cortex hormones to PA-deficient rats,
-er cortisone, ACTH, thyroxine, growth hormone, nor anterior pituitary
-ct was able to improve their resistance to a cold environment (64). Thy-
-e was suggested as a possible regulator of the CoA level in the tissues
-on the basis of an observed increase in CoA after administration of the
-one to thyroidectomized animals.

[3] For a discussion of ascorbic acid-adrenal relationships, cf. section on ascorbic
-

A colorimetric assay has been developed for PA (66) which depend
acid hydrolysis and coupling of the liberated pantoic acid with 2,7-naph
lenediol. The method is rapid, and good precision and agreement with
biological values are claimed, among the high potency vitamin mix
tested. The method was apparently not intended for use with most
materials, and indeed it seems probable that many interfering comp
normally present would invalidate the assay.

Alloxan inactivation of CoA (nonenzymatic) has been reported (67),
the suggestion that the diabetogenic effect of alloxan may in part be attr
able to its reactivity with the sulfhydryl group of CoA in the beta cell
the pancreas.

In an effort to obtain a better understanding of the chemistry of the
ester bond, numerous model compounds and reactions have been exam
including the reaction between amines and thiocarboxylic acids (68), an
cleavage of various thioesters by hydrolysis or aminolysis (69, 70). Sub
uent groups in the compounds were varied considerably. The thioac
thioester groups were all highly reactive, with aminolysis (including rea
with hydroxylamine) being more rapid than alkaline hydrolysis, and th
turn more than acid hydrolysis. The competition among these reactio
aqueous solution thus greatly favored the hydroxylamine reaction as
been regularly observed with acetyl CoA or acetyl LBF. The reaction
for ethylthioacetate and β-acetaminoethyl thioacetate were similar (70)
the presence of an amide group on the β-carbon therefore exerted only
influence on the reactivity. It would appear that the activity of the S
bond in CoA derivatives may not differ greatly from those in the models
ied, as would also be expected from the use of similar model compound
acyl-N-acetylthioethanolamines) by Lynen in fatty acid oxidation st
(3, 71). CoA preparations have been found stable for 2 years at room te
ature in the dry state, and several months in neutral aqueous solution. I
presence of 0.5 N H_2SO_4, 20 to 30 per cent was destroyed in a few mi
(72). Replacement of acyl by aryl in LBF derivatives (S-phenyl LBF
duced a growth antagonist for *Lactobacillus helveticus* (73).

Virtually all of the known biochemistry of CoA at present centers
the —SH group, because of its importance and the novelty of many
functions. Although, as pointed out by Lipmann (74), there may be a
cy to overemphasize this group, it has so far yielded extremely valua
formation. A possible further property of CoA that has scarcely been
ined is a potential role in phosphate energy transfer (75), where a sugg
intermediate compound is an enzyme-phosphoryl~S-CoA. This int
observation deserves major attention in future research.

THIAMINE

Crude allithiamine was first prepared (76) by heating thiamine
with an alcoholic extract of garlic (*Allium sativum* L. var. *japonicum*
mura). The product was negative to the thiochrome test but positive

...atment with cysteine. The pure compound was later isolated (77) and ...wn to have the structure given in Figure 1, formula I. The homologues ...h other alkyls, such as methyl, propyl, were also formed during the reac-...n at pH 8 and 50 to 60°C., especially with other species of *Allium* (78). Al-...hiamine and its homologues could be reduced by cysteine into a thiol form ...thiamine (V), as shown in Figure 1.

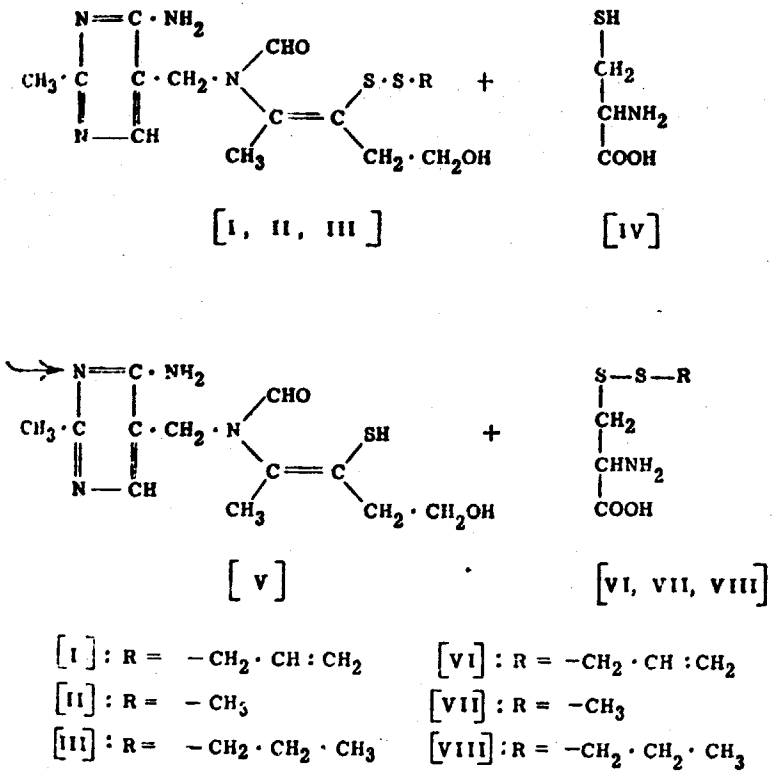
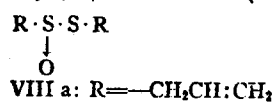


FIG. 1. Reduction of allithiamine and homologues by cysteine.

...he byproduct S-allyl mercaptocysteine from the allithiamine reaction ...been obtained previously (79) from allicin (VIII a) and cysteine.



...he formation of allithiamine (I) could thus be explained by the reaction ...en the thiol form of thiamine and allicin (VIII a). The latter was found ...an enzymatic decomposition product of alliin (80). Since other ho-...ues were also produced, it is likely that the plants contain other allicin-...pounds.

Since allithiamine was freely convertible to thiamine by cysteine, the-...should possess vitamin activity. This was found to be true for rats on a ...mine-free diet (76, 81). It was also active for many fungi and was believe... be converted to thiamine (81). Because allithiamine and its homologues ... quite soluble in many organic solvents, their uptake by blood cells *in* ... could be readily followed (82). Under the same conditions, thiamine, ... mine disulfide, or thiamine pyrophosphate were not taken into rabbi... chick blood cells even in the presence of energy yielding compounds such ... ATP or glucose.

A number of model experiments, simulated to physiological conditi... were performed in order to elucidate the biochemical mechanism of thia... action (83). A compound, thiamine-cysteine, probably with the struc... (XI), was produced as shown in reaction 1 of Figure 2 from thiamine ... cystine in a phosphate buffer, or from the disulfide form of thiamine and ... teine as in reaction 2 of Figure 2. Thiamine in aqueous solution was in eq...

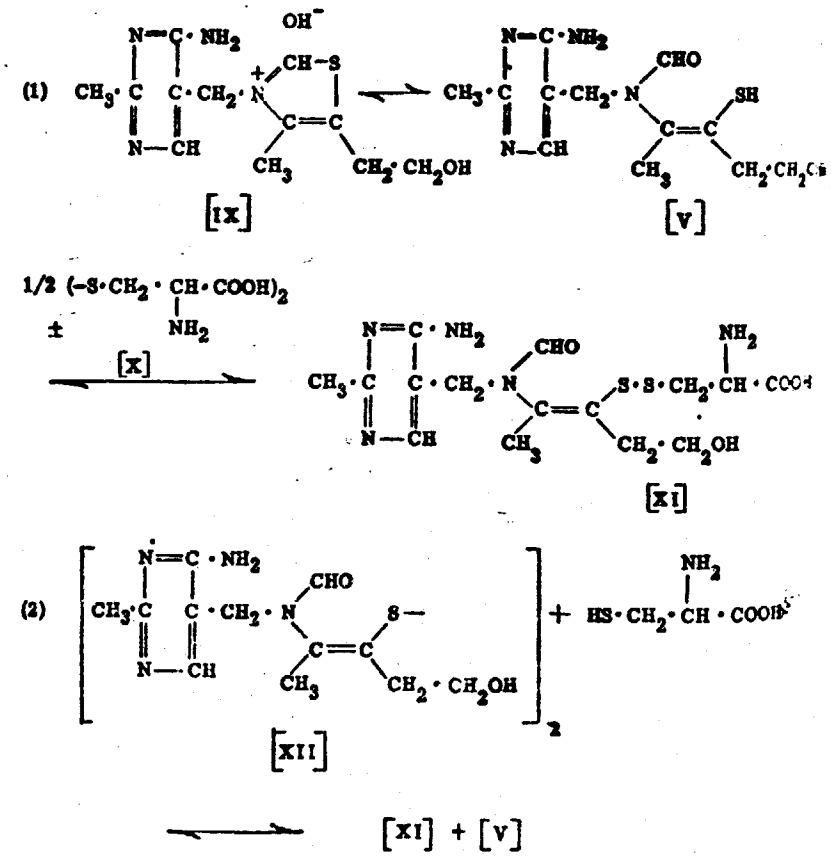


FIG. 2. Formation of thiamine-cysteine.

nium between the ammonium (IX) and thiol forms (V). The equilibrium of IX and V is not a simple reaction, however, for there is an internal redox reaction: the states of nitrogen, sulfur and carbon are altered. Lack of physical data prevents a prediction of the relative amounts of each form present. On the other hand, in the cysteine-cystine containing system, the over-all equilibrium could be depicted as (XI)$\xleftrightarrow{(V)}$(XII)$\xleftrightarrow{(IV)}$(XI). Based on these reasonings, the Japanese group believed that the biochemical action of thiamine *in vivo* was attributable to an equilibrium reaction similar to reaction 1, and that the catalytic action of the vitamin would be a result of the oxidation of certain substrates affected by thiamine-cysteine (XI) or its homologues. They felt that the vitamin action was not attributable to a redox equilibrium between the thiol form of thiamine and thiamine disulfide as had been proposed earlier (84). The observation is interesting and deserves further study. Judging from the known actions of thiamine (such as simple and oxidative decarboxylations, transketolations), the above proposal seems to be prematurely drawn. In living systems, many biologically active thiol compounds, especially those recently discovered, such as thioctic acid, pantetheine, and coenzyme A, would complicate the above relationships and could not easily conform to the suggestion. For example, it is believed that thiamine and lipoic acid are required for oxidative decarboxylation. The precise arrangement of the cofactors is not known (see lipoic acid section). Regardless of whether the suggestion (85) is correct, that two coenzymes are separately attached to animal pyruvic oxidase, or whether they exist in the conjugated form of "lipothiamide pyrophosphate," LTPP, (86), it seems cumbersome to speculate that yet another molecule, such as cysteine may be attached to the same apoenzyme for oxidative function. As a matter of fact, the "oxidative" aspect of the α-ketoacid oxidases is attributable to other cofactors than the thiamine moiety.

The action of many cofactors, such as flavin- and pyridine nucleotides and CoA, can now be explained in terms of organic chemistry. However, the function of thiamine pyrophosphate in simple decarboxylation is still unknown. Probably the CO_2 group of the substrate is first transferred to thiamine pyrophosphate; if so, the form of the carboxylated cofactor remains to be elucidated.

Bracken thiaminase has been resolved into two components, viz., a thermostable dialyzable cofactor and a theromolabile apoenzyme (87). The apoenzyme was purified to a certain extent (88). The thermostable portion was found to exist in many other natural materials, such as yeast, liver, urine, etc. The most active concentrates contained appreciable amounts of several amino acids. DL-proline and L-hydroxyproline were active *in vitro* as the cofactor, while many related amino acids showed less activity. Bracken thiaminase, like the other thiaminase systems present in carp, clams, and *Bacillus thiaminolyticus* (89, 90), could use many metasubstituted aromatic amines as cofactor for the action.

The apoenzyme of carp thiaminase was also purified (91) by fractionation

with ammonium sulfate. It was presumably not identical with the bracken apoenzyme (92). A cofactor was likewise required for its action. The thiaminase actions of both carp and bracken could thus be defined as in Figure 3. The products were the thiazole moiety of the vitamin and a conjugate which contained the pyrimidine portion of thiamine, although the products of bracken thiaminase were not definitely identified. Harris, in a study of biosynthesis of

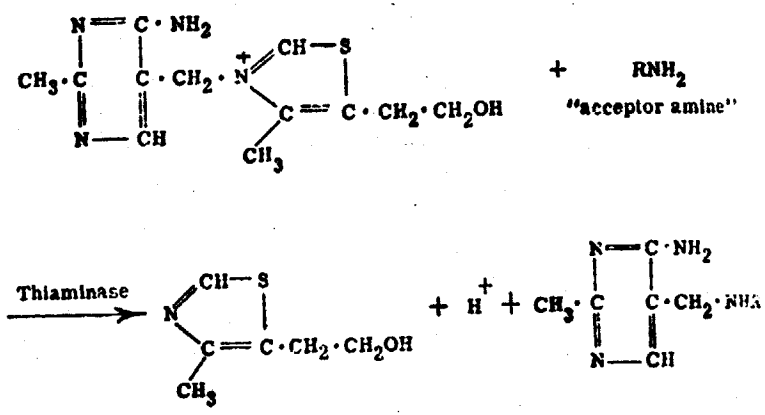


FIG. 3. Mode of thiaminase action.

thiamine in *Neurospora*, found that the pyrimidine moiety preferentially condensed with some unidentified precursor of thiazole to form a thiamine-like intermediate which was secondarily transformed to the vitamin (93). The strains which failed to carry out the latter reaction accumulated this intermediate, the nature of which was not determined. From the experiments described, one possibility may be pyrimidine-amine. Fujita and co-workers believed, however, that the thiaminase reaction was simply a base exchange. From this observation as well as his own, Woolley (91) proposed a very interesting hypothesis to supplement the phosphorus-involving mechanism of biosynthesis. According to this theory, the energy for many biosynthetic reactions which apparently do not involve phosphate could be derived from the reduction of quaternary bases, such as ammonium or sulfonium compounds. He estimated that the free energy change in the thiaminase reaction was about 7000 to 8000 cal. per. mole. Although this figure was only approximate, it was sufficiently high to drive many endergonic reactions. This class of action has received less attention, so that little information has accumulated. Future work will likely be shifted in this direction, to evaluate the C–C bond and other "onium" compounds, not only in thiamine but also in related structures. It is also interesting to speculate whether any of the steps of protein synthesis, which involves the conversion of ammonium to labile nitrogen, may have some connection with the foregoing hypothesis of action such as $\overset{+}{N}H_2CHRCOO^- + \overset{+}{N}H_3CHRCOO^- \rightarrow \overset{+}{N}H_3CHRCONHCH$

...uld be thermodynamically feasible under physiological conditions without ...tional driving force from other "energy-rich" compounds.

Horecker *et al.* (95, 96) observed that enzyme preparations from spinach ...at liver catalyzed the conversion of pentose phosphate to sedoheptulose ...sphate and glyceraldehyde-3-phosphate (G-3-P²). This conversion re-...d thiamine pyrophosphate. The preparation from spinach contained ...at 0.8 mole of thiamine pyrophosphate per mole (10,000 gm.) of protein. ...contrast to pyruvic carboxylase, the resolution of the spinach enzyme could ...e achieved by treatment at acid pH in the presence of 45 per cent saturated ...monium sulfate. This behavior suggests that the acid group of thiamine ...ophosphate is attached to the protein, whereas in carboxylase, the strong-...attachment should be through the basic group [as also shown experimen-... (97)]. The apoenzyme also catalyzed the reaction of L-erythrulose and ...G-3-P to form a mixture of pentose phosphate and heptulose phosphate. ...ther product was formed in the absence of thiamine pyrophosphate. Rac-... *et al.* (98) isolated a crystalline enzyme from bakers' yeast which resem-...d the spinach enzyme in pentose metabolism. It catalyzed the conversion ...ptulose-5-phosphate to D-G-3-P, as well as the decarboxylation of hydroxy-...vate. Both reactions required acceptor aldehyde, such as ribose-5-phos-...ate, glyceraldehyde, or glycolaldehyde. The yeast enzyme was also thia-...e pyrophosphate dependent. This enzyme was named transketolase (98).

...discoveries of the new roles of thiamine in oxidative decarboxylation and ...transketolation crystallize the importance of this vitamin in animal as well ...crobial nutrition. It has seemed surprising that these organisms required ...mine, even though they did not possess a major simple decarboxylative ...way for pyruvate, such as found in yeasts and higher plants.

A relationship between thiamine and pyridoxine has been recognized for ...time in certain strains of yeast and other microorganisms. Various theo-...ve been proposed. A thorough investigation has recently been made (99, ...on one strain of a top brewer's yeast which requires an exogenous supply ...r vitamin for the attainment of rapid growth, and of both vitamins ...aximum growth. The decarboxylation rate was also slower in the thia-...deficient yeast, but this could be corrected by the addition of thiamine ...hosphate. The requirement was attributable to its inability to synthe-...rimidine at a rate needed to support optimum growth and, to a lesser ...t, by its retarded rate of synthesis of thiazole. Pyrimidine was found to ...ce a sparing effect upon thiamine equivalent to that of pyridoxine, and ...xine was found to stimulate the rate of synthesis of thiazole. The data ...interpreted by postulating a reversible interrelationship between thia-... and pyridoxine with pyrimidine as the key intermediate.

...ridoxineless mutants of *Neurospora crassa* and *N. sitophila* (101) grew ...when thiamine was also added to the culture medium. The stimulatory ...of thiamine in this case was found to be a result of competitive inhi-...by thiamine of the destruction of pyridoxine. Pyridoxine, in turn, in-...d with thiamine synthesis, but sufficient endogenous thiamine was ...t to satisfy the normal growth requirements. In *Lactobacillus fermenti*

36, it was found that ascorbic acid has thiamine sparing action (102). The ...dition of both ascorbic acid and thiamine gave a greater growth resp... than either one alone. Pyrithiamine inhibited the utilization of ascorbic ... for growth in a thiamine-free medium. The microorganism could synthe... thiamine when ascorbic acid was present. Therefore the thiamine sparing ...tion was thought (102) not to be attributable merely to the reducing po... of ascorbic acid. In the use of *L. fermenti* for thiamine determination in nat...ral materials, this effect should be considered.

After the intraperitoneal injection into rats of thiamine labeled with ... at position 2 of the thiazole ring, about 66 per cent of the radioactivity ... excreted in the urine (103). Five thiamine-containing compounds, includ... cocarboxylase, were observed. Another appeared to be a 6-thioctic acid ...jugate. The experiment employed rather large dosages of thiamine, and ...plicability to normal metabolism of the findings from such an experim... may be regarded with reservation. This is also true in an experiment with ... labeled thiamine (104), where radioactive sulfur was found in various fr...tions of sulfur in the urine, after administering the labeled vitamin to th...mine-deficient rats. Administration of oxythiamine reduced the excretion ... radioactive inorganic sulfate and increased the neutral sulfur fraction.

Intravenous injection of a large dose of the pyrimidine moiety of thiam... produced a transient but significant increase in the volume rate of gastric ...cretion but usually no effect on the acidity of the juice (105). The effe... thiamine to raise blood pressure and increase heart rate was clarified as att...utable to the acidity of the vitamin rather than its specific action (106).

Thiamine is unstable in usual laboratory rations and becomes more ... the presence of fat. It can be stabilized by addition of reducing compou... such as ascorbic acid, cysteine, hydroquinone, by sulfasuxidine or glyc... or by storing in a dry state or under an inert atmosphere (107). A mo... method for thiamine determination was claimed to be superior to pre... ones, by preliminary ferricyanide oxidation in acid to remove an interf... nicotinamide metabolite, F_2. Neopyrithiamine has been shown to po... antithiamine activity in the pigeon (108). Reviews on metabolic functio... thiamine (86) and on antithiamine (90) have appeared.

Lipoic Acid (6-Thioctic Acid)

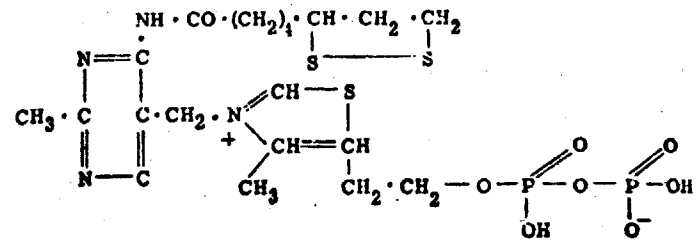
Because of the intimate role that lipoic acid has been found to pla... pyruvate oxidation, an extended discussion of this compound is given in ... chapter on carbohydrate metabolism.

Lipoic acid has perhaps not earned the title "vitamin" in the cla... sense, since it has not yet been shown to be required in animal diets ... Such an objection has little real value, however, since this compound co...ly qualifies as an essential biocatalyst in many systems. As has been p... out (110), enzymatic behavior may under some conditions be fully as va... criterion as growth in measuring the prosperity of an organism. Our k... edge of the chemistry and biochemistry of lipoic acid have proceeded ...

ate seldom equalled in vitamin research. Since last year's review (111), in which the structure of the factor (protogen; acetate replacement factor; pyruvate oxidation factor) was described as 6-thioctic acid, this compound has been shown by Gunsalus, Reed and others to occupy a vital role as a primary dehydrogenase in microbial oxidation of pyruvate and α-ketoglutarate (86, 112); see also other reviews on this subject (113, 114)]. A second role for lipoic acid appears as a transfer agent of acyl groups to CoA, whereby acetyl CoA is formed; whereas a third, and perhaps the most interesting function is as an energy transfer agent through the possession by lipoic acid of an S~ bond (86, 112, 114).

The conversion of oxidative energy to S-acyl "high" energy evidently occurs at a relatively low potential; the redox potential of lipothiamide pyrophosphate (86, 111) has been calculated at approximately −0.42 volt at pH 7.0 (86). Such a low value places this cofactor very close to hydrogen on the redox scale and adds credibility to the intriguing suggestion of Calvin and associates (115, 116, 117) that lipoic acid may operate very early in the acceptor chain of hydrogen from water during photosynthesis, and that the thioctic disulfide ring may participate in the primary quantum conversion act of photosynthesis. The reaction is viewed as chlorophyll (activated) + S—S → chlorophyll (ground state) + S S whereby the free thiyl radicals are produced by electronic excitation and can serve as the hydrogen acceptor system. A similar postulate has been made for the key reaction in the visual process [Strauss (118)], since Wald & Brown (119) had found that the bleaching of rhodopsin is accompanied by the liberation of two —SH groups per molecule of retinene released. Conversely, the retinene + opsin → rhodopsin conversion required —SH group participation. The liberation of these —SH groups during bleaching has been considered essential in the initiation of the electrical processes of vision (119). These hypotheses have not yet been supported by direct experiment, although indirect evidence that is in line with Calvin's suggestions has been described (117). Both schemes appear generally plausible and if they can be validated they will go far toward improving our understanding of both of these important photochemical changes.

The structure of the functional form of lipoic acid is of major interest. Gunsalus *et al.* (85) have suggested that the thiamine coenzyme may be separate from lipoic acid or other cofactor at the protein surface, whereas Reed & DeBusk (86, 111) consider the thiamine-lipoic conjugate, "lipothiamide pyrophosphate" (LTPP[2]) (see formula XIII!) to be the active codehydrogenase. Despite the differing conclusions, the experimental data in these papers may not be in serious conflict. The identity of LTPP has not yet been rigorously proven, and until it is more firmly established the structure proposed may be regarded with some reservation. Thus, as pointed out elsewhere (114), with the sulfur atoms essential for catalytic activity, only the pyrophosphate group remains available for attachment to the apoenzyme. Such a linkage



Formula XIII. "Lipothiamide pyrophosphate."

would be weaker than that known to exist in "bound" lipoic forms. In view of the discussion in the preceding section concerning open-chain thiamine forms, it seems possible that rupture of the thiazole ring may account for formation of LTPP, which would not only permit additional points of enzyme attachment but might also provide a more complex, loosely integrated coenzyme with separate centers of activity. Such a molecule might fill the alternate decarboxylation-oxidation roles described for the physically active substance (86). Further examination of this coenzyme should fully elucidate its role and solidify the excellent work that has been done by the various laboratories involved in the lipoic acid problem.

Coenzyme activity of thiamine without lipoic acid is well known; separate functions for lipoic acid without thiamine have been claimed from the reports (120, 121, 122) that in *Tetrahymena*, lipoic acid is required only as an acyl transfer agent (to CoA) and is not involved at the aldehyde level of oxidation. The findings are interesting, although somewhat unexpected in view of the supposed dehydrogenase function of lipoic acid. They depend in part upon the behavior of an alumina-treated pyruvic oxidase from *Tetrahymena*. This preparation reportedly free from lipoic acid, was found to exchange $C^{14}O_2$ with pyruvate carboxyl as well or better in the absence of lipoic acid than with the factor present. However, it appears possible that reaction (e.g., decarboxylation, exchange, etc.) between the pyruvate and CO_2 might have accounted for the results. The observation deserves additional study to establish unequivocally the lack of lipoate participation in dehydrogenase system in this organism.

A relationship, probably indirect, between thioctic and folic acids is suggested by the report (123) that the conversion of glycine to serine in *Tetrahymena*, which depends upon high levels of folic acid, is improved by levels of thioctic acid. In the absence of the factor, this organism accumulates α-keto acids, chiefly pyruvic, α-ketoglutaric, and possibly α-keto[illegible] (124). The latter, which was assumed to arise from leucine, may thus depend upon thioctic acid for oxidative degradation.

Riboflavin

By taking advantage of a riboflavin-producing strain of *A. gossypii* [illegible] could give a maximum yield of 500 γ per ml of culture medium, the bi[illegible]

...s of riboflavin has been examined (125, 126). With C^{14}-formate, the radio-activity was found almost completely in carbon position 2, but bicarbonate gave predominant labeling at carbon 4. Other substrates such as $C^{14}H_3COOH$, $CH_3C^{14}OOH$, $NH_2C^{14}H_2COOH$, $NH_2CH_2C^{14}OOH$ and uniformly labeled glycine led to a wider distribution of the isotopes. Ring C of the vitamin possessed a labeling pattern similar to that of the pyrimidine portion of purines. No mechanism was offered for the formation of the aromatic ring, but the side chain was thought to arise by a C_2+C_3 condensation.

The maximum formation of riboflavin by *Eremothecium ashbyii* was found to require two unidentified factors (127). Factor A was associated with oleic acid, some of its derivatives, and yeast extract. Factor B occurred in gliadin, ...ins, and "vitamin-free" casein. These factors were apparently not directly linked with the growth of the microorganism. The synthesis of riboflavin by *Aerobacter aerogenes* was believed to be inhibited by aureomycin and terramycin (128), while aureomycin competitively inhibited the utilization of riboflavin by a riboflavin mutant of *Bacillus subtilis* and other bacteria (128).

Bioluminescence has been recognized as an enzymatic reaction, but the mechanism has not been understood until recently. Cell-free and particle-free extracts of *Achromobacter fischeri* were prepared (129, 130) which showed continued luminescence. Diphosphopyridine nucleotide (DPN[2]) was required for the light-emitting process. Long chain (C_7—C_{18}) fatty aldehydes were also stimulatory (130, 131). Based on these observations, it was suggested that DPN was either closely linked to the light-emitting system or was identical with bacterial luciferin. However, other workers (132) found that a flavin was essential for reduced DPN to function in initiating light emission in ...ified bacterial extracts. Flavin mononucleotide (FMN[2]) was the only derivative that could function in this capacity. In addition to FMN and reduced diphosphopyridine nucleotide (DPNH[2]), at least one other factor which was presumably identical with bacterial luciferin was required for light production. This finding was in line with the fact (133) that certain dim strains of luminous bacteria would luminesce brightly when grown in the presence of riboflavin.

Riboflavin-5′-phosphate, flavin-adenine-dinucleotide (FAD[2]) and a new FAD with the structure of a 4′,5′-cyclic phosphate (134) are presumably the biochemically active forms of riboflavin. Four FAD enzymes were found to contain metals. They are iron in DPN-cytochrome reductase (135); molybdenum in xanthine oxidase (136, 137) and in nitrate reductase (138); and copper in acyl coenzyme A dehydrogenase (139). In this connection, it is interesting to observe the iron requirement in the phosphoroclastic degradation of pyruvate by *Clostridium butyricum in vitro*. This system required cocarboxylase, coenzyme A, and Fe^{++} (140). The latter could not be replaced by any other common bivalent cations of which Co^{++}, however, showed slight activity. Although the metals in these oxidases are nondialyzable, they evidently exist in a form which is able to accept and give up electrons. The mechanism of oxidation-reduction (135, 139) is believed to con-

sist of an intramolecular electron transport from FAD to the metal. The reduced metal is then oxidized by the subsequent enzyme. Two moles of FAD and one atom of molybdenum were found per mole of xanthine oxidase (136). If both FAD groups are initially reduced, then the valence change in molybdenum must be at least 4 in order to permit their reoxidation. However, such a change of valence would probably involve amphoterism in molybdenum unless peroxide formation is assumed. Alternately, intermolecular oxidation is complete. These restrictions need not apply to DPN-cytochrome reductase where the FE:FAD ratio is 4. Whether the attachment of metals in these flavoprotein enzymes is similar to that of iron in heme proteins is not known. It seems likely that the metal may be coordinated with FAD, or be attached to the protein adjacent to the FAD group, similarly to the proposed metal-protein attachments in certain proteolytic enzymes (141). In this way the metal could be dissociated, as well as easily reactivated.

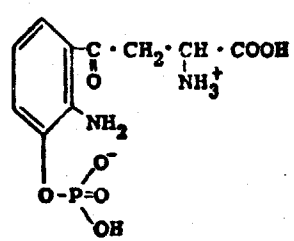
Quinacrine hydrochloride (Atabrine) at 0.04 to 0.08 per cent of the diet exerted a sparing action on riboflavin in rats (142). The drug increased the urinary excretion of riboflavin and enhanced the growth of young rats on rations with suboptimal amounts of the vitamin. The liver riboflavin content was not affected by quinacrine. Its riboflavin-sparing action was explained as possibly attributable either to inhibition of the metabolic destruction of riboflavin or to the structural similarity of the two substances, so that the drug might partially replace the vitamin in flavin-containing enzymes. Some other structural analogues such as phenazines, were found to function in the hexosemonophosphate system. However, it had been previously reported (143, 144) that quinacrine competitively antagonized FAD and FMN. It would be of interest to determine whether quinacrine can attach to an apoenzyme and thus function as a hydrogen transport catalyst. Meanwhile, although stimulation of intestinal synthesis of riboflavin was ruled out in the experiments, it appears possible that quinacrine may function to repress intestinal organisms that normally compete with the host for dietary flavins and hence improve the growth rate of the animal. This type of explanation is widely used to account for the growth-promoting effects of antibiotics.

Rabbits receiving continuous injections of sodium acetoacetate and β-hydroxybutyrate (145) over long periods exhibited riboflavin and niacin deficiencies. The concentrations of both vitamins in the blood were lowered. This fact was possibly a result of the extra demand upon flavo- and pyridinoproteins, which in turn depreciated these vitamins for other metabolic functions. However, this experiment does not necessarily indicate that diabetics would become dificient in these vitamins, since these keto acids are not completely dissimilated.

Tryptophan is believed to be a precursor of nicotinic acid in many plants although the biosynthetic pathway has not been completely explored. In the rat, riboflavin and pyridoxin are definitely involved in the conversion. Kynureninase, which has been shown to be pyridoxal phosphate-dependent (146), catalyzes the conversion of kynurenine to anthranilic acid, and of 3-hydroxy

kynurenine to hydroxyanthranilic acid. A purified kynureninase has now been obtained from Neurospora (147), which also is pyridoxal phosphate-dependent, which converted kynurenine, 3-hydroxykynurenine, and N-formylkynurenine to anthranilic, 3-hydroxyanthranilic and formylanthranilic acids respectively. The kynureninase was strongly inhibited by many amines (148). The inhibition could be reversed by pyridoxal phosphate, possibly as a result of the formation of Schiff bases with the amines. However, both the substrate and the coenzyme were also inhibitory. The mechanism was not clear; the inhibition could be attributable to the substrate-pyridoxal phosphate compound. The latter acted as an inhibitor (148) in the kynureninase system.

Riboflavin was previously suggested to be involved in the conversion of kynurenine to 3-hydroxykynurenine (149). This does not appear related to the fact that the excretion of tryptophan metabolites was in the form of hydroxykynurenine in tuberculotic patients (150) and in fever (151), but in the form of kynurenine in normal humans (151). No significant difference was found (152) in the metabolism of anthranilic acid by normal or riboflavin-deficient rats, whereas deficient rats after tryptophan administration excreted considerably more anthranilic acid and its derivatives than did the normal rats. These observations seem to indicate that riboflavin deficiency causes a rearrangement of the metabolism of tryptophan which brings about an excessive formation and excretion of anthranilic acid and its conjugates. A series of detailed studies were carried out (153) on the urinary metabolites after tryptophan and anthranilic administration in normal, riboflavin-deficient and riboflavin- and pyridoxine-deficient rats. Although riboflavin deficiency greatly disturbed the tryptophan metabolism with increased yields of xanthurenic acid, the formation of hydroxylated metabolites was not inhibited at all. From the available data, the authors suggested that kynurenine was



Structure XIV. Hydroxykynurenine phosphate.

oxidatively phosphorylated to hydroxykynurenine phosphate, XIV, and that the phosphorylated intermediate would normally be directed toward the formation of more hydroxyanthranilic acid and less xanthurenic acid. It was considered that riboflavin was involved in a phosphorylative, rather than an oxidative step in the disposition of kynurenine. This idea is in line with the findings (154) that tryptophan and kynurenine were converted to hydroxyan-

thranilic acid phosphate in rat liver preparations although hydroxykynurenine formed hydroxyanthranilic acid. Precise knowledge on the role of riboflavin in tryptophan metabolites will probably have to await the success of suitable *in vitro* experiments

Acetate or thioctic acid reversed propionate inhibition in *Streptococcus faecalis* in the presence and absence of riboflavin. In the presence of increasing amounts of lyxoflavin, extra riboflavin was necessary to restore the inhibition by thioctic acid (155, 156). It was concluded that L-lyxoflavin was competitively inhibitory to riboflavin in the organism. It is interesting to note that in the absence of the inhibitor (propionate), high dosages (up to μg per ml.) of lyxoflavin did not show any depression in growth. Thus, the reason why the competitive inhibition between these two flavins was demonstrable only in the presence of propionate remains to be discovered.

According to Snell *et al.* (157), different organisms showed different relationships between lyxoflavin and riboflavin. For example, L-lyxoflavin, galactoflavin, and isoriboflavin all increased the growth response of *Lactobacillus casei* to suboptimal amounts of riboflavin but had no effect in its absence. When riboflavin was present in excess, lyxoflavin did not increase the cell yield or growth rate. At high concentrations of lyxoflavin and galactoflavin, they inhibited the growth of the organism, as competitive antagonists of riboflavin. Both dichlororiboflavin and isoriboflavin were ineffective as inhibitors. L-Lyxoflavin promoted maximum growth of *Lactobacillus lactis* in the absence of riboflavin, and such growth continued indefinitely upon subculture. The cells from these cultures did not contain riboflavin, but lyxoflavin. It appeared, therefore, that in this organism, lyxoflavin could replace riboflavin in the latter's essential metabolic roles. In chicks, lyxoflavin stimulated growth when fed 2.5 to 10 times the amount of riboflavin in the diet. At higher levels (over 40:1) lyxoflavin inhibited growth. The inhibition could be reversed by increasing the riboflavin content in the diet. A somewhat different result was obtained by Sherwood *et al.* (158), who found that although L-lyxoflavin was inactive in chicks, it stimulated growth of male turkey poults on a corn-soybean diet. Judging from the different responses to lyxoflavin shown in animals (111) and in microorganisms depending upon slight variances in media, the deviation of the results described is not surprising. The metabolic relationship between lyxoflavin and riboflavin is at the present time far from clear. However, L-lyxoflavin can hardly be considered as a vitamin in spite of the earlier favorable findings (111).

A review on metabolic functions of riboflavin has appeared (159).

Vitamin B_6

The metabolically active form of the vitamin B_6 group is evidently the phosphoric ester of the 5-hydroxymethyl compound. If this hydroxy group is missing, the phosphoric ester can no longer be formed, although the 5-hydroxymethyl group in position 4 could presumably still take part in the normal biochemical reactions. Consequently, the 5-desoxy compounds were

expected to have no vitamin activity and might be inhibitors (160). For reasons, the 5-desoxy analogues of pyridoxine, pyridoxal, and pyridoxamine were prepared (160) by a method illustrated in Figure 4.

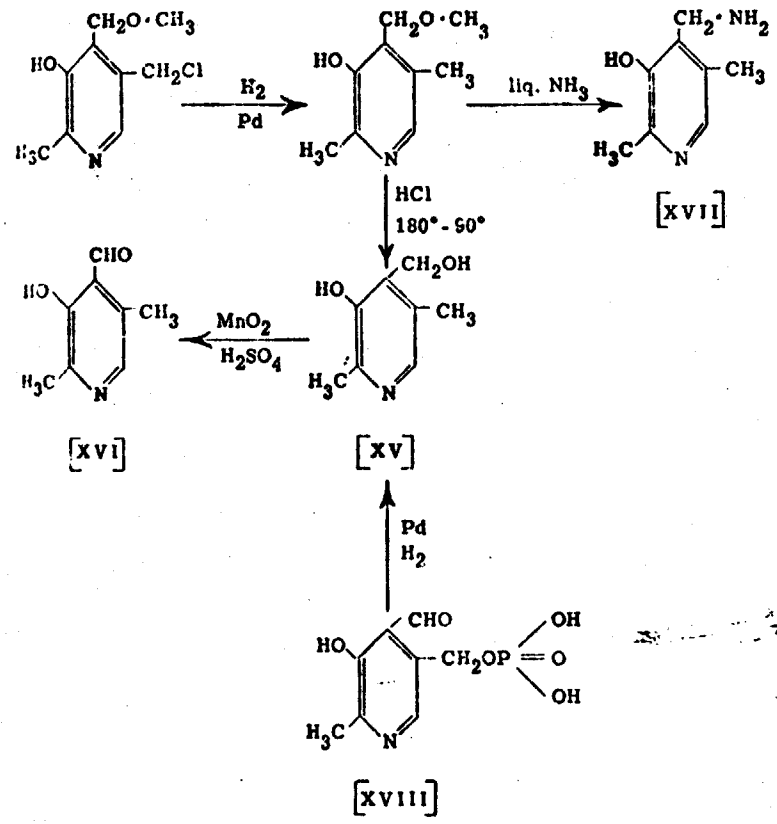
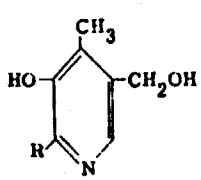


FIG. 4. Formation of 5-desoxy analogues of Vitamin B_6.

It is interesting to note that pyridoxal-5-phosphate, XVIII, could be directly hydrogenated to 5-desoxypyridoxine, XV. XV was resistant to further



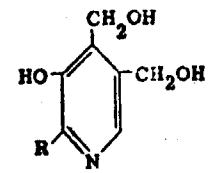
XIX : $R = CH_3-$

XX : $R = C_2H_5-$

XXI : $R = CH_3(CH_3)CHCH_2-$

XXII: $R = CH_3(CH_2)_3CH_2-$

Formulas XIX to XXII. 4-Desoxypyridoxine and its homologues.



XXIII : $R = CH_3(CH_3)CH^-$

XXIV : $R = CH_3(CH_3)CHCH_2^-$

XXV : $R = CH_3(CH_2)_3CH_2^-$

XXVI : R =

XXVII : $R = CH_3CH_2^-$

Formulas XXIII to XXVII. Analogues of pyridoxine.

hydrogenation. Under the same conditions, pyridoxal and pyridoxine produced a mixture of 4-desoxypyridoxine, XIX, and 5-desoxypyridoxine, XV. Another interesting property was the close similarity of the absorption spectra of XVIII and 5-desoxypyridoxal, XVI. This was evidently a result of the loss of ability to form hemiacetals in both compounds by the usual alteration of the hydroxy group in position 5.

Three homologues of 4-desoxypyridoxine were also synthesized, with changes in the 2-position: these were the ethyl, XX, isobutyl, XXI, and amyl, XXII, derivatives, respectively (161). No biological tests were reported. Several analogues of pyridoxine, with structures XXIII to XXVI, were synthesized by the Merck group (162) and by Davoll & Kipping (163). According to the Cambridge group, these compounds showed no biological activity except for the isopropyl compound. When the latter was tested in the presence of pyridoxine, the analogue gave only 1/400 of the activity of pyridoxine.

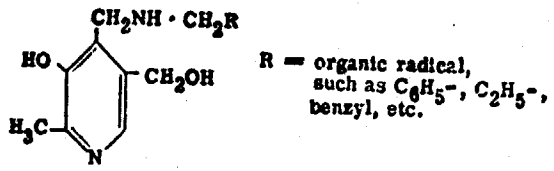
These desoxy compounds were tested microbiologically by Rabinowitz & Snell and found to be antagonists to the vitamin B_6 (164). 4-Desoxypyridoxine was relatively ineffective in organisms that could grow in the absence of vitamin B_6, such as *Lactobacillus arabinosus*. On the other hand, it greatly impaired the growth of vitamin B_6-dependent organisms, such as yeasts and molds (but not *L. casei*, *S. faecalis*, or *L. helveticus*). The comparative effectiveness of the various forms of the vitamin to counteract the inhibition was found to vary from one microorganism to another.

4-Desoxypyridoxine is also known to act as a pyridoxine antagonist in higher animals (cf. 165). It has also been used to hasten the onset of deficiency symptoms by incorporation into vitamin B_6-free diets (166). However, severe dermatitis and body weight drop of the rats fed 4-desoxypyridoxine were claimed (165) not to be a result of the disturbance of the transamination systems. Indeed, the liver glutamate-oxalacetate reaction remained practically the same, while the rate of the glutamate-pyruvate system increased markedly.

5-Desoxy analogues of vitamin B_6 were also antagonistic to vitamin B_6 in various microorganisms (167), as expected (160). The degree of activity

different in different organisms. For example, the decreasing effectiveness as inhibitor in *Saccharomyces carlsbergensis* was approximately "ethyl" pyridoxine, XXVII > 4-desoxypyridoxine, XIX > 5-desoxypyridoxine, XV ≧ 5-desoxypyridoxal, XVI > 5-desoxypyridoxamine, XVII. The effectiveness of reversal in this organism was pyridoxine > pyridoxal > pyridoxamine. In contrast to other pyridoxine inhibitors (164), 5-desoxypyridoxal and 5-desoxypyridoxamine also inhibited *S. faecalis*. The inhibition was, however, no longer demonstrable when D-alanine was incorporated into the medium. The result was interpreted (167) to indicate that in the absence of D-alanine the sole purpose of vitamin B_6 was for the synthesis of this amino acid. When the sole function of the vitamin was bypassed, the inhibitors no longer depressed growth.

The Merck group observed that several pyridoxylamines, XXVIII (168) were fairly active for rats. However, most of these compounds were found to have neglibible activity for *S. carlsbergensis*, *S. faecalis*, and *L. casei* (169). Pyridoxylethylamine (R = ethyl in XXVIII) was the only one to show as



Structure XXVIII. Pyridoxylamines.

much as 2 per cent of the activity of pyridoxal. For *N. sitophila*, many of these compounds had low activity except ethyl, benzyl, methyl, ethanol, β-phenylethyl, and 3-phenylpropyl which showed from 20 to 90 per cent equivalent activity. It was recalled that pyridoxylamino acids also exhibited some activity in the mold, although inactive in yeast or lactic acid bacteria (170).

Functions of vitamin B_6 in metabolism are believed to include coenzyme activity for transaminases, dehydrases and desulfhydrases, amino acid decarboxylases, racemases (159), thionase (171), and some other miscellaneous reactions. (Further details of the behavior of these enzymes should be consulted in the appropriate chapters on metabolism.) At one time transamination was thought to be limited to only two reactions, viz. aspartic plus α-ketoglutaric acid and alanine plus α-ketoglutaric acid. Now a large number of amino acids have been shown to participate in transamination (cf., for example, 172, 173, 174). Favorable evidences concerning the participation of B_6 in these reactions are still appearing. The liver glutamate-pyruvate system appeared to be more sensitive than glutamate-oxalacetate to dietary deficiency of vitamin B_6 in the rat (166, 174a); this also confirmed earlier Russian work. Glutamate-pyruvate transaminase and cysteine desulfhydrase activities were significantly reduced in livers of the vitamin-deficient rat, while the glutamine-pyruvate and glutamine-phenylpyruvate reactions proceeded at approximately equal

rates (166). The impairments were corrected by the addition of pyridoxal phosphate to the preparations. The glutamine systems were shown to be unaffected because of the strong affinity of the coenzyme for the protein. However, a marked fall of the fasting level of plasma glutamine and a significant increase of blood urea were found in the deficient rat (175). Transamination of lysine was claimed to be unaffected by dietary deficiency of B_6 (176). This apparent independence might well be attributable to the affinity of the coenzyme as in the case of glutamine.

Thionase (171), which can attack various thioethers, decreased rapidly in livers of rats on a vitamin B_6-deficient diet (177). Addition of casein or synthetic containing amino acids aggravated the effect. Incorporation of pyridoxal in the diet or addition of pyridoxal phosphate to the liver preparations restored the enzymic activity. This experiment supports the claim that this class of enzyme is pyridoxal-dependent (171). L-Cysteic acid was converted anaerobically to taurine by rat liver preparations. This conversion was diminished in the preparations from rats on a B_6-deficient diet. This was in line with the fact that taurine, which was a known constituent of normal rat urine, could not be detected in the urine of the deficient animals (178). In line with a previous microbial result (179), the incorporation of C^{14}-labeled formate into the β-carbon of serine was found to be lower in liver preparations of pyridoxine-deficient chicks (treated with 4-desoxypyridoxine) than in normals (180).

The transformation of keto acids to amino acids in growing microorganisms has been studied extensively (181) and found definitely dependent on vitamin B_6. A clear-cut demonstration was also shown *in vivo* (182) in the animals. Rats on a diet deficient in vitamin B_6 and an individual essential amino acid deficient diet did not respond to the addition of either the vitamin or the keto analogue of the amino acid. The addition of both resulted in prompt stimulation in growth comparable to those obtained in rats on a complete diet. α-Hydroxy and keto-isovaleric and isocaproic, β-imidazole pyruvic and β-indole pyruvic acids each were able to replace the corresponding amino acid only when B_6 was present. From a comparison in regenerating liver tissue of paritally hepatectomized, pair-fed control, and vitamin B_6-deficient rats, the vitamin deficiency did not alter significantly the constituents, including glutamic dehydrogenase, either qualitatively or quantitatively. Vitamin B_6 was, however, found to be mobilized to the liver from extrahepatic tissue under conditions of rapid synthesis of liver protein in partially hepatectomized rats (183). No difference was found, likewise, between the normal and deficient rats in the absorption of protein, fat, and carbohydrate (184). Although nitrogen retention in the deficient rat was found to be less, the animal was able to maintain body protein (175, 184).

The mechanism of B_6 participation in enzymatic systems has received further attention. An attractive theory which was considered in earlier (170) included a Schiff's base of structure XXIX as an intermediate, somewhat as shown in Figure 5. However, pyridoxyl glutamic, XXX, and pyridylidene glutamic acid, XXXI, were found completely devoid of activity

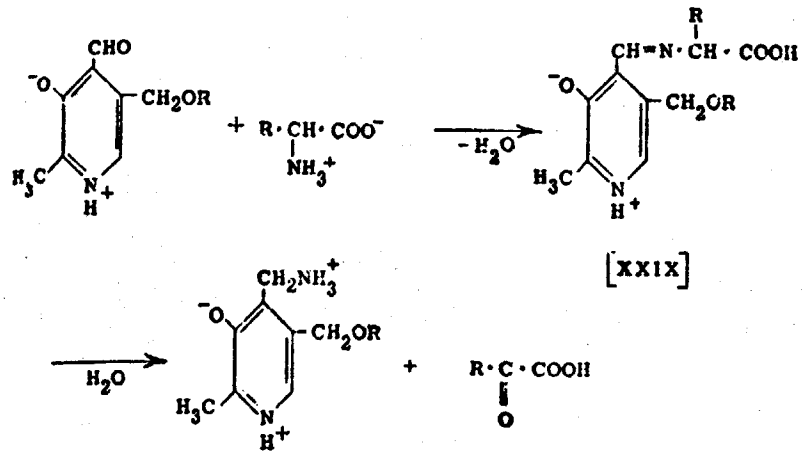
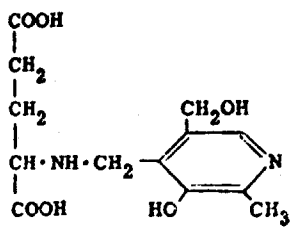
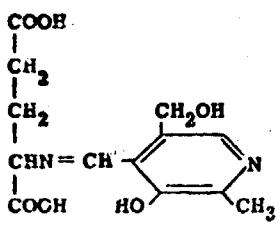


FIG. 5. Transamination via Schiff base formation.

ghly purified preparation of glutamate-oxalacetate transaminase from pig heart (187). Compounds with structures resembling XXIX evidently cannot serve as intermediates in enzymatic transaminations unless the phosphory-



XXX. Pyridoxyl glutamic acid.



XXXI. Pyridoxylidene glutamic acid.

form shows a different behavior. Recently nonenzymatic pyridoxal- and metal (Cu^{++}, Fe^{+++} or Al^{+++}) catalyzed cleavages of hydroxyamino acids (187, 188, 189) were found to be demonstrable at pH 3 to 12 at room temperature. Isolation of chelates with Schiff bases containing pyridoxal, amino acid and metal ion were reported (190). These as well as other observations on nonenzymatic reactions led Snell to propose the reaction sequence shown in Figure 6 as the common feature of pyridoxal-catalyzed reactions (159). He said:

"... resulting in an activation of the amino acid residue so that it may undergo ... a variety of reactions in groupings immediately surrounding the α-carbon ... The vacant coordination positions about the metal ion might well serve to ... coenzyme (and substrate) to the apoenzyme. In non-enzymatic reactions, these ... coordination positions are undoubtedly occupied by water or additional molecules ... reactants.

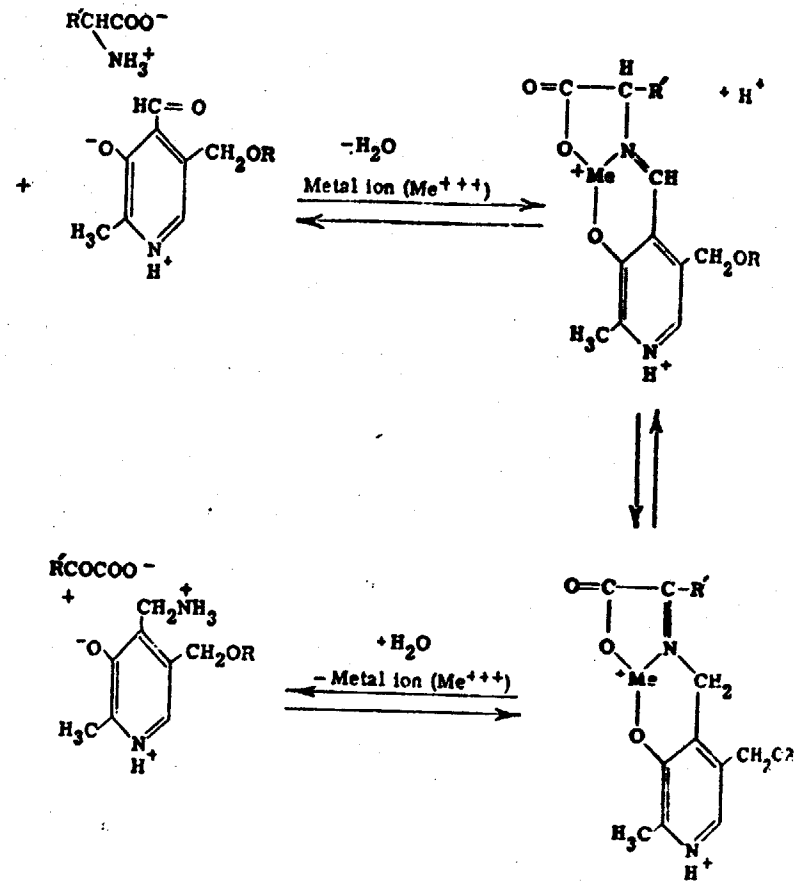


FIG. 6. Transamination via chelate formation.

The test of this hypothesis in enzymic reactions would be of interest. However, it should be pointed out that the phosphorylated vitamin is always required in enzymic reactions while the nonphosphorylated form can catalyze the nonenzymatic reactions. If, as seems logical, this fact indicates an attachment to protein through the phosphate bridge, then the 5-desoxy analogue should not be able to compete with the vitamin for the protein, i. e., it should remain inert, unless another bond between the coenzyme and protein is proposed. It will be seen that the mechanism in Figure 6 calls for the presence of a hydrogen atom on the α-carbon of the amino acid. α-Methylglutamic acid, in which no α-hydrogen exists is a potent inhibitor of glutamic carboxylase and can react with pyridoxal phosphate nonenzymatically. Presumably, the breakdown of normal enzymatic activity is caused by the need for a mobile hydrogen; yet it has been reported that the α-hydrogen of glutamic acid is probably not labilized during decarboxylation (192), where

stabilization of this atom would appear to occur in the reactions represented in Figure 6. For decarboxylation, at least, some refinements in this new attractive scheme may be required.

Since vitamin B_6 participates in a number of catalytic reactions, it is perhaps not surprising to discover further connections with some apparently unrelated systems. The similarity in syndromes attributable to pyridoxine and fat deficiency has been recognized for a long time. It was found (185) that linoleate was a precursor of arachidonate and that linolenate was a precursor of hexaenoate in rats. These conversions were stimulated by B_6. Vitamin B_6 plus linoleate relieved dermal symptoms and stimulated growth, fat synthesis, and arachidonate synthesis much more than either supplement. Pyridoxine plus linolenate stimulated growth, fat synthesis, and hexaenoate synthesis much more than did either supplement alone, but dermal symptoms were not relieved (185). These findings might account for a marked diminution in total crude fatty acids in the carcass of both old and young rats on a vitamin B_6 deficient diet (184, 186). When sodium salts of fatty acids were administered to rats together with tryptophan, the excretion of xanthurenic acid in the urine was greatly enhanced. Such increment could be checked by injection of pyridoxine (187a).

An interesting report has appeared concerning the relationship between pyridoxine and the gonadotrophic content of the anterior pituitary (188a). Rats on a vitamin B_6 deficient, 4-desoxypyridoxine containing diet had a great increase (5 to 8 times) in the gonadotrophic content of anterior pituitary. This was almost entirely a result of the change of follicle-stimulating hormone, while the luteinizing hormone content remained practically the same (188a). On a B_6-supplemented diet, growth hormone promoted weight gain and nitrogen retention while it did not promote these on the vitamin deficient ration. On the contrary, the hormone aggravated the symptoms of B_6 deficiency, such as increased severity of acrodynia, a further elevation of blood urea, and a further lowering of liver B_6 content (189a). This observation should belong to the same category as the one usually used in nutritional experiments which uses thyroid or iodoproteins to increase metabolic rate. The increase in metabolism can accelerate the depletion of a limiting dietary factor to be examined so that the hypophysectomized vitamin B_6-deficient rat does not show external signs or biochemical evidence of deficiency (189a).

The formation of indole, as well as the decarboxylating activity for arginine, ornithine, and glutamic acid in *E. coli* was decreased by treating resting cells with isonicotinic acid hydrazide. The inhibition could be reversed by addition of pyridoxal to the system (190a).

The aldehyde group of pyridoxal could be oxidized in the presence of liver aldehyde oxidase to pyridoxic acid. This reaction was observed in rat and rabbit liver preparations (191a). The significance of the reaction remains to be investigated.

A differential method for determination of pyridoxine, pyridoxal, and pyridoxamine was reported. A preliminary separation was based on the basicity

of pyridoxamine, and lability of pyridoxal (192a). A review of metabolic functions of vitamin B_6 appeared (159).

NIACIN

It is generally agreed that in many organisms tryptophan is a precursor of nicotinic acid, although the yield from the conversion is very low. Besides serving as a precursor of the vitamin, tryptophan is also one of the building blocks of proteins. The biosynthesis of protein is, however, a complex process which is dependent upon many factors, including the simultaneous availability of amino acids. It is logical to believe, and it has been demonstrated (193) that this synthesis is an all-or-none operation. Consequently, an intriguing interrelationship exists between these two biosynthetic processes. Its metabolic priority, if any, can be revealed by a suitable plan of experiment.

Increase of essential amino acids in diets containing marginal levels of tryptophan should be able to induce niacin deficiency by funneling the tryptophan toward protein synthesis. Henderson *et al.* found this to be true (194) by using an ingenious adjustment of the dietary amino acids. Growth of rats was severely suppressed by increasing threonine from 0.33 to 0.38 per cent in a diet which contained 10 per cent hydrolyzed casein and 0.1 per cent tryptophan. Levels of lysine above 0.5 per cent and valine above 0.7 per cent caused a growth suppression which could be corrected by niacin. Similar results were observed in a 10 per cent zein diet (194, 195). The observations also revealed that under these conditions tryptophan was preferentially used in protein synthesis at the expense of niacin formation. Thus in the establishment of a nutritional requirement, the amino acid levels should be considered (see also 196).

Many apparently conflicting results were obtained in studying niacin metabolism in different phyla. As pointed out (197), mammals may be divided into two groups in respect to niacin metabolism: one group, including man, dog, and cat, can aminate nicotinic acid, whereas the other, including guinea pig and rabbit, deaminates nicotinamide to nicotinic acid. However, N′-methyl nicotinamide (NMN^2) is very unstable in alkali. The alkalinity of rabbit urine may destroy the compound in the urinary tubules before it is excreted. It was found that the administration of mandelic acid to increase urinary acidity enhanced the excretion of NMN in rabbit (197). Besides NMN, nicotinic acid, nicotinuric acid, and N′-methyl-2-pyridone-5-carboxamide were also detected. The last formed the principal end-product of nicotinic acid metabolism. By an elaborate technique of chromatography, Ro & Kodicek, however, were not able to detect the pyridone in man and after administration of nicotinic acid, nicotinamide, or tryptophan (198). After a dose of 100 mg. nicotinic acid to man, the main end-products found in urine were nicotinuric acid > NMN > nicotinamide. Nicotinic acid did not appear in the urine until vasodilatory symptoms occurred. The pattern was the same in the rat, except that nicotinic acid was a normal constituent. After nicotinamide administration, nicotinuric acid was not regularly found in either species, otherwise the excretion was the same as after nicotinic acid (198).

using bioautography and radioautography on *Torula cremoris* cultures, John-son & Lin (199, 200) found essentially the same results following large doses of carboxyl-C^{14} labeled vitamin in rats. Nicotinic acid and NMN-like compounds accounted for 60 per cent of the C^{14} excreted after administration of nicotinic acid and nicotinamide respectively. At least seven and nine other products, respectively, were found in the urine to have radioactivity after nicotinic and nicotinamide addition. Of these unidentified compounds, the chief one followed the same chromatographic behavior as N′-methyl-2-pyridone-5-carboxylamide, which the Cambridge workers were unable to find.

In contrast to the rabbit and other mammals, the urinary excretion of NMN could not be detected following the subcutaneous injection of nicotinamide into either a herbivorous (*Bombyx mori*) or a carnivorous insect (*Lucilia caesar* L) (201). Niacin metabolism has thus been shown not only to exhibit species differences but also to vary according to hereditary factors (202). Two pure strains (albino and black hooded) of rats showed great differences in their daily elimination of nicotinamide methochloride and their response (i.e., the increase of this excretion) to extra dietary administration of nicotinamide. Both factors were remarkably stable. When they were crossbred, the output of the methochloride and the response to extra nicotinic acid were inherited multifactorially. The two sets of genes controlling the two phenomena had some, but not all, members in common. No evidence was obtained for a direct effect of sex, or that sex-linked genes, played an important part in the heredity of output and response (202). These studies on the end products contribute significantly in the advance of the knowledge of intermediary metabolism of the vitamin. The observations after large dosages, as pointed out with thiamine, should, however, be carefully regarded before application is made to normal metabolism. Other types of experimentation such as *in vitro* studies of purified systems, may provide the information that is needed, based on the urinary inventories.

Acetylpyridine has been recognized for some time as an inhibitor of niacin action. The same compound has recently been suggested as a precursor of the vitamin. 3-Acetylpyridine was converted in the adult dog to nicotinamide, NMN, and nicotinic acid, in yields of 20 to 30 per cent (203). At high levels (1 gm. per kilo of ration) however, acetyl pyridine was found very toxic to the animal. Further studies (204) showed that the conversion took place rapidly in blood cells, liver, and kidney slices, but not in muscle or blood plasma. The action of acetyl pyridine as a precursor was confirmed (205) by the finding that a daily dose of 60 mg. could prevent the symptoms of blacktongue in dogs on a niacin deficient diet.

Nicotinic acid or its ethyl ester was not used in the biosynthesis of nicotine by incubating the compounds with tobacco root, even over a period of three weeks (206). A summary of the metabolic functions of nicotinic acid appeared (159).

Ascorbic Acid

Evidence on the mechanism of ascorbic acid biosynthesis has been provided in the recent work by C. G. King, Isherwood, and their colleagues. The

Columbia school has employed the chloretone-treated rat. Chloretone increased ascorbic acid synthesis, and thus facilitated the characterization. Ascorbic acid isolated from the urine after administration of uniformly labeled C^{14}-glucose was uniformly radioactive (207). Glucose-1-C^{14} gave ascorbic acid containing C^{14} chiefly in position 6 (208), whereas glucose-6-C^{14} led principally to ascorbic acid-1-C^{14} (209). The suggestion of direct conversion of glucose to L-ascorbic acid induced the authors to study glucuronic acid in biosynthesis. By using uniformly labeled glucuronolactone, a uniformly labeled ascorbic acid was obtained. The yield in terms of radioactivity from glucuronolactone was many times greater than that from glucose (210). The formation of glucuronolactone from glucose was previously demonstrated in the rabbit *in vivo* (211), and in guinea pig liver *in vitro* (212). The synthesis might be summarized as: D-glucose→D-glucuronolactone→L-ascorbic acid. The second step is actually an intramolecular oxidation-reduction and was thought possibly not to need more than one enzyme (210).

Although this scheme was developed in the chloretone treated rat, chloretone *per se* was not a precursor of the vitamin. Moreover, Isherwood *et al.* employed the normal rat with similar results. The Cambridge workers (213) discovered that not only the γ-lactones of D-glucuronic acid, XXXIII, and L-gulonic acid, XXXIV, when fed to cress seedlings or injected into rats, were transformed into L-ascorbic acid, XXXV, but also D-galacturonic acid, XXXVII, methyl ester and L-galactonic acid γ-lactone, XXXVIII. No other sugar acid γ-lactone tested yielded L-ascorbic acid. L-Idono-γ-lactone, XXXIX, and L-talono-γ-lactone, which differ from L-gulono- and L-galactono-γ-lactones only in the configuration of carbon 5 (or 2′), were inactive. This fact led these investigators (213) to formulate two analogous sequences in the biosynthesis of ascorbic acid as shown in Figure 7.

A fundamental feature is the change from the D- to L- series at the second step with inversion of the whole molecule. The enzyme or enzymes catalyzing the transformation of the aldonic acid into L-ascorbic acid is specific for a configuration of the hydroxyl group on C-4 and an L-configuration of the hydroxyl groups on carbons 2′ and 5′ referred to ascorbic acid.

The configuration of carbon-3′ is also specific; thus D-araboascorbic acid, XLIII was produced in the rat from D-mannono-lactone, XLI, and in cress seedlings from D-altrono-γ-lactone only (Fig. 8). However, no similar difference was found in L-gulonic and L-galactonic acid-γ-lactone. It appears that organisms such as the guinea pig, which require the exogenous vitamin, are lacking the enzyme or enzymes to perform the second step.

The report by Nath *et al.* (214) raised a question whether the Isherwood schemes were the only pathways leading to ascorbic acid, although the Cambridge group did not find any evidence of involvement of L-glyceraldehyde, D-gluconic acid, sorbose, or sorbitol. Acetoacetate could decrease the vitamin in the animal (215) but induced a slight increase of ascorbic acid when administered simultaneously with glucose. This effect was also noticed in germinating mung beans (*Phaseolus mungo*). However, a much more striking increase was demonstrated in both organisms after administration of a con-

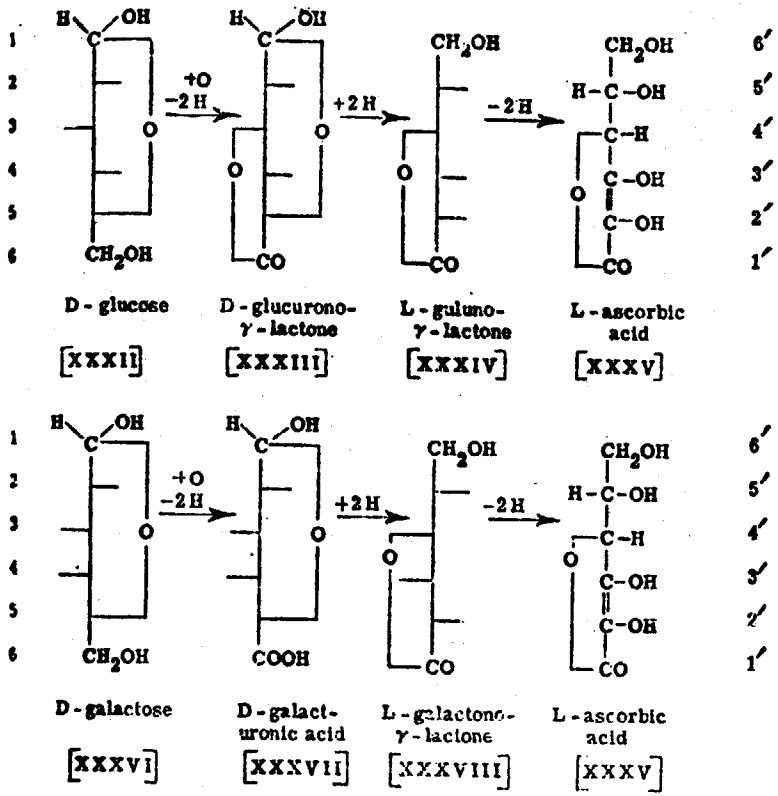
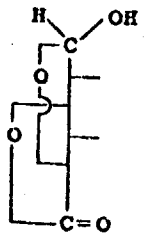
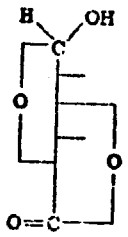


FIG. 7. Biosynthesis of L-ascorbic acid

[illegible] product of these compounds. This product was believed to have the [illegible]ture shown in XLIV. One hundred mung bean seeds gave 2.79, 4.12, and [illegible] 2.1 mg. of ascorbic acid in a medium containing supplements of 5 mg. [illegible] XLIV, 25 mg. of XLIV, and none, respectively. The conversion (about 10 [illegible]ent) of the condensation product to ascorbic acid was rather high rela[illegible] to the experiments by Horowitz *et al.* (210). Since the Indian authors did [illegible] report the method and purity of the preparation, the actual yield might



[illegible]ructure XXXIX. L-idono-γ-lactone



Structure XL. L-talono-γ-lactone

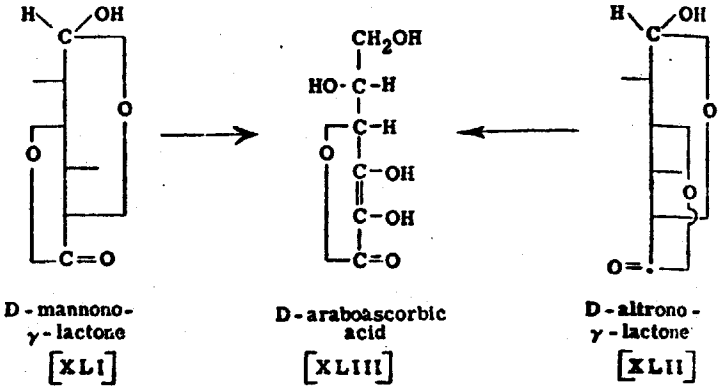
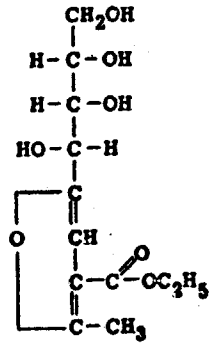


FIG. 8. Formation of D-araboascorbic acid.

even be higher. However, a direct comparison from the two groups can [illegible] be made. The high yield also probably eliminates the function of the conde[illegible]sation product as a coenzyme. It is evident that neither the legume nor t[illegible] rat can carry out the condensation effectively, since both compounds are [illegible]mal metabolites and since simultaneous injections did not increase as m[illegible] as the administration of XLIV. This argument could detract from the ov[illegible]all importance of the scheme as worked out by the Columbia and Cambri[illegible] investigators, in the biosynthesis of the vitamin in the organisms requiring exogenous ascorbic acid.



Structure XLIV. A condensation product of acetoacetate and glucose.

From the chemical viewpoint, ascorbic acid has a unique structure am[illegible] vitamins, since it contains a dienol group which not only contributes red[illegible] action to the compound, but also confers an acidic behavior upon the m[illegible]cule. In nonbuffered aqueous solution it was oxidized by *o*-iodosobenzoic [illegible] the rate following a second order reaction. The oxidized vitamin under[illegible] transformation to an equivalent molar quantity of a product which was r[illegible]

oxidized by iodine but not by iodosobenzoic acid. In phosphate buffer, extensive degradation occurred with formation of up to three equivalents of products capable of reducing *o*-iodosobenzoic acid (216).

D-Araboascorbic acid which differs from ascorbic acid only at the configuration of C-5 showed practically all the chemical properties of the vitamin (217). The two compounds showed almost the same cyanide- or phenol-combining ability. Either could prevent the toxic effects of cyanide in *E. coli*, of phenol in rats, or tetanus toxin in mice (217). Both showed a stimulatory effect for the formation of citrovorum factor from folic acid. Conversion of folic acid to citrovorum factor involved at least two steps, namely reduction and formylation. The nonspecificity of the reducing agent was shown in mice (217), in chick liver homogenates (218), in *S. faecalis* A (219), and in *L. casei* (220). In fact, tetrahydro- (221) or dihydropteroylglutamic acid (222) might be substituted for citrovorum factor in the absence of ascorbic acid or other reducing agents. D-Araboascorbic acid was demonstrated to be equally potent in most of the above investigations as a reductant. The pantothenic acid sparing action by ascorbic acid (223) could also be exhibited by D-araboascorbic acid. However, D-araboascorbic acid exhibited only about 5 per cent of the antiscorbutic potency (217), and it therefore does not seem reasonable to propose that the antiscorbutic activity is due to reducing properties.

The first oxidative step of the conversion of tyrosine to acetoacetic acid has been considered ascorbic acid dependent (224). More recently it has been shown (225) that the reaction could use, with equal activity, D-araboascorbic acid or hydroquinone, in acetone powder extracts of rat, dog, and rabbit livers. Furthermore, structurally unrelated compounds such as homogentisic acid, PAB, *p*-phenylenediamine, 2,6-dichlorophenolindophenol could also increase the tyrosine oxidation. The positive correlation of blood vitamin A levels with plasma ascorbic contents (226) might well be attributable to the antioxidant property of the vitamin. An observation (227) was reported that ascorbic acid inhibited the proteolysis by crude pepsin preparation but did not by crystalline pepsin. The reason might be attributable to inactivation of impurities in the crude preparations that were dependent upon the redox behavior of ascorbic acid.

The literature concerning the replacement by ascorbic acid of the vitamin requirement in lactic acid bacteria has been reviewed recently (228). It was suggested that ascorbic acid functioned by reactivating inactive oxidized fragments of vitamin B_{12} (229). The observations (102) of the growth of *L. casei* in a thiamine-free but ascorbic-containing medium might be an analogy of the B_{12} case. In these experiments on animals as well as microorganisms, the only requirement may well be simply a compound with a proper redox potential.

Some functions, however, exhibited by ascorbic acid are not easily explicable by its reducing action, but on the other hand are also obscure from a purely enzymological view. Adequate ascorbic acid intake was shown to be required not only for normal healing in the early post-wound period in the

guinea pig, but also for the maintenance of the scar tissue in later stages (2[illegible]). This might be a result of an increased ascorbic requirement during the formation of collagen-containing tissue, as observed in the same animals (231). The vitamin deficiency also caused the impairment of VEM (vasoexcitor material) (232) and decrease of liver protein content (233). The mechanism of stress is not very clear and the role of ascorbic acid in stress (234, 235, 236) is [illegible] obscure.

In 1951, Long and associates (237) found that more cholesterol was deposited in the adrenals of ascorbic acid deficient guinea pigs than in those of the normal controls. This fact was also reported by King *et al.* (238). Ascorbic acid-1-C^{14} was not appreciably incorporated into cholesterol. The vitamin exerted a marked effect upon the conversion of C-1 labeled acetate to cholesterol and other steroids in guinea pig *in vivo*. Compared with *ad libitum* and pair fed controls, a progressive increment in their fixation of acetate in cholesterol with the onset of scurvy was observed. The severely scorbutic animals incorporated six times as much C^{14} from acetate into cholesterol isolated from adrenals (238, 239). This fact may be explained by assuming that either the metabolism of acetate is deranged or the further utilization of sterols is depressed by the lack of ascorbic acid. It has been suggested by several groups that the biosynthesis of cortical hormones is inhibited by ascorbic acid deficiency (240). If this hypothesis is correct, cholesterol should obviously accumulate. Many previous negative results might be a result of difficulties in methodology. Recent findings (241) showed that ascorbic acid depressed the breakdown of cortical hormones to 17-ketosteroids determined as dehydroisoandrosterone. Simultaneous ascorbic acid treatment of adrenalectomized rats receiving cortisone acetate resulted in a decrease of urinary 17-ketosteroids and an increase of urinary corticosteroids (241). The same authors also found (242) in *in vitro* experiments that liver tissue of ascorbic acid-treated rats metabolized the 17-OH, as well as the 20,21-ketosteroids and the conjugated unsaturated systems of the corticosteroid structure at a slower rate than does liver tissue of control rats. The influence of ascorbic acid in sterol metabolism is further complicated by the effect of other compounds, such as insulin (243). Ascorbic acid enhanced the insulin tolerance of cortisone-treated, adrenal-demedullated rats. The vitamin alone did not affect insulin tolerance. The urinary glucose and nitrogen were increased after cortisone injection. These increases were further enhanced when ascorbic acid was used along with cortisone. This observation confirmed the view that ascorbic acid can enhance gluconeogenic action of cortical hormones. As a matter of fact the lack of the vitamin induced hypoglycemia in guinea pigs by repeated injection of acetoacetate (215). The injections also depleted glycogen storage in the deficient animal. No steroid determinations were made in this study. Possibly the steroid metabolism is greatly disturbed, so that a gross abnormality was observed.

Certain oxidases, such as potato polyphenol oxidase (244) and xanthine oxidase (245), were inactivated by ascorbic acid. The incubation of pu[illegible]

xanthine oxidase with trace amounts of ascorbic acid resulted in severe inhibition in the enzymic activity. However, blood levels and rate of excretion of uric acid and allantoin were independent of the ascorbic acid intake in guinea pigs. Contrary to expectations, when polyphenoloxidase was treated with ascorbic acid under anaerobic conditions, and the ascorbic acid was then removed by dialysis, the enzymic activity was greatly reduced. The treated enzyme could not be reactivated by cupric ions. No explanation was given. It would be of interest to determine whether the inactivation in both systems may be a result of the antioxidant action of the vitamin. On the other hand, an ascorbic oxidase of fungus spores was found to be completely specific (246). It was irreversibly inhibited by D-araboascorbic acid and was quite different from other described ascorbic oxidases being inert toward "copper poisons" and inhibitors for iron proteins (246, 247).

In spite of the many examples given in the foregoing pages which point to a relatively nonspecific reducing function for ascorbic acid, it seems compelling to regard this vitamin as specifically essential for other purposes. Thus, the scorbutic condition in the animal can not be non-specifically relieved by reducing agents. Such a specific role, however, should be referable to a participation in one or more enzyme systems, in a manner that can be interpreted biochemically. The present uncertainty regarding ascorbic acid function must continue to present a challenge to enzyme chemists to determine the proper place in the metabolic scheme for this important vitamin.

Improved methods for the determination were reported (248, 249), and reviews on the interaction of ascorbic acid with bacteria (228), its role in plants (250), and its physiological role in general (240) have appeared during the past year.

Miscellaneous

The beneficial effect of antibiotics on animal growth was considered (251) to be attributable to an alteration of the intestinal flora; growth rates of both [illegible]- and riboflavin-deficient rats were improved. This study confirms the general experience reported in a review (252), in which faster growth resulted when antibiotics were fed to calves, swine, turkey poults, and chicks, as well as to school children on all-vegetable diets. Sparing action of the growth promotant upon the dietary vitamin requirements was usually observed.

A purified diet was described for young guinea pigs (253) which was used for the production of specific vitamin deficiencies. It was composed of sucrose, corn starch, cerelose, cellophane, vitamin-free casein, corn oil, minerals, and vitamins. The complete diet was stated to support growth and physiological development as satisfactorily as the same diet supplemented with greens, or with a supplemented commercial rabbit chow. Omission of any of seven B vitamins (but not B_{12}, biotin, or inositol) produced clear-cut deficiencies of each.

Destruction of certain B vitamins within purified rations was traced to the salts being used (254): the Phillips-Hart mixture (255) employed was more deleterious than a U.S.P. XI formula. The effect was greatest on thia-

mine [see also Thiamine section (107)]. This was thought to be attributable to the alkalinity of the mixture rather than to the effect of specific ions.

New growth factors.—Research on new growth factors continues at a rapid pace. A recent tabulation (114) contained nearly 50 reports of still unidentified principles for animal or microbial growth or enzymatic action; since then at least another 20 papers have appeared. Most of these factors are probably duplications, and others may likely be artifacts or represent the combined effects of already known factors. Nevertheless, some of these, such as the xanthine oxidase factor [inorganic molybdate (136, 137, 256, 257, 258); see Riboflavin section], coprogen (259), and ferrichrome (260) have been carried to the point where they are known to be specific entities in pure form. Ferrichrome, a growth factor for *Pilobolus kleinii* and *Arthrobacter terregens*, has been isolated from the smut fungus *Ustilago sphaerogena*. The pure crystalline compound is 500 times more potent than hemin, which was previously found to be a growth requirement for the organism (261, 262). Its provisional formula was assigned as $C_{27-29}H_{42-46}O_{12}N_9Fe$, with a molecular weight of 7[illegible] to 800 calculated from diffusion data. Its structure is not known, but a hydrolysate of the compound was found to contain glycine and a dibasic amino acid (263). A specific spectrophotometric method of assay has been described which permits the assay of 3 μg. or more of the substance per ml., with a reproducibility of ± 4 per cent (264) It is interesting to note that addition of [illegible] to the smut fungus culture immediately reduces the ferrichrome concentration of the cells, while the metal is essential for the accumulation of cytochrome-*c*, as well as for rapid growth, in the same organism (265). The redox potential of oxidized-reduced ferrichrome was found to be considerably lower than that of cytochrome-*c*. The reduced form can be stabilized by cyanide to prevent air oxidation (261). Its role, if any, in electron transport, and its relationship with the cytochrome system and with coprogen (259) deserve additional study.

Another vitamin, B_{13}, has received further clarification (266) after a lapse of several years (267). It was originally concentrated from distillers' dry solubles. Orotic acid has now been isolated from that source and found to be active in the B_{13} (rat growth) assay. Based on this propinquity, together with a similarity in absorption spectra, the authors suggest (266) that B_{13} and orotic acid may be related, although not identical. The earlier work (267) reported growth responses from B_{13} with as little as 6 μg. per day, whereas [illegible] of orotic acid was required to produce a growth response (266).

Most difficult to evaluate are the factors which have been characterized only by a single growth or other physiological response. Into this category fall a score or more of "factors" that have been reported in recent years dealing with growth of chicks and poults. Opportunities seem excellent for duplication not only among each other but also with new forms of vitamin [illegible], pyrimidines (whey factor), and with unidentified microbial growth factors. In addition, strain and environmental differences may operate to [illegible] growth. In spite of these restrictions, it appears that two, and possibly [illegible]

stinct factors for poultry nutrition may now be under study. These are: (*a*) factor or factors in liver or fish solubles, active on chicks and poults, and in proving hatchability (268 to 277). This has been fractionated (278) from solubles and shown to be soluble in water and in phenol-water or metha-water mixtures, but not in ether. It is reportedly not identical with lyxo-in or dried whey. Concentrates were active at 0.05 per cent of the diet. another fractionation study, however, states that the factor is insoluble in 72 cent phenol (279), not adsorbed on Norite at acid pH and heat stable 280). (*b*) A factor from whey (270, 272, 281 to 284), which may also be pres-t in such materials as soybean (285) or yeast (286). Possible relationships orotic acid or B_{12} suggest themselves (cf. also 287). A third possibility is a factor in grass juice (288, 289). Whether the growth promoting effect of alfa meal (290) is related to the grass juice factor appears unknown. The arged hock disorder in turkeys (286) has been shown to be preventable by in and vitamin E (291).

Progress toward the identification of these factors has been relatively This has no doubt been attributable to many causes, including the long riod required for assay, the slight growth effects observable (often as low 10 per cent over the growth on the basal diet), and the effect of infection, tibiotics, and even the physical environment on growth responses. Several rkers have recorded better growth in clean cages or batteries than in quar-s that have seen prolonged use. In addition, only a few of the studies cited e employed purified basal diets or have extensively concentrated the sup-ents being fed. Until this is done, uncertainty will persist regarding both number and the nature of these stimulatory agents.

Other factors for higher animals include: an antipancreatic fibrosis factor ucks (292) and for rats (293, 294). "Stress factors" have been reported rats, which were required during feeding of thyroid extract or iodinated eins; these were thought to respond to lyxoflavin (295), but other reports to 299) have indicated a requirement for an unknown liver principle to ome the thyrotoxic effect.

ne factors for bacteria and other lower forms include: N-D-glucosylgly-and related glycosylglycines, for lactic acid bacteria (300); stimulatory s from marine algae extracts for both *S. faecalis* and *Leuconostoc citro-m* (301); growth factors for *Lactobacillus bifidus*, including strepogenin ons (302), poultry fecal extracts (303), papaic digest of soybean (304), ll as blood group mucoids (305, 306); histidine (307) and serine (308) les for *Lactobacillus delbrückii*; *Staphylococcus albus* growth factors were previously thought (309, 310) to be special peptide structures, lly those of cystine, but which were later reported (311) to be active after removal of all amino acids; a growth factor for *Fusobacteria* (312); stable factors for lactobacilli, which may be related to B_{12}, but are ht not to be identical (313, 314); a growth factor for pleuropneumonia-anisms, which is reported to be a low molecular weight basic protein owth factors for Clostridia (316) which may be strepogenin-like; a

growth factor for *Trypanosoma cruzi*, which is believed to be a complex hem globin derivative (317); and a growth factor for Pythiogeton (318). Mycoba tin (empirical formula: $C_{47}H_{76}O_{30}N_5$), which is essential for the growth of *M cobacterium johnei*, has been isolated into a crystalline aluminum comp from a nonpathogenic *Mycobacterium* (319). Unknown materials, probal nucleotides, appear active as a coenzyme for alcoholic fermentation (320) for citrate oxidation (321). A bacterial deaminase cofactor has been descri which is produced by heating carbohydrates with H_2SO_4 (322). This deco position product was thought to be functionally related to biotin and ad nylic acid.

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THE ROLE OF PYRIDOXINE IN THE EXPERIMENTAL CHEMOTHERAPY OF TUBERCULOSIS

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Shortly after the beginning of the use of isoniazid, there appeared in the literature the information that with prolonged treatment with this preparation, side effects are observed (Sh. A. Alimov, N. A. Shmelev, F. L. Elinson, B hl and Nimitz, Irkens, and Lazarou) which are diminished with the use of pyridoxine (R. A. Radkevich, Biehl and Vilter, McDermott, Riemensnider, and Zbinden.

Apparently, due to the toxic effect of isoniazid on the nervous system, there is a disruption in exchange of the vitamin B group. With the prescription of pyridoxine (vitamin B_6), the occurrence of peripheral neuropathy and a number of toxic effects caused by isoniazid are prevented.

The toxicity of isoniazid is also explained by the fact that it is a source of ammonia, which in its free form is extremely harmful to the nervous system.

E. S. Stepanyan and R. A. Radkevich and co-workers showed that vitamin B_6 dispels side effects only in patients treated with isoniazid, and in no single case is this observed during treatment with para-aminosalicylic acid or streptomycin.

B. Ya. Stukalova, Boone, Zbinden, and Ungar state that vitamin B_6 does not affect the bacteriostatic activity of the products of hydrazide of isonicotinic acid, The data of McCune and McDermott contradict this.

We conducted a number of experiments in order to investigate the toxicity of vitamin B_6, its bacteriostatic action, and its effect on the tuberculostatic action of isoniazid. At the same time we determined the action of vitamin B_6 with large doses of isoniazid in a tuberculosis experiment.

In a number of repeated experiments *in vitro*, we ascertained the action of vitamin B_6 on the tuberculostatic activity of isoniazid. It turned out that in the presence of a nutritive medium of horse serum, vitamin B_6 did not inhibit the growth of tuberculosis mycobacteria and did not influence the bacteriostatic activity of isoniazid.

The toxicity of vitamin B_6 and isoniazid was studied in 19 experiments on 169 white mice, with the administration of these preparations *per os* and parenterally. The maximum permissible doses were determined in acute and chronic experiments. It was noted that with subcutaneous administration of vitamin B_6, the permissible doses for mice were from 1 to 30 mg in acute cases, and from 0.1 to 1 mg in chronic cases. These vitamin B_6 dosages were also permissible for *per os* administration. With subcutaneous administration, 3-4 mg of isoniazid turned out to be a lethal dose. In the chronic experiment, permissible doses of isoniazid were 0.1, 0.2, 0.3, 0.4, and 0.5 mg.

In a histological study of the mouse organs in this group of experiments, no sort of pathological changes was ascertained.

Vitamin B_6 in doses of 3, 5, and 10 mg prevented death in mice which had received 3 mg of isoniazid. These same data were obtained with the administration of vitamin B_6 and isoniazid *per os*.

The mice in one group received *per os* 3 mg of isoniazid and at the same time 3 mg of vitamin B_6; the other group received the same dosage under the skin.

After 45 minutes, part of the mice showed twitching of the extremities; the animals fell on their side, the back feet dragged and were immobile. Such a state continued for 2 hours, and after 24 hours the mice returned to normal.

We studied the action of vitamin B_6 on experimental tuberculosis of mice which were treated with isoniazid. 90 mice were infected internally with 0.1 mg of $H_{37}RV$ culture in 0.5 ml of physiological solution.

The animals were divided into 4 groups of 18 mice each and one of 16: in the first group, 18 mice received 0.6 mg of isoniazid, in the second - 0.6 mg isoniazid and 0.2 mg vitamin B_6, in the third - 1 mg isoniazid, in the fourth - 1 mg isoniazid and 0.2 mg vitamin B_6, and the 16 mice in the fifth group served as a control.

The treatment of all the experimental mice was begun after 24 hours and lasted 1 month, after which all the animals remaining still alive were killed.

The times the animals were alive, weights of body and organs, and the seeding of tuberculosis mycobacteria were determined, and histological studies of the organs were conducted.

TIMES THE ANIMALS LIVED

Group of Mice Studied	Experiment Day 7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	# of Animals Living
1												1								1	16 out of 18
2												1						2			15 out of 18
3							3			1		3		5				1		4	0 out of 18
4					1						2				5					2	7 out of 18
5	1							1				3		5				3			4 out of 16

In the table are presented data on the length of time the mice lived (in days) in the experimental and control groups.

As is evident from the table, 12 control mice died before the end of the experiment. The mice which received 1 mg of isoniazid began to die from the 13th day on, all the way up to the 27th day.

Thus, isoniazid apparently had a cumulative effect and in a large dose caused the mice which were infected with the tuberculosis mycobacteria to die.

It is interesting that the mice of the second group were less affected, in which the animals recieved 0.6 mg of isoniazid or 0.6 mg isoniazid and 0.2 mg vitamin B_6.

The cases deserved attention in which, in the group of animals which received 1 mg isoniazid and 0.2 mg vitamin B_6, 7 out of 10 mice lived, while in the group which received only isoniazid in a 1-mg dose, all the mice died.

The mean weight of lungs and spleen of the control mice significantly exceeded the weight of these organs in the experimental mice in all groups. The spleen weighed the least in animals which received 0.6 mg of isoniazid or 0.6 mg isoniazid and 0.2 vitamin B_6.

We determined the seeding of tuberculosis mycobacteria from the organs of the experimental and control animals. 100% growth of mycobacteria was noted in seeding the organs of the control mice. In the experimental mice, the percentage of lack of growth varied from 32 to 52%. The best results were in the group of mice which received 0.6 mg of isoniazid and 0.2 mg vitamin B_6.

The small percentage of lack of growth for the tuberculosis mycobacteria in the experimental animals is explained by the fact that they were infected with a very virulent strain, due to which, after only 13 days, the

control animals began to die. Considering this, we internally infected another group of 65 mice with 0.1 mg of a human-type culture of moderate virulency (K_1).

In this experiment, the animals were divided into 5 groups, 3 of 15 mice each and 2 of 10. The first group of 15 served as a control; the second group received 1 mg of isoniazid and 0.2 mg vitamin B_6 subcutaneously. The third group received 0.6 mg isoniazid and 0.2 mg vitamin B_6. The fourth group, 10 mice, received 0.6 mg isoniazid and the fifth - 0.2 mg vitamin B_6. The animals were killed on the 30th day after the beginning of the experiment.

In the seedings from the lungs and spleen of the control animals, in 98% there was growth of tuberculosis mycobacteria. The same results were obtained in the mouse group which received 0.2 mg of vitamin B_6.

In seedings from the organs of mice which received 0.6 mg of isoniazid, growth was absent in 65% of the cases, and in 67% for mice which received 0.6 mg isoniazid and 0.2 mg vitamin B_6. In the animal group which received 1 mg isoniazid and 0.2 mg vitamin B_6, lack of growth was noted in 70% of the cases.

Thus, in this experiment using the infection of the moderately virulent strain of tuberculosis mycobacteria, there was a greater suppression of the ability of the mycobacteria to live.

In a histological study, a specific tuberculotic process was expressed in all the control mice with an expressed tendency to an exudative-necrotic type of reaction.

With the use of isoniazid in the 0.6-mg doasage, specific changes in the lungs occurred only in 2 out of 15 animals which received treatment and resulted in the development of isolated foci of catarrhal-desquamative pneumonia without caseous necrosis.

Changes in the liver were characterized by the development of lymphocide-histocytic foci; in the livers of 5 animals, particles of coagulated tissue necrosis were noted. In the spleen of the majority of the animals, hyperplasia of the lymphatic follicles occurred; the spleens of 4 mice had fine particles of coagulated tissue necrosis.

In the organ study of animals which received isoniazid in a 0.6-mg dose along with vitamin B_6, in the lungs and spleen of all except one mouse, tuberculotic changes were absent. Only in the liver of several of the animals was the tuberculotic process expressed.

With the use of isoniazid in higher dosages, rare foci of tuberculotic pneumonia without caseous necrosis were noted in the lungs of 8 out of 11 mice which received treatment. In the liver and spleen, tuberculotic changes were expressed which were similar to those in the group of untreated (control) mice, and in a number of animals, changes in the liver were more severe than in the control mice.

In the group of animals which received 1 mg of isoniazid together with vitamin B_6, changes in the liver had a limited character and resulted in the development of rare foci of catarrhal-desquamative pneumonia. Changes in the liver were characterized by the development of fine lyphatic-histocytic foci. In the spleen of the majority of animals, hyperplasia of the lymphatic follicles was expressed. However in the spleen and liver of 5 out of 15 animals, particles of caseous tissue necrosis were present.

Conclusions

1. In the presence of a nutritive medium of horse serum, vitamin B_6 does not affect the growth of tuberculosis culture.

2. Vitamin B_6 does not affect the bacteriostatic property of isoniazid.

3. Vitamin B_6 is non-toxic for mice and pigs. In dosages of 3, 5, and 10 mg, it increases the tolerance of mice to a lethal dose of isoniazid.

4. In in vivo experiments on infected animals, vitamin B_6 in combination with 1 mg of isoniazid prevents the premature death of half of the animals.

5. The greatest suppression in ability of the tuberculosis mycobacteria to live in the in vivo experiment is observed in the group of mice which received 0.6 mg of isoniazid and 0.2 mg of vitamin B_6.

6. The combined use of isoniazid and vitamin B_6 contributes to the restriction of the tuberculotic process and the decrease in exudative-necrotic changes in the organs. The most therapeutic effect is obtained with use of 0.6 mg isoniazid in combination with 0.2 mg vitamin B_6.

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РОЛЬ ПИРИДОКСИНА В ЭКСПЕРИМЕНТАЛЬНОЙ ХИМИОТЕРАПИИ ТУБЕРКУЛЕЗА

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Вскоре после начала применения изониазида в литературе появились сообщения о том, что при продолжительном лечении этим препаратом наблюдаются побочные явления (Ш. А. Алимов; Н. А. Шмелев; Ф. Л. Элинсон; Biehl и Nimitz; Irkens, Lazaron), которые уменьшаются при применении пиридоксина (Р. А. Радкевич; Biehl и Vilter; McDermott, Riemensnider; Zbinden).

Очевидно, в основе токсического влияния изониазида на нервную систему лежит нарушение обмена витаминов группы В. При назначении пиридоксина (витамин B_6) предупреждается возникновение периферических невропатий и ряда токсических явлений, обусловленных изониазидом.

Токсичность изониазида объясняют также тем, что он является источником аммиака, который в свободном виде весьма вредно действует на нервную систему.

Э. С. Степанян, Р. А. Радкевич с соавторами показали, что витамин B_6 устраняет побочные явления только у больных, лечившихся фтивазидом, и ни в одном случае этого не наблюдалось при лечении ПАСК и стрептомицином.

Б. Я. Стукалова, Boone, Zbinden, Ungar указывают, что витамин B_6 не влияет на бактериостатическую активность производных гидразида изоникотиновой кислоты. Этому противоречат данные McCune, McDermott

В доступной нам литературе мнения о действии пиридоксина при терапии гидразидом изоникотиновой кислоты в эксперименте и клинике туберкулеза разноречивы.

Мы поставили ряд опытов для исследования токсичности витамина B_6, бактериостатического действия его и влияния на туберкулостатическое действие изониазида, определили действие витамина B_6 одновременно с большими дозами изониазида в эксперименте при туберкулезе.

В ряде повторных опытов in vitro мы выясняли действие витамина B_6 на туберкулостатическую активность изониазида. Оказалось, что при наличии в питательной среде лошадиной сыворотки витамин B_6 не задерживал роста микобактерий туберкулеза и не влиял на бактериостатическую активность изониазида.

В 19 опытах на 169 белых мышах исследовалась токсичность витамина B_6 и изониазида при введении этих препаратов per os и парентерально. Были определены переносимые дозы в острых и хронических опытах. Отмечено, что при подкожном введении витамин B_6 переносим в острых опытах на мышах в дозах от 1 до 30 *мг*; в хронических опытах в дозах от 0,1 до 1 *мг*. Эти дозы витамина B_6 были переносимы и при введении per os. При подкожном введении 3–4 *мг* изониазида оказались смертельной дозой. В хроническом опыте были переносимы 0,1, 0,2, 0,3, 0,4 и 0,5 *мг* изониазида.

При гистологическом исследовании органов мышей этой группы опытов никаких патологических изменений не выявлено.

Витамин B_6 в дозах 3, 5, 10 *мг* спасал от смерти мышей, получивших 3 *мг* изониазида. Эти же данные были получены и при введении витамина B_6 и изониазида per os.

Мыши одной группы получили per os, а другой — под кожу по 3 *мг* изониазида и одновременно по 3 *мг* витамина B_6.

Через 45 мин. у части мышей начались подергивания конечностей, животные падали на бок, тянули задние лапки, были неподвижны. Такое состояние продолжалось 2 часа, а затем через 24 часа мыши приходили в нормальное состояние.

Мы изучали действие витамина B_6 на экспериментальный туберкулез мышей, которых лечили изониазидом. Внутривенно было заражено 90 мышей 0,1 *мг* культуры H_{37} RV в 0,5 *мл* физиологического раствора.

Животных разделили на 5 групп: в 1-й группе 18 мышей получили по 0,6 *мг* изониазида, во 2-й группе 18 мышей получили по 0,6 *мг* изониазида и 0,2 *мг* витамина B_6, в 3-й группе 18 мышей получили по 1 *мг* изониазида; в 4-й группе 18 мышей получили по 1 *мг* изониазида и 0,2 *мг* витамина B_6 и 16 мышей 5-й группы служили контролем.

Лечение всех подопытных мышей было начато через 24 часа и продолжалось 1 месяц, после чего все оставшиеся в живых животные были забиты.

Определялись сроки переживаемости животных, вес тела и органов, высеваемость микобактерий туберкулеза, проводились гистологические исследования их органов.

Сроки переживаемости животных

Группа исследованных мышей	День опыта																			Число выживших животных
	7-й	8-й	9-й	10-й	11-й	12-й	13-й	14-й	15-й	16-й	17-й	18-й	19-й	20-й	21-й	22-й	23-й	24-й	25-й	26-й	
1-я												1								1	16 из 18
2-я												1						2			15 из 18
3-я							3			1		3		5				1		4	0 из 18
4-я					1						2				5					2	7 из 18
5-я	1							1				3		5				3			4 из 16

В таблице представлены данные о переживаемости мышей (в днях) в группе подопытных и контрольных животных.

Как видно из таблицы, 12 контрольных мышей погибли до конца опыта. Мыши, получавшие по 1 *мг* изониазида, начали погибать с 13-го дня опыта и погибли все до 27-го дня.

Таким образом, изониазид обладал, очевидно, кумулирующим действием и в большой дозе привел мышей, зараженных микобактериями туберкулеза, к гибели.

Интересно, что меньше всего погибло мышей из 2 групп, в которых животные получили по 0,6 *мг* изониазида и 0,6 *мг* изониазида и 0,2 *мг* витамина B_6.

Заслуживает внимания то обстоятельство, что в группе животных, получавших по 1 *мг* изониазида и 0,2 *мг* витамина B_6, из 10 мышей выжили 7, в то время как в группе животных, получавших один изониазид в дозе 1 *мг*, погибли все мыши.

Средний вес легких и селезенки контрольных мышей значительно превосходил средний вес этих органов у подопытных мышей во всех группах. Минимальным был вес селезенки у животных, получивших 0,6 *мг* изониазида, 0,6 *мг* изониазида и 0,2 *мг* витамина B_6.

Мы определяли высеваемость микобактерий туберкулеза из органов подопытных и контрольных животных. Отмечен 100% рост микобактерий при посеве органов контрольных мышей. У подопытных мышей процент отсутствия роста колебался от 32 до 52. Лучшие

результаты были в группе мышей, получавших 0,6 *мг* изониазида и 0,2 *мг* витамина B_6.

Небольшой процент отсутствия роста микобактерий туберкулеза у подопытных животных объясняется тем, что они были заражены весьма вирулентным штаммом, от которого уже через 13 дней стали погибать контрольные мыши. Учитывая это, мы заразили другую группу в 65 мышей внутривенно 0,1 *мг* культуры человеческого типа средней вирулентности (K_1).

В этом опыте животных разделили на 5 групп: в 1-й 15 мышей служили контролем, во 2-й 15 мышей получили по 1 *мг* изониазида и 0,2 *мг* витамина B_6 подкожно; в 3-й группе 15 мышей получили по 0,6 *мг* изониазида и 0,2 *мг* витамина B_6, в 4-й группе 10 мышей получили по 0,6 *мг* изониазида; в 5-й группе 10 мышей получили по 0,2 *мг* витамина B_6. Животные были забиты через 30 дней после начала опыта.

В посевах из легких и селезенки контрольных животных в 98% имелся рост микобактерий туберкулеза. Такие же результаты получены в группе мышей, получавших по 0,2 *мг* витамина B_6.

В посевах из органов мышей, получавших по 0,6 *мг* изониазида, рост отсутствовал в 65% случаев, получавших 0,6 *мг* изониазида и 0,2 *мг* витамина B_6 - в 67%. В группе животных, получавших 1 *мг* изониазида и 0,2 *мг* витамина B_6, отсутствие роста отмечено в 70% случаев.

Таким образом, в этом опыте при заражении животных штаммом микобактерий туберкулеза средней вирулентности было обнаружено большее угнетение жизнеспособности микобактерий.

При гистологическом исследовании у всех контрольных мышей выявлен специфический туберкулезный процесс с выраженной наклонностью к эксудативно-некротическому типу реакции.

При применении изониазида в дозе 0,6 *мг* специфические изменения в легких выявлены лишь у 2 из 15 получавших лечение животных и заключались в развитии единичных очажков катарально-десквамативной пневмонии без казеозного некроза.

Изменения в печени характеризовались развитием редких лимфоидно-гистиоцитарных очажков; у 5 животных в печени отмечались участки творожистого некроза ткани. В селезенке у большинства животных выявлена гиперплазия лимфатических фолликулов; у 4 мышей в селезенке обнаруживались мелкие участки творожистого некроза ткани.

При исследовании органов животных, получавших изониазид в дозе 0,6 *мг* в сочетании с витамином B_6, в легких и селезенке у всех (за исключением одной мыши) туберкулезные изменения отсутствовали. Лишь в печени у нескольких животных выявлен туберкулезный процесс.

При применении изониазида в более высокой дозировке в легких у 8 из 11 получавших лечение мышей отмечены редкие очажки туберкулезной пневмонии без казеозного некроза. В печени и селезенке были выявлены туберкулезные изменения, сходные с таковыми в группе нелеченных (контрольных) животных, а у ряда животных изменения в печени были более тяжелые, чем у контрольных мышей.

В группе животных, получавших 1 *мг* изониазида в сочетании с витамином B_6, изменения в легких носили ограниченный характер и заключались в развитии редких очажков катарально-десквамативной пневмонии. Изменения в печени характеризовались развитием мелких лимфоидно-гистиоцитарных очажков. В селезенке у большинства животных выявлена гиперплазия лимфатических фолликулов. Однако в селезенке и печени у 5 из 15 животных обнаружены участки казеозного некроза ткани.

Выводы

1. При наличии в питательной среде лошадиной сыворотки витамин B_6 не влияет на рост туберкулезной культуры.

2. Витамин B_6 не влияет на бактериостатическую способность изониазида.

3. Витамин B_6 нетоксичен для мышей и свинок. В дозе 3, 5 и 10 *мг* он повышает толерантность мышей к смертельной дозе изониазида.

4. В опытах in vivo на зараженных животных витамин B_6 в комбинации с 1 *мг* изониазида спасает от преждевременной гибели половину животных.

5. Наибольшее угнетение жизнеспособности микобактерий туберкулеза в опыте in vivo наблюдается в группе мышей, получавших 0,6 *мг* изониазида и 0,2 *мг* витамина B_6.

6. Комбинированное применение изониазида с витамином B_6 способствовало ограничению туберкулезного процесса и уменьшению экссудативно-некротических изменений в органах. Наилучший терапевтический эффект получен при применении 0,6 *мг* изониазида в сочетании с 0,2 *мг* витамина B_6.

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ROLE DE LA PYRIDOXINE DANS LA CHIMIOTHERAPIE EXPERIMENTALE DE LA TUBERCULOSE

E. I. Tchertkova et *N. N. Udilova*, candidats ès sciences médicales

Les auteurs se sont proposés d'étudier le rôle de la pyridoxine (vitamine B_6) dans la chimiothérapie expérimentale de la tuberculose.

Les recherches ont prouvé qu'en présence de sérum équin dans le milieu de culture, vitamine B_6 n'influence point sur la poussée de M. tuberculosis, n'exerçant non plus aucun effet sur l'action bactériostatique de l'isoniazide.

Dans les expériences faites sur les souris blanches et les cobayes vitamine B_6 n'est pas toxique et administrée à raison de 3,5, 10 mg elle est capable d'élever la tolérance des souris, saines et infectées, à la dose léthale de l'isoniazide.

La suppression maximum de la viabilité des M. tuberculosis se fait observer dans les expériences où les souris recevaient 0,6 mg de l'isoniazide et 0,2 mg de la vitamine B_6.

L'administration combinée de la vitamine B_6 d'ensemble avec l'isoniazide contribue à la restriction du processus tuberculeux et tend à réduire les altérations exsudatives et nécrotiques dans les organes des animaux.

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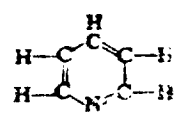
Action of Vitamin B_6 and its Antivitmain on the Development of the Fowl Embryo

by G. Conti and G. Milio

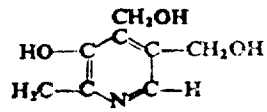
The group of B_6 vitamins contain three distinct substances: pyridoxine which is a derivitave of pyridine; pyridoxamine and pyridoxal which are derived from pyridoxine. All organisms utilize pyridoxal; certain ones equally utilize pyridoxine, others pyridoxamine, and finally, others (the rat, for example) utilize the three substances. This statement makes one think that the truly active substance would be pyridoxal, and that only certain organisms would be able to form pyridoxine, pyridoxamine, or both substances. Furthermore, pyridoxal in the form of phosphoric ester (pyridoxalphosphate) acts importantly as co-enzyme in the number of complex enzymatic actions in the metabolism of amino acids. Among the most complex are the trans aminase, the amino acid-decarboxylase, the deshydratases, the desulfhydrases, the racemases, the histaminase, and the tryptophanases.

In aminals, the lack of B_6 vitamins provokes varied disorders. In rats, characteristic dermatitis associated with an epileptic consulsive syndrome, a muscular fatigue and a stoppage of growth is observed. In the dog, a hypochromic anemia is observed; in the pig, a hypochromic anemia with convulsions, movement disorders, and lesions of the nervous system. In baby chickens, the lack of B_6 vitamins provokes a stoppage of growth. Finally, in man, the real syndrome provoked by the lack of B_6 vitamins has not het been verified; the first deficiency syndrome has been obtained thereapeutically following the administration of deoxypyridoxine, antagoinst of pyridoxine.

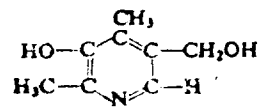
Among the antivitamins of the B_6 group, the most active is deoxypyridoxine (DPX), discovered by Ott in 1945. DPX has a structure chemically analagous to that of vitamin B_6, and more precisely, a structure analagous to that of pyridoxime (PX); in DPX, the OH group is replaces by an ++ atom in position 4.



Pyridine



Pyridoxine
(Vitamine B_6)



Désoxypyridoxine
(Antivitamine B_6)

In 1946, Ott studied the biological action of DPX in baby chickens. This compound proved to be the strongest among the inhibitives of B_6 vitamins. Ott was able, in effect, to establish that a molecule of PX neutralizes the effects of two molecules of DPX. While for the other inhibitives, one molecule of vitamin was capable of neutralizing 40 or more of them. DPX showed a lethal effect even at very weak doses (16 γ) in baby chickens who suffered from a nutritional lack of vitamin B_6. The lethal effect did not occur in the baby chickens who received DPX after having been previously fed a normal meal containing vitamin B_6. Under these conditions, no slackening of growth was observed.

Emerson (1947) discovered that in weaned rats receiving food not containing vitamin B_6, the period of time necessary for the appearance of acrodynie turned out to be considerably reduced by the addition of a certain quantity of DPX to the food.

Cravens and Snell (1949) injected doses of DPX in fertilized chicken eggs. 1 mg of substance revealed toxic effects that provoked the death of the embryos in 100% of the cases when the injection was given before incubation. If the dose of 1 mg or 0.5 mg was injected 4, 5 or up to 15 hours after the beginning of incubation, the number of deaths would have been very modest and would have varied between 5 and 21% of the embryos treated. By injecting, before incubation, 1 mg of DPX combined with different vitamins in the B_6 group (2 to 1000 γ), the lethal effects have appreciably diminished parallel to the increase in the quantity of PX. These authors have discovered that in order to obtain the survival of 50% of the embryos, one must combine either 1 part pyridoxal to 20 parts DPX, or 1 part pyridoxamine to 50 parts DPX, or finally, 1 part pyridoxine to 100 parts DPX. In this manner, it can be established that pyridoxine has the strongest neutralizing power. These results are, however, very different from those obtained previously by Ott. Quantities of DPX exceeding 1 mg, injected on the 6th day of incubation are toxic; however, this toxicity has not been able to be prevented by any of the three forms of vitamin B_6. Moreover, even pyridoxine and pyridoxal are shown to be toxic at very high doese (5 and 10 g. per egg), opposed to pyridoxamine which doesn't appear to show a lethal action even in strong doses.

The data cited in the bibliography on the action of PX and DPX on the fowl embryo was not complete or in agreement. But it was interesting to find fault with this study that, above all, posed the following questions:

(a) Can DPX be considered a teratogenic activity?

(b) Does there positively exist in the fowl embryo a specific period of teratogenic sensitivity for DPX?

(c) Is the injection of PX harmful, and at what dose, for the development of the fowl embryo?

(d) Are the eventual harmful effects caused by an adequate dose of PX, administered contemporaneously?

Materials and Technique

Our experiments were carried out on a total of 2463 chicken eggs, all fertilized. The eggs came from the Leghorn chickens, all of the same breeding.

We have seen from prior experiments that an injection of 0.1 cc of liquid Tyrode must be considered practically harmless in the development of the fowl embryo. This is why the different doses of matter we have injected have always been disolved in this quantity of liquid Tyrode.

As for the details of the injection, we have operated in the following manner: holding the egg in the left hand, slightly tilted, with the end pointed towards the top, and the main part on the top of the work table, we have injected the matter into the albumen on level with the small end. To do this, the needle is put in obliquely at a depth of about 2 cm. This way there is no chance of injuring the embryo or perforating the yellow part, and one is certain that the matter is put into the albumen. That is particularly demonstrated for us in the fact that, while injecting the matter, a small drop of albumen comes out the hole in the shell where the needle was placed.

After the injection, we close the hold with a drop of paraffin. When the injection is made after incubation, the liquid injected is not warmed first, but is at room temperature. When the injection is made after incubation, the liquid is heated to the temperature of 37-38°C. Before the incubation procedure, the eggs are left in a horizontal position for at least 12 hours at the temperature of the laboratory. In order to control the eggs in the experiment, we have observed them after 3 days of incubation and in each section, we have eliminated the unfertilized eggs. At the end of the 3rd day, we have systematically controlled the eggs every 24 hours by observing them. The dead embryos have been immediately removed, observed under a stereomicroscope, eventually photographed, and finally the peculiarities or interesting malformations set in a 10% formaldehyde solution, are presented. The dead embryos, by the 15th day or later, have been systematically weighed. To evaluate the stage of development of the embryo we followed the classification proposed by Hamburger and Hamilton.

Our experiments are divided into 4 groups; diagram 1 that follows gives us a quantitative idea of the number of embryos treated in each of the four groups:

Control eggs (several series)	256
Eggs pricked in the white part	109
Eggs injected with 0.1 cc of Tyrode	106
Eggs treated with DPX:	
1 mg. before incubation	102
1 mg. after 24 hours	102
1 mg. after 36 hours	107
1 mg. after 48 hours	100
1 mg. after 60 hours	104
0.6 mg. after 24 hours	104
0.4 mg. after 24 hours	94
0.2 mg. after 24 hours	101
0.1 mg. after 24 hours	100
Eggs treated with PX:	
1 mg. after 24 hours	105
0.6 mg. after 24 hours	106
0.4 mg. after 24 hours	103
0.2 mg. after 24 hours	103
0.1 mg. after 24 hours	105
Eggs with combined treatment DPX + PX	
1:1 before incubation	92
1:10 before incubation	92
1:100 before incubation	91
1:1 after 24 hours	92
1:10 after 24 hours	94
1:100 after 24 hours	95
Total number of fowl eggs used in the experiments	2,463

Results and Discussion

Effect of different doses of DPX injected at the 24th hour of incubation

In the first group of experiments we injected DPX into the fertilized eggs at doses of 1, 0.6, 0.4, 0.2, and 0.1 mg. For each of these doses the injection was always made at the 24th hour of incubation. The results obtained can be seen in Table 1. (See next page for Table 1.)

If we consider the figures in the first line, we see the values which indicate the number (in percent) of dead embryos in the first 3 days following treatment. These values give us an idea of the immediate effect of the treatment. For the control group embryos, not having been submitted to treatment (c), the values refer to the number (in percent) of dead embryos from the moment they are first placed in the incubator up to the 72nd hour of incubation. It can be seen already, that in the control group not treated, there are 6.9% of the embryos who died early, and this percentage is almost equal in the embryos merely pricked, or having received 0.1 cc of liquid Tyrode (T) after 24 hours of incubation. If we now compare the percentage of deaths among the control embryos and the embryos treated by different doses of DPX, we discover that, among the treated, the values increase, doubling, tripling or more, but without ever reaching very important figures. It is interesting to note that the different values are not proportional to the doses of DPX used. In effect, the relatively weak doses (0.4 mg) are those that have provoked the highest number of early deaths (22.3%) while the highest dose (1 mg) has only caused 14.7% of early deaths.

The figures cited in the second line give us an idea of the late death rate of the embryo after the 3rd day and up to the moment of hatching. In the groups of control embryo, the number which died during this period is not high; in the embryos treated the percentage is obviously higher. Here, the figures correspond to the different doses resulting in a regularly descending curve, which underlines the importance of the DPX in the late death rate.

An examination of the figures in the third line of the table furnish us with data equally significant. These figures indicate the number (in percent) of embryos suceeding in living until they are hatched. The figures of this line confirm those of the second, and shows once more that the survival of the embryos increases with the decrease in dosage. It definately seems as if the DPX does not have an immediate lethal effect, but rather a delayed effect harmful to the vitality of the embryo, provoking its death (line 2) or stopping its development (line 4) in the periods in between.

TABLE 1

	Control Group (%)			Embryos (%) treated by DPX after 24 hours of incubation				
	C	P	T	1 mg.	0,6 mg.	0,4 mg.	0,2 mg.	0,1 mg
Early Death	6,9	4,5	6,5	14,7	13,5	22,3	12,9	16,0
Late Death	11,1	16,9	7,9	48,0	30,7	21,3	12,8	11,0
Living the first day	82,0	78,6	85,6	37,3	55,8	56,4	74,3	73,0
Underdeveloped	2,5	3,4	2,1	13,8	17,7	13,5	10,2	10,8
Malformed	5,5	4,8	3,5	14,9	14,4	14,9	2,3	3,6

C = Control group without any treatment

P = Control group pricked without injection

T = Control group submitted to an injection of 0.1 cc of liquid Tyrode after 24 hours incubation

Among the embryos who died beyond the third day after treatment, a number of cases of underdevelopment are found. The extend of maturity and the weight of these underdeveloped embryos is appreciably inferior to that of the control group. In the fourth line, the values indicate the numbers of embryos which undergo a retardment of their development. These embryos present at the moment of death or hatching, a stage of development and a weight obviously inferior to the average values obtained in normal embryos. In regard to the baby chicks and the underdeveloped embryos, we are able to affirm that when the baby chick is capable of hatching spontaneously, it is never underdeveloped.

We have never been able to observe living underdeveloped baby chicks. Among the dead embryos at the end of the incubation period, between the 19th and 21st day, the number of underdeveloped is more apparent than during the rest of incubation. These embryos correspond apparently (formation of limbs, development of feathers, etc.) to the normal embryo, but they are thinner and smaller. Among those, we have frequently discovered the weight to be less than 25 g. (Sometimes even 16-17 g.). The grest margin between the extreme weights of the living chicks is astonishing. It is necessary to think, however, that the eggs themselves can greatly vary in size (the average weights of our eggs were from 48 to 57 g.). Evaluating the degree of underdevelopment, presented certain difficulties sometimes, especially in embryos who died in the relatively early phases of development.

The figures in the 4th line indicate to us that DPX manifests, at all the doses used, an inhibitory effect on growth, expressed by a certain percent of underdeveloped embryos.

Finally, a comparative examination of the first 4 lines seems to indicate clearly that DPX exerts inhibitory effects on the development of the fowl embryo. These effects manifest themselves threefold: by the premature death of the embryos, by a slow death rate, and finally by a retardment of development. Among these three effects, only one seems to be connected with the quantity of matter used: the slow death rate, while the two other inhibitory effects are seen in modest percentages and are totally independent of the quantity of matter used.

Finally, in the last line, we report the values (in percent) of the deformed embryos. Since one can't recognize well enough the malformations in the dead embryos during the first 3 days, in this calculation, as in the others on the underdeveloped, we have not paid attention to the total number of embryos treated in each series, but rather we have calculated the percentage of embryos living beyond the third day. In effect, the highest does have doubled or tripled the number of malformations in regard to the normal; but this number is of little importance (14-15%). In contract, the weakest doses have shown no teratogenic effect, and the number of malformations is less than that observed in the control embryos. Among the different malformations, we are not able to identify any malformation that can be attributed to a specific characteristic.

Action of 1 mg. of DPX injected at different periods of incubation

Studies on the chemo-teratogenicity indicates to us that the fowl embryo reacts differently to different stimuli and that it possesses a period of increasing tenderness in the course of early development. This critical period varies according to the nature of the materials used and the outline of malformations by Ancel. We have consequently carried out a second group of experiments with DPX. It was injected at different periods of development, that is to say, before incubation or 24, 36, 48, and 60 hours after the beginning of incubation.

In this 2nd group of experiments we have constantly maintained the dose at 1 mg. The results are summarized in the table below (Table 2). (See next page for Table 2).

The figures in the first line show that the early death rate in the control group (embryos having received a liquid injection of Tyrode) is a little higher in the group of embryos injected before incubation, and that it diminishes appreciably in the other groups. This fact is confirmed by observing the results obtained with DPX: the number of early deaths is very high (44.1%) when the injection is made before incubation; while in the following days, the early death rate is reduced to appreciably lower values. These values always remain superior to the corresponding control series.

TABLE 2

	Control Group (%)			Embryos treated (%) with 1 mg. of DPX at different periods				
	TO	T24	T48	Avant	24 hr.	36 hr.	48 hr.	60 hr.
Early death	10,4	6,5	6,1	44,1	14,7	8,4	13,0	19,2
Late death	14,6	7,9	2,1	40,2	48,0	38,3	22,0	19,3
Living the 21st day	75,0	85,6	91,8	15,7	37,3	53,3	65,0	61,5
Underdeveloped	7,0	2.1	2,2	21,1	13,8	17,4	9,1	11,9
Malformed	16,3	3,5	2,2	8,8	14,9	7,2	5,7	8,3

TO = Control embryos having received an injection of liquid Tyrode before incubation

T24 = Control embryos having received an injection of liquid Tyrode after 24 hours incubation

T48 = Control embryos having received an injection of ;iquid Tyrode after 48 hours incubation

The late death rate (late effect) in the embryos treated with DPX, confirms for us that the most critical period for the embryo is before incubation. In effect, if the figures in the first 2 columns are added up, one finds that 84.3% of the embryos died before hatching when the injection was given before incubation. This figure gradually decreases to about 35% when the injection is made after 48 or 60 hours of incubation. In all the cases, we can observe that the late death rate is more important than the early one, and that the dose of 1 mg. DPX exerts an important enough inhibitory effect to allow a limited number of embryos to reach the day of hatching (line 3).

Another aspect of the retarded effect on treatment is represented by the underdevelopment. Here again, we see values superior to the control group, but which stay the same even though the limits are relatively lower. These values confirm again that the most critical period for the embryo is expressed by the early and late death rates, and the underdeveloped displayed before incubation.

In regard to the teratogenic effect, the 5th line shows that the matter did not produce the deformities. Only when DPX is injected after 24 hours of incubation, the number ofdeformities is a little higher, but only reaches 15% of the embryos treated. This value is not sufficient enough to attribute a specific teratogenic effect ot DPX. More than an equal percentage of malformed embryos was obtained by injecting Tyrode before incubation.

The second group of experiments permits us to draw the following conclusions: (a) DPX exerts an inhibitory effect on the development of the fowl embryo; (b) Such an effect is more or less indicated and more or less rapid: in a certain percentage, it is manifested by the early death of the embryo; in other cases by the late death; in still other cases by a simple retardment of development, which in many cases is associated with the death of the embryo. In other words, the inhibitory effect of DPX is represented by a stoppage or retardment of development, which most times is followed by death; (c) The inhibitory effect appears more evident, the earlier the period of treatment: the maximal intensity is observed when administering the DPX before incubation; and (d) a 1 mg. dose of DPX injected at different periods of incubation does not have a specific teratogenic effect.

Action of different doses of PX injected at the 24th hour of incubation

Before carrying out combined treatment with the two substances, we have accomplished a 3rd group of experiments to study the tolerance of the fowl embryo with respect to PX. The matter was injected into the fowl embryos in even doses, and exactly the same details as those used in the first group of experiments were followed.

Table 3 summarizes the results obtained. (See next page for Table 3). One can easily observe that the treatment with PX is well endured by the embryos, evenat a 1 mg. dose. The variation in values (in percent) between the maximal and minimal doses are always very moderate, either in the early or late death rates of the embryos, or the frequency of the underdeveloped or deformed. The values always come near the values observed in the control series. Consequently, the conclusions drawn can not be positive, that is to say, that PX must be considered at least at the doses we have used, as absolutely harmless.

Action of DPX and PX in different ratios and injected before and after 24 hours of incubation

To assure outselves that the effects obtained by the administration of DPX in the first and second group of experiments are truly caused by DPX, we have carried out a 4th group of experiments which will be used as a biological counter-test. That is to say, we have united with the DPX fixed doses of PX, its antagonist, capable of neutralizing its effects when it is administered similtaneously. The two substances were injected in the ratios of 1:1, 1:10, and 1:100. The dose of DPX was maintained constant at 1 mg. The PX was added respectively in doses of 1 mg., 0.1 mg., 0.01 mg. The two substances were injected either before incubation, or after 24 hours, after having been disolved in 0.1 cc of liquid Tyrode. The table below (Table 4) shows the results obtained. As for the control series, we used the series that helped us during the preceeding experiments, taking into consideration the effect of 1 mg. DPX injected before and 24 hours after incubation. (See next page for Table 4).

TABLE 3

	Control Group (%)			Embryos treated (%) with PX after 24 hours incubation				
	C	P	T	1 mg.	0,6 mg.	0,4 mg.	0,2 mg.	0,1 mg.
Early death	6,9	4,5	6,5	6,7	5,7	6,8	7,8	8,6
Late death	11,1	16,9	7,9	19,0	21,7	14,6	9,7	13,3
Living the 21st day	82,0	78,6	85,6	74,3	72,6	78,6	82,5	78,1
Underdeveloped	2,5	3,4	2,1	3,1	5,0	5,2	2,1	2,1
Malformed	5,5	4,8	3,5	7,1	6,0	5,2	2,1	4,2

TABLE 4

	Control Group (%)		treatment with combined DPX-PX before incubation			after 24 hours		
	DPXO	DPX24	1:1	1:10	1:100	1:1	1:1	1:11
Early death	44,1	14,7	13,0	27,2	22,0	15,2	30,9	20,0
Late death	40,2	48,0	28,3	23,8	32,9	21,8	14,8	24,5
Living the 21st day	15,7	37,3	58,7	49,0	45,1	63,0	54,3	55,5
Underdeveloped	21,2	13,8	8,8	7,4	12,7	6,4	7,7	8,0
Malformed	8,8	14,9	13,7	11,9	12,7	9,0	4,6	6,6

DPXO = Embryos treated with 1 mg. DPX dissolved in 0.1 cc of liquid Tyrode before incubation

DPX24 = Embryos treated with 1 mg. DPX dissolved om 0.1 cc of liquid Tyrode, after 24 hours

Concerning the early death rate, the figures in the first line indicate clearly that the combination of the two antagonists in the ratio of 1:1 shows the most favorable results; and that either the combination was injected before incubation of the antagonists were injected 24 hours after. In comparing these values with those of the control series injected with 1 mg. DPX, one can observe that, in the first case (DPX administered before incubation), the high death rate is appreciably reduced by the addition of PX. Such a reduction of mentality reaches maximal values in the ratio of 1:1; the reduction of mortality is less in the ratios of 1:10 and 1:100. In the second case, on the other hand, (DPX injected after 24 hours incubation), no reduction in mortality is observed. Quite the contrary with the ratios of 1:10 and 1:100, one notices an increase of mortality in proportion to treatment with DPX alone. These factors indicate that DPX injected before incubation is directly responsible for the high, early death rate, and this rate can be appreciably reduced by the simultaneous administration of an adequate dose of PX. But that equally shows that when DPX is administered after 24 hours of incubation, the early mortality rate that it provokes, can be connected to the independent causes of a specific inhibitive action. In effect, this death rate, which is not completely elevated, is not reduced by the simultaneous administration of DPX.

Analagous considerations can be made regarding the figures relating to the late death rate. Even in this case, the addition of PX is capable of reducing to some extent the mortality rate and that, either the combined treatments be made before incubation, or after 24 hours of incubation. In the latter case, the reduction of mortality sometimes seems more important.

The effectiveness of the combination of PX and DPX again seems confirmed by observing the values indicating the percentage of embryos capable of reaching hatching. It is noticed that the 3 ratios are capable alone of reducing the inhibitory effect of DPX.

The antagonist effect of PX vis-a-vis the DPX, is again witnessed by the obvious reduction in the percentage of under-developed embryos observed in the different ratios.

Finally, concerning the number of malformed, this group of experiments does not contribute any singificant data. What is well understood, is the fact that each of the two substances, injected separately, does not reveal any teratogenic effects with the doses we have used.

In conclusion, we can draw the following conclusions from this group of experiments:

(a) The addition of PX to DPX appreciably reduced the early and late mortality rate provoked by the treatment with DPX alone. Consequently the survival rate increases notably.

(b) To neutralize the high, early mortality rate provoked by the DPX injected before incubation, it is necessary to use a high concentration of PX. In our experiments, the ratio of 1:1 is revealed to be more effective. To neutrilize the late mortality rate, and in order to lower the rate of underdeveloped embryos, even the concentrations of PX (1:10 and 1:100) seem to be sufficient.

Considerations on the teratogenic power of different solvents, of DPX, and of PX

In the preceeding studies, we have studied the action of different solvents on the development of the fowl embryo. Having established the absence of a teratogenic power on the part of these solvents, represented a fundamental fact that now allows us to examine the teratogenic power of substances dissolved in these solvents. The information we have drawn up is connected to a quantitative criteria, and this criteria alone seems insufficient to excluse an eventual teratogenic power. To validate this opinion, the qualitative criteria must not be neglected. That is to say, it is necessary to specify the fact that there is an interdependent connection between the administration of one given substance and the appearance of malformations, repeating themselves with a certain frequency and persistence. With this purpose, we have compared the results furnished by all the groups of experiments carried out; considering not only the total percentage of malformed, but equally and, above all, the partial percentage of different types of malformations. It is evident that such a comparison could

not be total, or rather carried out on the total number of embryos treated with all the substances, and without taking account of either the dose used, or of the period when the substance was injected. In the table on the following page (Table 5), the values (in percentage) are very similiar and not that much different from the values obtained in control embryos not having undergone treatment. The table clearly shows that any of the two substances, DPX and PX, and any of the three solvents have no teratogenic effect.

Always taking into account that the DPX has provoked, in many cases, a high, early mortality, and in equally realizing the difficulties encountered in establishing the accurate existence of the malformations in dead embryos during the first 3 days, we have made other calculations. These were made only taking into account those embryos who have survived beyond the 3rd day after treatment. As one can see in Table 6 (on the following page), the percentage of malformed always remains very low, and presents values which are similar enough to those of the control embryos.

Finally, we have made another calculation, taking into account the kind of malformation presented by each embryo. This calculation implies the difficulties that only one group of limited embryos, with isolated deformities, presented. Most of the deformities are presented in combination with one or several other deformities. Resulting in, in certain cases, some very complex monstrous appearances. Consequently the embryos present several deformities, appearing two or several times under the name of corresponding deformities.

All the figures in Table 7 represent percentages relating to the total malformation obtained with treatment corresponding to the respective column. (See next page for Table 7). For example, the figure 8 in the red twisted beaks column (figure observed in control embryos), corresponds to 8% of the 25 embryos found deformed, among the control group embryos.

As the figures in the table show, there are no substances used with a specific frequency of a type of malformation. There are types of malformations who do repeat themselves more frequently than others, but this frequency is not connected to treatment with a single substance when the malformation generally repeats itself with all the substances used (for example, malformations of the eyes, omencephalohemia, etc.). One example of a specific malformation is double monsters, which out of all the malformed embryos, only exists in embryos treated with DPX. But apart from the insignificant number of cases (2), there are other considerations to show us that this malformation must not be considered as specific to DPX.

TABLE 5

Treatment	# of embryos treated	True # of malformed embryos	No. (%) of malformed embryos
Control group	473	25	5.3
Injections	374	17	4.6
Water distribution	1,031	46	4.5
Sol. Physiol.	1,034	56	5.4
Tyrode	1,241	84	6.7
DPX	914	68	7.5
PX	522	26	4.9
DPX-PX	556	44	7.9

TABLE 6

Treatment	No. of embryos living at end of the 3rd day	No. of malformed embryos at end of 3 rd day	
		No.	%
Control	440	25	5,7
Pricked	359	17	4,7
Water Dist.	735	46	6,3
Sol. Physiol.	727	50	6,8
Tyrode	902	73	8,1
DPX	749	66	8,8
PX	485	24	5,0
DPX-PX	437	43	10,0

TABLE 7

	C	P	ED	SP	TY	DPX	PX	DPX-PX
Brachygnathia sup. & inf.	16	0	11	7	4	4	4	2
Retroversion of the beak	0	5	11	4	4	9	15	11
Twisted beak	8	23	13	19	18	7	8	18
Parrot beak	12	12	15	7	11	17	35	7
Micro-and anopthalmia	52	53	38	50	39	28	12	23
Deviation of beak	0	0	0	0	1	6	0	2
Anencephalia, exencephalia	48	23	19	23	27	25	16	16
Deviation of spinal column	0	0	2	7	2	4	4	5
Spina bifida	0	0	2	0	0	1	0	0
Celosomia	20	41	35	19	46	19	20	41
Ectrosomia	0	0	0	0	1	0	0	0
Ectromelia	8	0	0	0	0	0	0	0
Brachymelia	0	0	0	0	0	0	4	0
Hemimelia	0	0	2	0	1	0	0	2
Phocomelia	0	0	0	0	0	2	0	0
Symelia	0	0	0	0	1	0	0	0
Ectrodactyly	0	0	2	2	0	0	0	0
Mouth Defects	0	0	0	0	0	4	0	0
Anourous (tailless)	0	0	0	0	0	0	0	2
Oedemia	4	0	4	4	1	9	16	10
Cysts on feathers	0	0	0	2	0	0	0	0
Anomalies of Feet	0	0	0	6	0	0	0	5
Double Monsters	0	0	0	0	0	3	0	0
Other monstrosities	0	12	4	2	5	0	0	5

C = Control Group
P = Injected
ED = Distilled Water
SP = Physiological Solution
TY = Liquid Tyrode

This malformation is connected with, in effect, a normal deviation process of egg segmentation, that reveals itself in very early phases of development. In our case, the two embryos were injected with DPX 24 hours after the beginning of incubation. It is then possible that when the treatment was made, the abnormal segmentation had already begun. The mouth defect is another variety of a malformation of specific value. We have only discovered it in embryos treated with DPX. In this case, however, the value is too low (4% of total malformations provoked by DPX) to consider this malformation as specific to the substance.

Finally, the values reported in the last table confirm the conclusions drawn from the preceeding tables: DPX and PX, as the 3 solvents, don't seem to reveal any specific teratogenic action.

SUMMARY

The action of vitamin B_6 and its antivitamin upon the development of the fowl embryo

1. Antivitamin B_6 (deoxypyridoxine) has an inhibitory effect upon the development of the fowl embryo.

2. This effect, which occurs with doses of 1·0 mg., may be shown by early death, or by arrested or delayed development which in many cases is followed by death at later stages.

3. The effect is greater the earlier the administration of the antivitamin. The greatest effect was obtained after administration to the egg before incubation.

4. Vitamin B_6 is without adverse effect upon the development of the fowl embryo.

5. Joint administration of vitamin B_6 and antivitamin B_6 reduces the early and the late mortality produced by the antivitamin alone.

6. Vitamin B_6, antivitamin B_6, and the three solvents used (distilled water, physiological saline and Tyrode solution) showed no specific teratogenic activity.

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Action de la vitamine et de l'antivitamine B_6 sur le développement de l'embryon de poulet

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Le groupe des vitamines B_6 comporte trois substances distinctes: la pyridoxine, qui est un dérivé de la pyridine; la pyridoxamine et le pyridoxal qui dérivent de la pyridoxine. Tous les organismes utilisent le pyridoxal; certains utilisent également la pyridoxine, d'autres la pyridoxamine, d'autres enfin (le rat, par exemple), utilisent les trois substances. Cette constatation a fait penser que la substance réellement active serait le pyridoxal, et que seuls certains organismes pourraient former de la pyridoxine, de la pyridoxamine ou les deux substances. De plus, le pyridoxal, sous forme d'ester phosphorique (pyridoxalphosphate) déploie une action importante comme co-enzyme dans de nombreux complexes enzymatiques actifs dans le métabolisme des acides aminés; parmi ces complexes, il faut citer les transaminases, les aminoacide-décarboxylases, les déshydratases, les désulfhydrases, les racémases, l'histaminase et les tryptophanases.

Chez les animaux, la carence en vitamines du groupe B_6 provoque des troubles variés. Chez les rats, on observe une dermatite caractéristique, associée à un syndrome convulsif épileptiforme, une fatigue musculaire et un retard d'accroissement. Chez le chien, on observe une anémie hypochrome; chez le porc, une anémie hypochrome s'accompagnant de convulsions, de troubles des mouvements et de lésions du système nerveux. Chez les poussins, la carence en vitamines du groupe B_6 provoque un arrêt de la croissance. Enfin, chez l'homme, on n'a pas encore constaté de véritable syndrome provoqué par la carence en vitamines B_6; le premier syndrome carentiel a été obtenu thérapeutiquement, à la suite d'administration de désoxypyridoxine, antagoniste de la pyridoxine.

Parmi les antivitamines du groupe B_6, la plus active est la désoxypyridoxine (DPX), découverte par Ott en 1945. La DPX a une structure chimiquement analogue à celle de la vitamine B_6, et plus précisément, une structure analogue à celle de la pyridoxine (PX); dans la DPX, un groupe OH est remplacé par un atome H en position 4.

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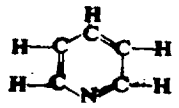
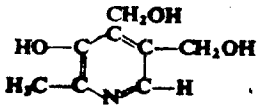
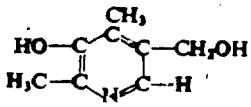


Pyridine



Pyridoxine
(Vitamine B_6)



Désoxypyridoxine
(Antivitamine B_6)

En 1946, Ott étudia l'action biologique de la DPX sur les poussins. Ce composé se révéla être le plus puissant parmi les inhibiteurs des vitamines du groupe B_6. Ott put en effet établir qu'une molécule de PX neutralisait les effets de deux molécules de DPX, tandis que, pour les autres inhibiteurs, une molécule de vitamine était capable d'en neutraliser 40 ou plus. La DPX montra un effet léthal même à des doses très faibles (16 γ), chez des poussins qui souffraient de carence alimentaire en vitamine B_6. L'effet léthal ne se produisit pas chez des poussins qui reçurent de la DPX après avoir été préalablement alimentés avec une nourriture normale contenant de la vitamine B_6; dans ces conditions, on n'observa aucun ralentissement de la croissance.

Emerson (1947) constata, chez des rats sevrés, recevant une nourriture ne contenant pas de vitamine B_6, que le temps nécessaire à l'apparition de l'acrodynie se trouvait sensiblement réduit par l'adjonction d'une certaine quantité de DPX à la nourriture.

Cravens et Snell (1949) ont injecté des doses de DPX dans des oeufs fécondés de poule. La quantité de 1 mg de substance a révélé des effets toxiques prononcés en provoquant la mort des embryons dans 100% des cas lorsque l'injection était faite avant l'incubation; si la dose de 1 mg. ou de 0,5 mg. était injectée 4, 5, jusqu'à 15 jours après le début de l'incubation, le nombre des morts s'est montré très modeste et a varié entre 5 et 21% des embryons traités. En injectant, avant l'incubation, 1 mg. de DPX associé à différentes quantités de vitamines du groupe B_6 (de 2 à 1000 γ), les effets léthaux ont sensiblement diminué parallèlement à l'augmentation de la quantité de PX. Ces auteurs ont constaté que, pour obtenir la survie de 50% des embryons, on devait associer soit une partie de pyridoxal à 20 parties de DPX, soit encore une partie de pyridoxamine à 50 parties de DPX, soit enfin, une partie de pyridoxine à 100 parties de DPX. De cette manière, on a pu établir que la pyridoxine possède le pouvoir de neutralisation le plus puissant. Ces résultats sont toutefois très différents de ceux obtenus par Ott auparavant. Des quantités de DPX dépassant 1 mg., injectées au 6ème jour d'incubation, se sont révélées toxiques; cependant, cette toxicité n'a pu être prévenue par aucune des trois formes de vitamine B_6. D'ailleurs, même la pyridoxine et le pyridoxal se sont révélés toxiques à des doses très élevées (5 et 10 g. par oeuf), contrairement à la pyridoxamine qui ne semble avoir montré aucune action léthale même à de si fortes doses.

Les données rapportées dans la bibliographie sur l'action de la PX et de la DPX sur l'embryon de poulet n'étant pas complètes ni concordantes, il nous a

paru intéressant de reprendre cette étude en nous posant surtout les questions suivantes:

(*a*) Peut-on considérer la DPX comme un agent tératogène?

(*b*) Dans l'affirmative, existe-t-il chez l'embryon de poulet une période de sensibilité tératogène spécifique pour la DPX?

(*c*) L'injection de PX est-elle nuisible, et à quelle dose, pour le développement de l'embryon de poulet?

(*d*) Les effets nuisibles éventuels exercés par la DPX sont-ils spécifiques? peuvent-ils être annulés par une dose adéquate de PX, administrée contemporanément?

MATERIEL ET TECHNIQUE

Nos expériences portent sur un ensemble de 2463 oeufs de poule, tous fécondés. Les oeufs provenaient de poules de race Leghorn du même élevage.

Des expériences antérieures nous avaient montré que l'injection de 0,1 cc de liquide de Tyrode doit être considérée comme pratiquement inoffensive pour le développement de l'embryon de poulet; c'est pourquoi les différentes doses de substance que nous avons injectées ont toujours été dissoutes dans cette quantité de liquide de Tyrode.

Quant à la modalité de l'injection, nous avons opéré de la manière suivante: en tenant de la main gauche l'oeuf légèrement incliné, avec le bout pointu vers le haut et le gros bout sur la table de travail, nous avons injecté la substance dans l'albumen au niveau du petit bout; pour ce faire, on introduit l'aiguille obliquement à une profondeur de 2 cm. environ. De cette façon, il n'y a aucun risque de léser l'embryon ou de perforer le jaune, et l'on est ainsi certain d'introduire la substance dans l'albumen. Cela nous est en particulier démontré par le fait que, en injectant la substance, une petite goutte d'albumine sort à travers le trou de la coquille par lequel l'aiguille a été introduite. Après l'injection, nous fermons le trou avec une goutte de paraffine. Lorsque l'injection est faite avant l'incubation, le liquide injecté n'est pas préalablement chauffé, mais se trouve à la température de la pièce de travail; lorsque les injections sont faites après l'incubation, les liquides sont chauffés à la température de 37–38°C. Avant la mise en couveuse ou le traitement avant l'incubation, on laisse les oeufs en position horizontale pendant 12 heures au moins, à la température du laboratoire. Pour contrôler les oeufs en expérience, nous les avons mirés après 3 jours d'incubation et, dans chaque série, nous avons éliminé les oeufs qui n'étaient pas fécondés. A partir du troisième jour, nous avons systématiquement contrôlé les oeufs toutes les 24 heures en les mirant; les embryons morts ont été immédiatement prélevés, observés au stéréomicroscope, éventuellement photographiés et enfin, s'ils présentaient des particularités ou des malformations intéressantes, fixés au formol à 10%. Les embryons morts au 15ème jour ou plus tard, ont tous été systématiquement pesés. Pour évaluer le stade de

développement des embryons, nous nous sommes tenus à la classification proposée par Hamburger et Hamilton.

Nos expériences se divisent en 4 groupes; le schéma qui suit nous donne une idée quantitative du nombre d'embryons traités dans chacun des 4 groupes:

Oeufs de contrôle (plusieurs séries)	256
Oeufs simplement piqués dans le blanc	109
Oeufs injectés avec 0,1 cc de Tyrode	106
Oeufs traités avec la DPX:	
1 mg. avant l'incubation	102
1 mg. après 24 heures	102
1 mg. après 36 heures	107
1 mg. après 48 heures	100
1 mg. après 60 heures	104
0,6 mg. après 24 heures	104
0,4 mg. après 24 heures	94
0,2 mg. après 24 heures	101
0,1 mg. après 24 heures	100
Oeufs traités avec la PX:	
1 mg. après 24 heures	105
0,6 mg. après 24 heures	106
0,4 mg. après 24 heures	103
0,2 mg. après 24 heures	103
0,1 mg. après 24 heures	105
Oeufs avec traitement combiné DPX + PX:	
1:1 avant l'incubation	92
1:10 avant l'incubation	92
1:100 avant l'incubation	91
1:1 après 24 heures	92
1:10 après 24 heures	94
1:100 après 24 heures	95
Nombre total des embryons de poulet qui ont servi pour ces expériences	2463

RESULTATS ET DISCUSSION

Action de différentes doses de DPX injectées à la 24ème heure de l'incubation

Dans ce premier groupe d'expériences, nous avons injecté à des oeufs fécondés la DPX à des doses de 1, 0,6, 0,4, 0,2, et 0,1 mg.; pour chacune de ces doses, l'injection a toujours été faite à la 24ème heure d'incubation. Les résultats obtenus se trouvent résumés dans le tableau suivant (Tableau 1).

Si nous considérons les chiffres de la première ligne, nous voyons des valeurs qui indiquent le nombre en pour-cent des embryons morts dans les trois premiers

jours qui ont suivi le traitement; ces valeurs nous donnent une idée de l'effet immédiat du traitement. Pour les embryons de contrôle n'ayant subi aucun traitement (C), les valeurs se rapportent au nombre en pour-cent des embryons morts durant la période allant du moment de la mise en couveuse jusqu'à la 72ème heure de l'incubation. On peut remarquer que, déjà chez les contrôles non traités, il y a 6,9 % d'embryons qui meurent précocement, et ce pourcentage est à peu près équivalent chez les embryons simplement piqués (P) ou ayant

TABLEAU I

	Contrôles (%)			*Embryons (%) traités par la DPX après 24 heures d'incubation*				
	C	*P*	*T*	*1 mg.*	*0,6 mg.*	*0,4mg.*	*0,2 mg.*	*0,1 mg.*
Mortalité précoce	6,9	4,5	6,5	14,7	13,5	22,3	12,9	16,0
Mortalité tardive	11,1	16,9	7,9	48,0	30,7	21,3	12,8	11,0
Vivants le 21ème jour	82,0	78,6	85,6	37,3	55,8	56,4	74,3	73,0
Hypodéveloppés	2,5	3,4	2,1	13,8	17,7	13,5	10,2	10,8
Malformés	5,5	4,8	3,5	14,9	14,4	14,9	2,3	3,6

C = Contrôles sans aucun traitement.
P = Contrôles piqués sans injection.
T = Contrôles ayant subi une injection de 0,1 c.c. de liquide de Tyrode après 24 heures d'incubation.

reçu 0,1 cc de liquide de Tyrode (T) après 24 heures d'incubation. Si nous comparons maintenant le pourcentage des morts parmi les embryons de contrôle et parmi les embryons traités par les différentes doses de DPX, nous constatons que, parmi les traités, les valeurs augmentent, doublent, triplent ou plus, mais sans jamais atteindre des chiffres très importants. Il est intéressant de remarquer que les différentes valeurs ne sont pas directement proportionnelles aux doses de DPX employées; en effet, les doses relativement faibles (0,4 mg.) sont celles qui ont provoqué le nombre le plus élevé de mortalité précoce (22,3 %) tandis que la dose la plus élevée (1 mg.) n'a déterminé que le 14,7 % de mortalité précoce.

Les chiffres reportés dans la deuxième ligne nous donnent une idée de la mortalité tardive qui frappe les embryons après le troisième jour et jusqu'au moment de l'éclosion. Dans les groupes des embryons de contrôle, le nombre des embryons qui meurent pendant cette période n'est pas élevé; chez les embryons traités, le pourcentage est sensiblement supérieur. Ici, les chiffres correspondant aux différentes doses suivent une courbe régulièrement descendante; qui souligne d'une manière évidente l'importance de la dose de DPX sur la mortalité tardive.

L'examen des chiffres de la troisième ligne du tableau nous fournit des renseignements également significatifs. Ces chiffres indiquent le nombre en pour-cent des embryons arrivés vivants à l'éclosion. Les chiffres de cette ligne

confirment ceux de la deuxième et montrent encore une fois que la survie des embryons augmente avec la diminution de la dose. Il nous semble en définitive que la DPX n'a pas un effet léthal immédiat, mais plutôt un effet retardé nuisible à la vitalité de l'embryon, en provoquant sa mort (chiffre de la deuxième ligne) ou en arrêtant son développement (chiffre de la quatrième ligne) dans des périodes intermédiaires.

Parmi les embryons qui sont morts au-delà du troisième jour après le traitement, on trouve de nombreux cas d'hypodéveloppement; le degré de maturité et le poids de ces embryons hypodéveloppés est sensiblement inférieur à celui correspondant des contrôles. Dans la quatrième ligne, les valeurs indiquent le nombre des embryons qui ont subi un retard dans leur développement; ces embryons présentaient au moment de la mort ou au moment de l'éclosion un degré de maturité et un poids sensiblement inférieurs aux valeurs moyennes obtenues chez des embryons normaux. A propos des poussins et des embryons hypodéveloppés, nous pouvons affirmer que, lorsque le poussin est capable d'éclore spontanément, il n'est jamais hypodéveloppé.

Nous n'avons donc jamais pu observer de poussins vivants hypodéveloppés. Par contre, parmi les embryons morts à la fin de la période d'incubation, entre le 19ème et le 21ème jour, le nombre des hypodéveloppés est plus marqué que durant le reste de l'incubation. Ces embryons correspondent apparemment (formation des membres, développement des plumes ... etc.) aux embryons normaux, mais ils sont plus chétifs, plus petits. Parmi ceux-là, nous avons fréquemment constaté des poids de moins de 25 g. (parfois même 16–17 g.). On pourrait s'étonner, à propos du poids des poussins vivants, de la grande marge entre les poids extrêmes (plus de 10 g.); il faut cependant penser que les oeufs eux-mêmes peuvent varier assez fortement de grandeur et de poids (la moyenne des poids des oeufs que nous avons utilisés va de 48 à 57 g.). A propos de l'évaluation du degré d'hypodéveloppement, nous devons encore dire qu'elle présente parfois certaines difficultés et cela surtout chez les embryons qui meurent dans des phases relativement précoces du développement.

Les chiffres de la quatrième ligne nous indiquent que la DPX manifeste, à toutes les doses employées, un effet inhibiteur sur la croissance, se traduisant par un certain pour-cent d'embryons hypodéveloppés.

En définitive, l'examen comparatif des quatre premières lignes semble nous indiquer d'une manière assez nette que la DPX exerce des effets inhibiteurs sur le développement de l'embryon de poulet. Ces effets se manifestent selon une triple modalité: par la mort précoce des embryons, par une mortalité tardive ou enfin par un retard du développement. Parmi ces trois effets, un seul semble être lié à la quantité de substance employée: la mortalité tardive, tandis que les deux autres effets inhibiteurs se manifestent dans des pourcentages modestes et qui sont tout à fait indépendants de la quantité de substance employée.

Dans la dernière ligne, enfin, nous reportons les valeurs en pour-cent des embryons malformés. Puisque, en général, on n'arrive pas à reconnaître assez

bien les malformations chez les embryons morts au cours des trois premiers jours, dans ce calcul comme d'ailleurs dans les calculs sur les hypodéveloppés, nous n'avons pas tenu compte du nombre total des embryons traités dans chaque série mais nous avons calculé le pour-cent sur le nombre des embryons qui vivaient au-delà du troisième jour. En effet, les doses plus élevées ont fait doubler ou tripler le nombre des malformés par rapport à la normale, mais ce nombre reste toujours dans des pourcentages très peu importants (14–15%). Par contre, les doses les plus faibles n'ont montré aucun effet tératogène, et le nombre des malformations obtenues est inférieur à celui observé chez les embryons de contrôle. Parmi les différentes malformations que nous avons obtenues, nous n'avons pu identifier aucune malformation à laquelle on puisse attribuer un certain caractère de spécificité.

Action de 1 mg. de DPX injecté à différentes époques de l'incubation

Les études sur la chimio-tératogénèse nous indiquent que l'embryon de poulet réagit différemment aux différentes stimulations et qu'il possède une période accrue de sensibilité au cours de son développement précoce; cette période critique varie, d'une part selon la nature des substances employées et d'autre part, en rapport avec les ébauches qui pourraient être impliquées pour les malformations (Ancel). Nous avons par conséquent réalisé un deuxième groupe d'expériences avec la DPX. Celle-ci a été injectée à des périodes différentes du développement, à savoir avant l'incubation ou bien 24, 36, 48 et 60 heures après le début de l'incubation.

Dans ce deuxième groupe d'expériences, nous avons maintenu constante la dose de 1 mg. Les résultats sont résumés dans le tableau qui suit (Tableau 2).

Les chiffres de la première ligne nous montrent que la mortalité précoce chez les contrôles (embryons ayant reçu une injection de liquide de Tyrode) est un peu plus élevée dans le groupe des embryons injectés avant l'incubation et

TABLEAU 2

	Contrôles (%)			*Embryons traités* (%) *avec 1 mg. de DPX à différentes époques*				
	TO	*T24*	*T48*	*Avant*	*24 hr.*	*36 hr.*	*48 hr.*	*60 hr.*
Mortalité précoce	10,4	6,5	6,1	44,1	14,7	8,4	13,0	19,2
Mortalité tardive	14,6	7,9	2,1	40,2	48,0	38,3	22,0	19,3
Vivants le 21ème jour	75,0	85,6	91,8	15,7	37,3	53,3	55,0	61,5
Hypodéveloppés	7,0	2,1	2,2	21,1	13,8	17,4	9,1	11,9
Malformés	16,3	3,5	2,2	8,8	14,9	7,2	5,7	8,3

TO = Embryons de contrôle ayant reçu une injection de 0,1 cc de liquide de Tyrode avant l'incubation.

T24 = Embryons de contrôle ayant reçu une injection de 0,1 cc de liquide de Tyrode après 24 heures d'incubation.

T48 = Embryons de contrôle ayant reçu une injection de 0,1 cc de liquide de Tyrode après 48 heures d'incubation.

qu'elle se réduit sensiblement dans les autres groupes. Ce fait nous est confirmé en observant les résultats obtenus avec la DPX: le nombre des morts précoces est très élevé (44,1 %) lorsque l'injection est faite avant l'incubation, tandis que dans les jours suivants, la mortalité précoce se réduit à des valeurs sensiblement inférieures; ces valeurs restent toutefois supérieures par rapport aux séries correspondantes de contrôle.

La mortalité tardive (effet retardé) chez les embryons traités avec la DPX nous confirme que la période la plus critique pour l'embryon se situe avant l'incubation. En effet, si l'on additionne les chiffres des deux premières colonnes, on trouve que 84,3 % des embryons meurent avant l'éclosion lorsque l'injection se fait avant l'incubation; ce chiffre va graduellement se réduire pour arriver à environ 35 % lorsque l'injection est faite après 48 ou 60 heures d'incubation. Dans tous les cas, nous pouvons observer que la mortalité tardive est plus importante que la mortalité précoce, et que la dose de 1 mg. de DPX exerce un effet inhibiteur assez important qui permet à un nombre assez restreint d'embryons d'atteindre le jour de l'éclosion (chiffre de la troisième ligne).

Un autre aspect de l'effet retardé du traitement est représenté par l'hypodéveloppement. Ici encore, nous voyons des valeurs qui sont supérieures aux contrôles, mais qui restent quand même dans des limites relativement basses. Ces valeurs confirment encore une fois que l'époque la plus critique pour l'embryon, se traduisant par la mortalité précoce, la mortalité tardive et les hypodéveloppés, se situe avant l'incubation.

En ce qui concerne l'effet tératogène, les chiffres de la cinquième ligne montrent que la substance ne produit pas de malformations. Seulement lorsque la DPX est injectée après 24 heures d'incubation, le nombre des malformés est un peu plus élevé mais atteint seulement 15 % des embryons traités; cette valeur n'est pas suffisante pour attribuer à la DPX un effet tératogène spécifique, d'autant plus qu'un pourcentage égal de malformés a été obtenu en injectant du Tyrode avant l'incubation.

En définitive, ce deuxième groupe d'expériences nous permet de tirer les conclusions suivantes:

(*a*) La DPX exerce un effet inhibiteur sur le développement de l'embryon de poulet.

(*b*) Un tel effet inhibiteur peut être plus ou moins marqué et plus ou moins précoce: dans un certain pourcentage il se manifeste par la mort précoce de l'embryon; dans d'autres cas, par la mort plus tradive; dans d'autres cas encore, par un simple retard du développement qui, dans beaucoup de cas, est associé à la mort de l'embryon. En d'autres termes, l'effet inhibiteur de la DPX est représenté par un arrêt ou un retard du développement qui est, dans bien des cas, suivi par la mort.

(*c*) L'effet inhibiteur paraît d'autant plus marqué que la période du traitement est plus précoce: l'intensité maximale s'observe en administrant la DPX avant l'incubation.

(*d*) La DPX, injectée à la dose de 1 mg à différentes époques de l'incubation, n'a pas d'effet tératogène spécifique.

Action de différentes doses de PX injectées à la 24ème heure de l'incubation

Avant de réaliser le traitement combiné avec les deux substances, nous avons réalisé un troisième groupe d'expériences dans le but d'étudier la tolérance de l'embryon de poulet vis-à-vis de la PX. La substance a été injectée aux embryons de poulet aux mêmes doses et en suivant exactement les mêmes modalités que celles employées dans le premier groupe de nos expériences.

TABLEAU 3

	Contrôles (%)			*Embryons traités (%) avec la PX après 24 heures d'incubation*				
	C	*P*	*T*	*1 mg.*	*0,6 mg.*	*0,4 mg.*	*0,2 mg.*	*0,1 mg.*
Mortalité précoce	6,9	4,5	6,5	6,7	5,7	6,8	7,8	8,6
Mortalité tardive	11,1	16,9	7,9	19,0	21,7	14,6	9,7	13,3
Vivants le 21ème jour	82,0	78,6	85,6	74,3	72,6	78,6	82,5	78,1
Hypodéveloppés	2,5	3,4	2,1	3,1	5,0	5,2	2,1	2,1
Malformés	5,5	4,8	3,5	7,1	6,0	5,2	2,1	4,2

Le tableau ci-dessous (Tableau 3) résume les résultats obtenus. Comme on peut facilement le remarquer, le traitement avec la PX est très bien supporté par les embryons, même à la dose de 1 mg. Les écarts des valeurs en pour-cent entre les doses maximales et minimales sont toujours très modestes, soit en ce qui concerne la mortalité précoce ou tardive des embryons, soit en ce qui concerne la fréquence des hypodéveloppés ou des malformés; les valeurs obtenues se rapprochent toujours des valeurs observées dans les séries de contrôle. Par conséquent, les conclusions que l'on peut tirer ne peuvent être que positives, c'est-à-dire que la PX doit être considérée, au moins pour les doses que nous avons employées, comme absolument inoffensive.

Action de la DPX et de la PX associées en différents rapports et injectées avant et après 24 heures d'incubation

Afin de nous assurer que les effets obtenus par l'administration de DPX dans le premier et le deuxième groupe d'expériences sont vraiment déterminés par la DPX, nous avons réalisé un quatrième groupe d'expériences qui servirait de contre-épreuve biologique: à savoir, nous avons associé à la DPX des doses bien déterminées de PX, son antagoniste, capable d'en neutraliser les effets lorsqu'elle est administrée simultanément. Les deux substances ont été injectées aux rapports 1:1, 1:10 et 1:100. La dose de DPX a été maintenue constante à 1 mg.; la PX a été ajoutée respectivement aux doses de 1 mg., 0,1 mg. et

0,01 mg. Les deux substances ont été injectées soit avant l'incubation, soit après 24 heures, après avoir été dissoutes ensemble dans 0,1 cc de liquide de Tyrode. Le tableau ci-dessous (Tableau 4) nous montre les résultats obtenus. Quant aux séries de contrôle, nous avons employé les séries qui nous avaient servi au cours des expériences précédentes en prenant en considération l'effet de 1 mg. de DPX injecté avant et 24 heures après l'incubation.

En ce qui concerne la mortalité précoce, les chiffres de la première ligne nous indiquent clairement que l'association des deux antagonistes dans le rapport 1 : 1 montre les résultats les plus favorables; et cela, soit que l'association soit injectée avant l'incubation, soit que les antagonistes soient injectés 24 heures après. En comparant ces valeurs avec celles des séries de contrôle injectées avec 1 mg. de DPX, on observe que, dans le premier cas (DPX administrée

TABLEAU 4

	Contrôles %		*Traitement avec le complexe DPX-PX*					
			avant l'incub.			*après 24 heures*		
	DPXO	*DPX24*	*1:1*	*1:10*	*1:100*	*1:1*	*1:10*	*1:100*
Mortalité précoce	44,1	14,7	13,0	27,2	22,0	15,2	30,9	20,0
Mortalité tardive	40,2	48,0	28,3	23,8	32,9	21,8	14,8	24,5
Vivants le 21ème jour	15,7	37,3	58,7	49,0	45,1	63,0	54,3	55,5
Hypodéveloppés	21,1	13,8	8,8	7,4	12,7	6,4	7,7	8,0
Malformés	8,8	14,9	13,7	11,9	12,7	9,0	4,6	6,6

DPXO = Embryons traités avec 1 mg de DPX dissous dans 0,1 c.c. de liquide de Tyrode, avant l'incubation.

DPX24 = Embryons traités avec 1 mg. de DPX dissous dans 0,1 c.c. de liquide de Tyrode, apres 24 heures.

avant l'incubation), le taux élevé de mortalité (44,1 %) est sensiblement réduit par l'adjonction de PX; une telle réduction de la mortalité atteint des valeurs maximales dans le rapport 1:1, la réduction de la mortalité est inférieure dans les rapports 1:10 et 1:100. Dans le deuxième cas, par contre (DPX injectée après 24 heures d'incubation), on n'observe aucune réduction de la mortalité; bien au contraire, avec les rapports 1:10 et 1:100, on remarque même une augmentation de la mortalité par rapport au traitement avec la DPX seule. Ces données nous indiquent que la DPX injectée avant l'incubation est directement responsable du taux élevé de mortalité précoce, et que ce taux peut être sensiblement réduit par l'administration simultanée d'une dose adéquate de PX. Mais cela nous montre également que lorsque la DPX est administrée après 24 heures d'incubation, le taux de mortalité précoce qu'elle provoque peut être lié à des causes indépendantes d'une action inhibitrice spécifique: en effet, ce taux de mortalité, qui d'ailleurs n'est pas du tout élevé, n'est pas réduit par l'administration simultanée de DPX.

[illegible] faites au moyen des chiffres relatifs

à la mortalité tardive. Même dans ce cas, l'adjonction de PX est capable de réduire plus ou moins largement le taux de mortalité et cela, soit que les traitements associés soient faits avant l'incubation, soit qu'ils le soient après 24 heures d'incubation; dans ce dernier cas, la réduction de la mortalité paraît même parfois plus importante.

L'efficacité de l'association de la PX à la DPX se trouve encore confirmée en observant les valeurs indiquant le pour-cent des embryons capables d'arriver à l'éclosion: on remarque que les trois rapports sont capables de réduire l'effet inhibiteur provoqué par la DPX seule.

L'effet antagoniste de la PX vis-à-vis de la DPX est encore témoigné par la réduction sensible du pour-cent des embryons hypodéveloppés que l'on observe dans les différents rapports.

Enfin, en ce qui concerne le nombre de malformés, ce groupe d'expériences ne nous apporte pas de données significatives. Mais cela se comprend bien, en tenant compte du fait qu'aucune des deux substances, injectée séparément, n'a révélé d'effets tératogènes aux doses que nous avons employées.

En définitive, ce groupe d'expériences nous permet de tirer les conclusions suivantes:

(*a*) L'adjonction de PX à la DPX réduit sensiblement le taux de mortalité précoce et de mortalité tardive provoquée par le traitement avec la DPX seule. Par conséquent, le taux de survie augmente notablement.

(*b*) Pour neutraliser le taux élevé de mortalité précoce provoqué par la DPX injectée avant l'incubation, il est nécessaire d'utiliser une concentration de PX élevée: dans nos expériences, le rapport 1:1 s'est révélé le plus efficace. Pour neutraliser le taux de mortalité tardive et pour faire baisser le taux des embryons hypodéveloppés, même des concentrations inférieures de PX (1:10 et 1:100) se sont révélées largement suffisantes.

Considérations sur le pouvoir tératogène de différents solvants, de la DPX et de la PX

Dans des recherches précédentes, nous avons étudié l'action de différents solvants sur le développement de l'embryon de poulet. Avoir établi l'absence de pouvoir tératogène de la part de ces solvants, représentait une donnée fondamentale qui nous a permis d'examiner, par la suite, le pouvoir tératogène de substances dissoutes dans ces solvants. Les renseignements que nous en avons tirés sont liés à un critère quantitatif et ce critère, à lui seul, nous paraît encore insuffisant pour exclure un pouvoir tératogène éventuel; pour rendre un tel jugement plus valable, il ne faut pas négliger le critère qualitatif; à savoir, il faut préciser s'il existe un lien d'interdépendance entre l'administration d'une substance donnée et l'apparition de malformations bien déterminées, se répétant avec une certaine fréquence et constance. Dans ce but, nous avons comparé les résultats fournis par tous les groupes d'expériences que nous avons réalisés,

et cela, en considérant, non seulement les pour-cents totaux des malformés, mais également et surtout en étudiant les pour-cents partiels des différents types de malformations. Il est évident qu'une telle comparaison ne pourra qu'être globale, c'est-à-dire réalisée sur le total des embryons traités avec toutes les substances et sans plus tenir compte ni de la dose employée ni de l'époque à laquelle la substance a été injectée. Dans le tableau qui suit (Tableau 5), on remarque que les valeurs en pour-cent sont très semblables entre elles et ne s'éloignent pas beaucoup des valeurs obtenues chez les embryons de contrôle n'ayant subi aucun traitement. Le tableau montre donc d'une manière très claire qu'aucune des deux substances, DPX et PX, et qu'aucun des trois solvants n'a d'effet tératogène.

TABLEAU 5

Traitement	*Nombre d'embryons traités*	*N réel d'embryons malformés*	*N. en % d'embryons malformés*
Contrôles	473	25	5,3
Piqûre	374	17	4,6
Eau dist.	1031	46	4,5
Sol. physiol.	1034	56	5,4
Tyrode	1241	84	6,7
DPX	914	68	7,5
PX	522	26	4,9
DPX-PX	556	44	7,9

En tenant compte, toutefois, que la DPX a provoqué, dans bien des cas, un taux élevé de mortalité précoce, et en tenant compte également des difficultés que l'on rencontre pour établir avec certitude l'existence de malformations chez les embryons morts au cours des trois premiers jours, nous avons fait d'autres calculs. Ceux-ci ont été faits en tenant compte seulement des embryons qui avaient survécu au-delà du troisième jour après le traitement. Comme on le remarque dans le Tableau 6, les pour-cents des malformés restent toujours très bas et présentent des valeurs qui sont assez semblables à celles des embryons de contrôle.

TABLEAU 6

Traitement	*Nombre d'embryons vivants à partir du 3ème jour*	*Nombre d'embryons malformés à partir du 3ème jour*	
		N. réel	*N. en %*
Contrôles	440	25	5,7
Piqûre	359	17	4,7
Eau dist.	735	46	6,3
Sol. physiol.	727	50	6,8
Tyrode	902	73	8,1
DPX	748	66	8,8
PX	485	24	5,0
DPX-PX	337	[illegible]	[illegible]

Nous avons enfin réalisé un autre calcul, en tenant compte du type de malformation présenté par chaque embryon. Ce calcul implique des difficultés, étant donné que seul un nombre restreint d'embryons présentent des malformations isolées. La plupart des malformations se sont présentées en association avec une ou plussieurs autres malformations, il en est résulté, dans certains cas,

TABLEAU 7

Cadre synoptique des différents types de malformations observés chez les embryons de contrôle et chez les embryons traités avec les solvants physiologiques, la DPX et la PX

	C	*P*	*ED*	*SP*	*TY*	*DPX*	*PX*	*DPX-PX*
Brachygnathie sup. et inf.	16	0	11	7	4	4	4	2
Rétroversion du bec inf.	0	5	11	4	4	9	15	11
Bec croisé	8	23	13	19	18	7	8	18
Bec de perroquet	12	12	15	7	11	17	35	7
Micro- et anophtalmie mono- et bilat	52	53	38	50	39	28	12	23
Déviation du bec	0	0	0	0	1	6	0	2
Anencéphalie, exencéphalie	48	23	19	23	27	25	16	16
Déviation de la colonne vert.	0	0	2	7	2	4	4	5
Spina bifida	0	0	2	0	0	1	0	0
Célosomie	20	41	35	19	46	19	20	41
Ectrosomie	0	0	0	0	1	0	0	0
Ectromélie	8	0	0	0	0	0	0	0
Brachymélie	0	0	0	0	0	0	4	0
Hémimélie	0	0	2	0	1	0	0	2
Phocomélie	0	0	0	0	0	2	0	0
Symélie	0	0	0	0	1	0	0	0
Ectrodactylie	0	0	2	2	0	0	0	0
Gueule de loup	0	0	0	0	0	4	0	0
Anourie	0	0	0	0	0	0	0	2
Oedème	4	0	4	4	1	9	16	10
Kystes des plumes	0	0	0	2	0	0	0	0
Anomalies de la position des pattes	0	0	0	6	0	0	0	5
Monstres doubles	0	0	0	0	0	3	0	0
Autres monstruosités	0	12	4	2	5	0	0	5

C = Contrôles. *SP* = Solution physiologique.
P = Piqûre. *TY* = Liquide de Tyrode.
ED = Eau distillée.

des images monstrueuses très complexes. Par conséquent, les embryons présentant plusieurs malformations figurent deux ou plusieurs fois sous le nom des malformations correspondantes.

Tous les chiffres du Tableau 7 représentent des pour-cents qui se rapportent au total des malformés obtenus avec le traitement correspondant à la colonne respective. Par exemple, le chiffre de 8 dans la rubrique des becs croisés, chiffre observé chez les embryons de contrôle, correspond à 8 % de 25 embryons trouvés malformés parmi les embryons de contrôle

Comme les chiffres du tableau le montrent, pour aucune des substances employées, il n'existe de fréquence spécifique d'un type de malformation. Il existe, il est vrai, des types de malformations qui se répètent plus fréquemment que d'autres, mais cette fréquence n'est pas liée au traitement par une seule substance puisque la malformation se répète de façon générale avec toutes les substances employées (voir par exemple les malformations des yeux, la célosomie, l'anencéphalie). L'un des exemples de malformation spécifique pourrait être représenté par les monstres doubles qui—sur le total des embryons malformés—existent seulement chez les embryons traités avec la DPX. Mais, mis à part le nombre insignifiant de cas (2), il y a d'autres considérations qui nous montrent que cette malformation ne doit pas être considérée comme spécifique pour la DPX. Cette malformation est liée, en effet, à une déviation des processus normaux de segmentation de l'oeuf, se réalisant à des phases très précoces du développement. Dans notre cas, les deux embryons ont été injectés avec la DPX 24 heures après le début de l'incubation; il est donc vraisemblable que, lorsque le traitement fut réalisé, la segmentation anormale avait déjà débuté. La gueule de loup est une autre variété de malformation qui pourrait avoir une valeur spécifique. Nous ne l'avons constatée que chez des embryons traités avec la DPX. Dans ce cas, cependant, la valeur est trop basse (le 4% du total des malformés provoqués par la DPX) pour considérer cette malformation comme spécifique de la substance.

En définitive, les valeurs rapportées dans le dernier tableau confirment les conclusions tirées des deux tableaux précédents: la DPX et la PX, comme les trois solvants, ne semblent révéler aucune action tératogène spécifique.

RÉSUMÉ

1. L'antivitamine B_6 (DPX) exerce un effet inhibiteur sur le développement de l'embryon de poulet.

2. Un tel effet inhibiteur se manifeste à la dose de 1 mg. et il est plus ou moins marqué et plus ou moins précoce. L'effet inhibiteur se traduit par la mort précoce des embryons ou par l'arrêt ou un retard du développement, qui, dans bien des cas, est suivi par la mort de l'embryon à un stade plus avancé.

3. L'effet inhibiteur paraît d'autant plus marqué que la période du traitement est plus précoce: l'intensité maximale s'observe en administrant la DPX avant l'incubation.

4. La vitamine B_6 (PX) doit être considérée comme absolument inoffensive pour le développement de l'embryon de poulet.

5. L'adjonction de PX à la DPX réduit sensiblement le taux de mortalité précoce et de mortalité tardive provoqué par le traitement avec la DPX seule.

6. La vitamine B_6, l'antivitamine B_6 et les trois solvants étudiés (eau distillée, solution physiologique et liquide de Tyrode) n'ont pas révélé d'action tératogène spécifique sur l'embryon de poulet.

SUMMARY

The action of vitamin B_6 and its antivitamin upon the development of the fowl embryo

1. Antivitamin B_6 (deoxypyridoxine) has an inhibitory effect upon the development of the fowl embryo.

2. This effect, which occurs with doses of 1·0 mg., may be shown by early death, or by arrested or delayed development which in many cases is followed by death at later stages.

3. The effect is greater the earlier the administration of the antivitamin. The greatest effect was obtained after administration to the egg before incubation.

4. Vitamin B_6 is without adverse effect upon the development of the fowl embryo.

5. Joint administration of vitamin B_6 and antivitamin B_6 reduces the early and the late mortality produced by the antivitamin alone.

6. Vitamin B_6, antivitamin B_6, and the three solvents used (distilled water, physiological saline and Tyrode solution) showed no specific teratogenic activity.

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ESTIMATION OF VITAMIN B_6 COMPOUNDS IN HUMAN BLOOD AND URINE

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SUMMARY

A sensitive and specific method for the estimation of B_6 compounds in blood and urine is described. It utilises the fluorescence of their cyanohydrin and lactone derivatives after separation of the compounds on a phosphocellulose ion exchanger. No significant variation was found in the levels of B_6 compounds in blood and urine of normal female subjects at different stages of the menstrual cycle.

Oral administration of pyridoxol HCl raised the levels of B_6 compounds in blood and urine; blood pyridoxal phosphate levels were still elevated after four days.

Methods for the estimation of B_6 compounds (pyridoxal phosphate—PALP; pyridoxal—PAL; pyridoxamine phosphate—PAMP; pyridoxamine—PAM; pyridoxol—POL; 4-pyridoxic acid—PIC) in biological fluids fall into three categories: microbiological, enzymatic and chemical, and have been comprehensively reviewed[1]. The microbiological assays[1] give values with wide variation and scatter, presumably owing to a lack of specificity exhibited by the bacterial organisms. Enzymatic methods are excellent but are specific only for PALP[2]. Chemical methods[3,4] have been described but the results obtained are very much higher than those from microbiological and enzymatic methods; we believe this could be due to interference from nonspecific fluorophores obtained as a result of the extraction procedures employed. A fluorometric method has therefore been developed which is sufficiently sensitive and specific to give greater accuracy in the estimation of B_6 compounds in biological fluids.

Blood and urine, after preliminary processing, are adsorbed on to a phosphocellulose ion exchanger and the eluted B_6 compounds are measured by the fluorescence of their cyanohydrin[5,6] and lactone[7] derivatives. Using this method, B_6 levels in blood and urine were measured at different stages of the menstrual cycle in four normal females and after oral administration of POL HCl (100 mg) in two normal females.

These techniques are being used to study vitamin B_6 metabolism in pregnancy and its complications.

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MATERIALS AND METHODS

Phosphocellulose Pll, H. Reeve Angel & Co. Ltd., London.
PALP, POL HCl, PAM 2 HCl, Koch Light Lab. Ltd., London.
PAMP HCl, PAL HCl, Sigma Chem. Co.
PIC was prepared by the method of Heyl[8].
Sodium glyoxylate monohydrate was prepared by the method of Metzler *et al.*[9].
Manganese dioxide was prepared by the method of Toepfer *et al.*[10].

Peroxide-free ether was obtained by percolating it through a column of alumina (CAMAG MFC, Alkaline, Brockman Activity 1). It was stored for not more than 24 h in an amber glass bottle.

Water was glass-distilled followed by deionisation on a Permutit column.

Preparation of chromatography columns

Glass columns (1.2 cm internal diameter, 30 cm long) with one end drawn to a tip and fitted with a glass sinter (porosity 0), and the other end in the shape of a thistle funnel, to serve as a reservoir, were filled with 0.01 *N* acetic acid. A rubber tube with a screw clip prevented the solution from running out. Pll cellulose, treated for the removal of fines etc.[11], was equilibrated in 0.01 *N* acetic acid and pipetted into the columns to give a bed height of 4 cm with the screw clips open. The columns were washed with 0.01 *N* acetic acid (20 ml) before use. Precautions normally taken to prevent columns running dry were found to be unnecessary and it was therefore found convenient to remove the rubber tubes from the columns.

Preparation of samples for chromatography

All procedures were carried out under subdued or red light.

Blood. Heparinised blood samples can be stored at −15° for up to a week. Blood (10 ml) was haemolysed with water (20 ml) and the protein precipitated by mixing with 10% (w/v) trichloroacetic acid (30 ml). The mixture was warmed at 50° for 20 min and centrifuged at 4000 rev./min for 20 min at room temperature. A known volume of the supernatant (40–50 ml) was extracted twice with equal volumes of peroxide-free ether to remove most of the trichloroacetic acid. The ethereal extracts were washed twice with water (5 ml) and the ether discarded. The washings were added to the aqueous extracts. The aqueous layer was evaporated down to approximately 1 ml under reduced pressure at 50° and transferred quantitatively with 0.01 *N* acetic acid to the column. The total volume applied (5–6 ml) was allowed to drain in, and the column effluent discarded.

PALP and PIC were eluted with 0.01 *N* acetic acid (10 ml). The column was then eluted with 0.1 *M* acetate buffer pH 4.7 (50 ml), the first fraction (30 ml) discarded, and the next (20 ml) collected for the estimation of PAL and PAMP. The elution was completed by the removal of adsorbed PAM from the column with 0.1 *M* phosphate buffer pH 7.4 (25 ml), the first fraction (10 ml) was discarded and the next (15 ml) collected. For the estimation of POL in blood, the column was eluted with 0.1 *M* acetate buffer pH 4.7 (55 ml), the first fraction (30 ml) discarded and the next (25 ml) collected for the estimation of POL, PAL, and PAMP.

Urine. 24-h urine collections can be stored under a layer of toluene (10 ml) at 4° for 48 h or −15° for 2 weeks. The volumes and pH were measured and a portion

TABLE I

PREPARATION OF PYRIDOXAL AND PYRIDOXAL PHOSPHATE CYANOHYDRINS FROM B_6 ANALOGUES AFTER SEPARATION INTO FRACTIONS ON P11 CELLULOSE COLUMNS

Fraction	*PALP* 2 ml	*PAL* 2 ml	*PAMP* 2 ml	*PAM* 2 ml	*POL* 2 ml
Manganese dioxide	—	—	—	—	Approx. 0.02 g. Shake 30 min at room temperature. Centrifuge. Collect supernatant
0.005 *M* potassium alum.	—	—	0.1 ml	0.1 ml	—
0.05 *M* glyoxylate	—	—	0.1 ml	0.1 ml	—
1.0 *M* phosphate buffer pH 7.4	2 ml[a]	2 ml	2 ml	2 ml	2×1 ml used to wash ppte. Washings added to supernatant
Heat	—	—	100°	15 min	—
Potassium cyanide (in 0.1 *M* phosphate buffer pH 6.9)	0.1 ml	0.05 *M*	0.1 ml	0.5 *M*	0.1 ml 0.05 *M*
Warm 50°	30 min	120 min	30 min	120 min	120 min
Cool to room temperature					
Adjust pH to	3.5–4[b,c] (1 ml 1.6 *N* HCl)	10 (1 ml 1.5 *N* NH_4OH)[c]	3.5–4 (1 ml 1.62 NHCl)[c]	10 (1 ml 1.5 *N* NH_4OH)[c]	10 (1 ml 1.5 *N* NH_4OH)[c]
Blanks prepared by omitting	potassium cyanide		glyoxylate		manganese dioxide
Volume compensated by	0.1 ml 0.1 *M* phosphate buffer pH 6.9		0.1 ml H_2O		—

[a] 0.2 *M* phosphate buffer used for blood PALP fraction.
[b] 0.33 *N* HCl used to adjust pH of blood PALP fraction.
[c] These solutions allowed the pH's to be routinely adjusted to the required values with 1 ml reagent.
All test and blank solution preparations were carried out in duplicate.

(10 ml) was adjusted to pH 3.3–3.6 with *N* acetic acid (5–10 ml), the total volume being recorded. A portion (10 ml) of this solution was allowed to drain through the column and the effluent collected. The column was eluted with 0.01 *N* acetic acid (10 ml) and the combined effluents (20 ml) were used for the determination of PALP and PIC. The column was further eluted with 0.1 *M* acetate buffer pH 4.7 (40 ml) the first fraction (15 ml) was discarded and the next (25 ml) collected for the determination of PAL, POL and PAMP. The elution was completed by the removal of adsorbed PAM with 0.1 *M* phosphate buffer pH 7.4 (11–25 ml).

Estimation of column fractions

The fluorescence of the B_6 compound derivatives was measured on an Aminco Bowman Spectrophotofluorometer. The instrument was standardised to a given sensitivity by means of the sensitivity control using a fluorescent glass block (C. Zeiss Optical Co. $\lambda_{excit} = 415$ mμ, $\lambda_{fluor} = 515$ mμ).

Instrument characteristics were:

Slit arrangement: No 3.

Lamp: Xenon 150 watt.

Detector: 1P21 or 1P28 photomultiplier tube.

PAL, POL and PAM[12] were estimated as the cyanohydrin derivative of PAL[5,6] ($\lambda_{excit} = 350$ mμ, $\lambda_{fluor} = 434$ mμ). PALP and PAMP were estimated as the cyanohydrin derivative of PALP[5,6] ($\lambda_{excit} = 318$ mμ, $\lambda_{fluor} = 417$ mμ). Table I describes the procedures employed for the conversion of B_6 compounds to their respective cyanohydrin derivatives.

PIC was estimated as its highly fluorescent lactone derivatiwe (λ_{excit} = 350 mμ, λ_{fluor} = 434 mμ) by a slight modification of the method of Woodring *et al.*[7].

Water (1.6 ml) and 2 *N* NaOH (0.4 ml) were mixed with an aliquot (0.2 ml) of the PIC fraction and heated at 100° for 5 min. After cooling to room temperature, 6 *N* HCl (0.4 ml) was added and the solution heated at 100° for 20 min. The addition of 6 *N* NH_4OH (1 ml) gave a pH between 9–10. Blanks were prepared by omitting the second heating step and adding 6 *N* NH_4OH immediately after addition of 6 *N* HCl. Test solutions were prepared in triplicate.

Standard curves of all derivatives were prepared by plotting relative fluorescent intensity (RFI) against the concentration of the B_6 compound.

RESULTS

Recoveries of B_6 compounds added to blood and urine are shown in Tables II and III. Duplicates of B_6 levels in blood differed from each other by less than 5% and in urine by less than 10%.

TABLE II

RECOVERIES OF A MIXTURE OF B_6 COMPOUNDS FROM P11 CELLULOSE COLUMNS

Compound	*Column fraction*	*RFI of column fraction*	*% Recovery*
PALP (RFI = 2.6)	10 ml 0.01 *N* acetic acid	2.5	96
PAMP (RFI = 2.11)	25 ml 0.1 *M* acetate buffer pH 4.7	2.0	95
PAL (RFI = 7.0)	25 ml 0.1 *M* acetate buffer pH 4.7	7.5	107
POL (RFI = 7.5)	25 ml 0.1 *M* acetate buffer pH 4.7	6.75	90
PAM (RFI = 6.3)	15 ml 0.1 *M* phosphate buffer pH 7.4	6.45	102
PIC (RFI = 5.2)	10 ml 0.01 *N* acetic acid	5.3	102

TABLE III

RECOVERIES OF B_6 COMPOUNDS FROM BLOOD AND URINE

Compound	*Concentration (ng/ml)*			*Recovery of compound from blood (%)*	*Concentration (ng/ml)*			*Recovery of compound from urine*
	Compound	*Blood*	*Blood + compound*		*Compound*	*Urine*	*Urine + compound*	
PALP	34.3	17.5	48.6	91	206*	20	197	86
PAMP	31.7	15	49.2	108	190*	0	156	82
PAL	28.6	26.4	53.5	95	86	49.7	125	88
POL	30.6	0	27	88	61	74.9	124	80
PAM	33.7	45.2	75.4	90	202	272	448	87
PIC	50	11.4	56.1	89	300	237	516	93

* Low concentrations of PALP and PAMP (<100 ng/ml) added to urine resulted in erratic recoveries.

Variation of B_6 compound levels during the menstrual cycle

Blood samples were withdrawn between 10.00 a.m. and 10.30 a.m. at weekly intervals over a 5-week period. Urine samples were collected over a 24-h period preceding the withdrawal of blood.

Fig. 1 shows the variation in blood levels of B_6 compounds in four normal females in the reproductive age group. Fig. 2 shows the urinary B_6 output over the same period of time.

Pyridoxol loading tests

Blood samples (11 ml) were collected from two normal females between 9.30 a.m. and 10.30 a.m. This was followed by the oral administration of POL HCl (100 mg). Blood samples were collected at 2, 6, 24 and 96 h after the dose and 24 h urine samples were collected the day before, the same day, and the day following the loading

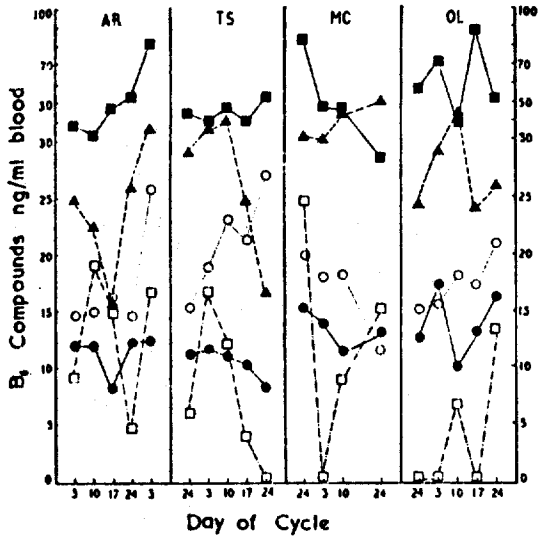

Fig. 1. Levels of B_6 compounds in blood of four normal females at different stages of their menstrual cycle (28–29 days). ●——● PALP, ■——■ PAL, □- - - - -□ PAMP, ○· · · · · · ○ PAM, ▲- - - - - -▲ PIC.

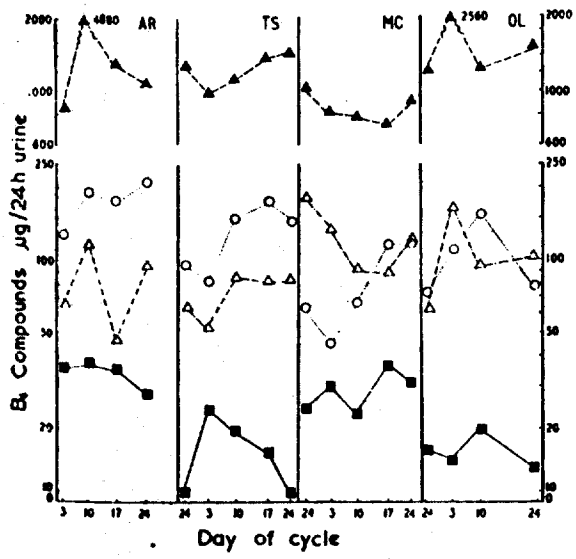

Fig. 2. Levels of B_6 compounds in urine of four normal females at different stages of their menstrual cycles (28–29 days). ■——■ PAL, ○· · · · · · ○ PAM, △- - - - - -△ POL, ▲- - - - - -▲ PIC.

with pyridoxol. Fig. 3 shows the variation in blood levels of B_6 compounds after the loading.

Table IV shows the variation in urinary B_6 output before and after the loading with pyridoxol.

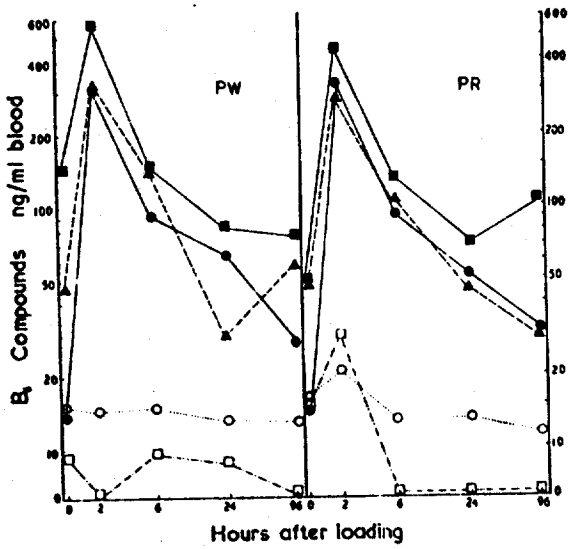

Fig. 3. Levels of B_6 compounds in blood of two normal females after oral dose of POL HCl (100 mg). ●——● PALP, ■——■ PAL, □------□ PAMP, ○·····○ PAM, △------△ PIC.

TABLE IV

COMPARISON OF URINARY OUTPUTS (mg/24 h) OF B_6 COMPOUNDS IN SUBJECT P.W. BEFORE AND AFTER DOSING WITH PYRIDOXOL HYDROCHLORIDE (100 mg)

Compound	*Day before loading*	*Day of loading*	*Day after loading*
PALP	0.040	0.258	0.045
PAMP	0	0	0
PAL	0.054	2.25	0.206
POL	0.072	2.94	0.044
PAM	0.130	0.127	0.112
PIC	0.970	19.8	3.16

DISCUSSION

Using the described method good recoveries of the B_6 compounds (Table II, III) were obtained from blood and urine samples. Although this study was carried out with whole blood, the method can be applied to the estimation of levels of B_6 compounds in plasma or blood cells. Total levels of B_6 compounds estimated (Fig. 1) were comparable to those obtained by some microbiological methods[1] but lower than those obtained by other chemical methods[3,4].

Preliminary studies showed that high levels, similar to those found by published chemical methods, could be obtained but were later found to be due to the interference from nonspecific fluorophores.

PALP levels in blood (Fig. 1) were comparable to those found by enzymatic techniques[8]. Estimation of blood PALP by the described method takes less time than other published methods and six estimations can be carried out in a normal working day.

A study of B_6 compounds during different stages of the menstrual cycle (Figs. 1, 2) showed no significant variations, which agreed with the results published by Diding *et al.*[13]. Blood PALP levels (Fig. 1) remained fairly constant over the course of the study and could be used as an index for the study of B_6 metabolism.

Loading the subjects with POL raised their levels of PALP, PAL and PIC in blood and urine and POL in urine. PAM and PAMP levels were unaffected (Fig. 3, Table IV). This pattern in blood is in agreement with the pattern of POL metabolism suggested for tissue[14], where POL is converted to POLP or PAL and metabolised to PALP or PIC. The very high levels of PALP, PAL and PIC found in blood after 2 h (Fig. 3) suggest a fairly rapid absorption of POL from the gut. The high urinary levels (Table IV) on the day of the loading suggested that the concentrations of the B_6 compounds in blood were above the renal threshold levels, and would account, together with tissue uptake, for the large drop in levels after 6 h (Fig. 3). The absence of POL from blood, even 2 h after its administration, could be explained by its complete metabolism in blood, its storage in tissues, its excretion in urine, or by its complete metabolism during absorption through the gut tissues. About 30% of the administered POL in terms of excess B_6 metabolites was accounted for in the blood and urine after 48 h suggesting a large tissue uptake of the vitamin. Blood PALP levels were over twice the normal after four days (Fig. 3).

Studies are in progress for assessing the role of B_6 metabolism in normal pregnancy and toxaemia of pregnancy.

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Blood and urine levels of vitamin B_6 in the mother and fetus before and after loading of the mother with vitamin B_6

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A specific chemical method was employed to measure B_6 compounds in men, pregnant and nonpregnant women, and fetal blood and urine before and after loading with pyridoxol. The blood levels of pyridoxal phosphate, the coenzyme form of the vitamin, were significantly lower in pregnancy, while fetal cord blood levels were high, indicating the existence of a relative deficiency state in pregnant women. In the various subjects examined, no differences were found in the urinary excretion of pyridoxic acid, the main metabolite of the vitamin, which suggests that intestinal absorption of the vitamin was unimpaired during pregnancy. Phosphorylation of pyridoxol was rapid in all subjects as blood levels of pyridoxal phosphate reached a peak 2 hours after oral and 10 minutes after intravenous loading. Pyridoxal phosphate levels in fetal blood reached a peak several hours after maternal blood indicating that the phosphorylated forms of the vitamin crossed the placental barrier to the fetus. Pyridoxol metabolism was unaffected during pregnancy, suggesting that the major cause of a relative maternal deficiency was as a result of the large fetal uptake of the vitamin.

THE EXISTENCE OF a vitamin B_6 deficiency has been suspected in pregnancy ever since Willis, Morris, and Massey[1] used this compound for alleviating symptoms of morning sickness in pregnant women. The use of the vitamin in this case was purely empirical and better evidence of a deficiency state was presented by McGanity and associates.[2] These workers showed that patients with hyperemesis gravidarum had low blood urea levels which exhibited an abnormal response following the administration of alanine and could be corrected by giving pyridoxol orally. The clinical symptoms of a deficiency state for this vitamin become apparent only in severe cases of B_6 deprivation, and the milder forms have to be detected by indirect means such as the increase in excretion of xanthurenic acid following oral loading with tryptophan.[3]

Several attempts have been made for a more direct approach to the problem of this deficiency state in pregnancy by studying the metabolism of vitamin B_6.[4-7] Most of the published data have been arrived at by the use of microbiologic methods for the estimation of B_6 metabolites in tissues and body fluids. The accuracy of these methods has been in some doubt[8] and may account for the confused situation of conflicting results which exists at present. This study has been devised to reinvestigate the problem and relies on specific chemical methods for the quantitative estimation of B_6 metabolites in body fluids. Fetal uptake and metabolism of vitamin B_6 when the mother is given loading doses of the vitamin have been included in the study.

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Materials and methods

Among the materials used were phosphocellulose P11,* 50 mg. pyridoxol HCl tablets, and 50 mg. of pyridoxol HCl for injection in saline. †Sodium glyoxylate monohydrate was prepared by the method of Metzler, Olivard, and Snell.[9] Manganese dioxide was prepared by the method of Polansky, Gamarra, and Toepfer.[10] Peroxide-free ether was obtained by percolating it through a column of alumina (CAMAG MFC, Alkaline, Brockman Activity 1). It was stored for not more than 24 hours in an amber glass bottle. Water was glass-distilled followed by deionization on a Permutit column.

Blood samples (11 ml.) were collected in heparinized containers and stored in a deep freeze at -20° C. for up to one week. Fetal blood was obtained from the cord. Urine samples (24 hour) were stored in a deep freeze at -20° C. for not longer than one week.

Administration of B_6. Pyridoxol HCl (50 mg.) was administered to the subjects orally or by injection in an antecubital vein.

Estimation of B_6 compounds. B_6 compounds in blood and urine were estimated by the method of Contractor and Shane[11] with the following modification. For the estimation of urine, 10 ml. samples were adjusted to pH 3.3 to 3.5 with normal acetic acid and then diluted with water until 0.33N with respect to the added acid. After noting the volume, 10 ml. aliquots were applied to a 4 by 1.2 cm. P11 phosphocellulose column and the described procedure followed. This technique permits the estimation of pyridoxal 5′-phosphate, pyridoxamine 5′-phosphate, pyridoxal, pyridoxol, pyridoxamine, and 4-pyridoxic acid, after separation on a phosphocellulose ion exchanger, by the strong fluorescence of their cyanohydrin[12, 13] or lactone derivatives.[14] Recent work[15] suggests that the "cyanohydrin" derivatives of pyridoxal and pyridoxal 5′-phosphate formed under weak alkaline conditions are actually 4-pyridoxic acid lactone and 4-pyridoxic acid 5′-phosphate, respectively.

*H. Reeve Angel & Co., Ltd., London, England.
†BDH Biochemicals, Poole, Dorset, England.

Results

Table I shows blood levels of B_6 compounds in men, pregnant and nonpregnant women, and fetuses.

There was a highly significant difference between blood pyridoxal 5′-phosphate levels of pregnant and nonpregnant women ($P < 0.005$), mother and fetus ($P < 0.005$), and blood pyridoxamine 5′-phosphate levels of mother and fetus ($P < 0.005$). Pyridoxol was not detected in the blood of any of the subjects. Levels of B_6 compounds in cord artery and vein were identical.

Table II shows the 24 hour urinary excretion of B_6 compounds in men, nonpregnant women, and women in the last trimester of pregnancy.

Pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate were not detected in the urine of any of the subjects.

Fig. 1 shows blood pyridoxal 5′-phosphate levels in men and in pregnant and nonpregnant women following oral administration of 50 mg. pyridoxol HCl.

Pyridoxal and 4-pyridoxic acid levels show a pattern similar to that of pyridoxal 5′-phosphate.

Pyridoxamine and pyridoxamine 5′-phosphate levels were unaffected by the loading.

Significant differences were found between blood pyridoxal 5′-phosphate levels of pregnant and nonpregnant women at 2 hours ($P < 0.02$), 24 hours ($P < 0.02$), and 48 hours ($P < 0.025$) after the oral loading. Ten minutes after intravenous loading with 50 mg. of pyridoxol HCl, blood levels of pyridoxol were approximately 1 per cent of the administered dose while blood pyridoxal 5′-phosphate levels rose to 155 ± 13 (3) mμg per milliliter in nonpregnant women. At the end of 6 hours, the blood levels of B_6 metabolites were similar to those obtained after oral loading.

Table III shows blood pyridoxal 5′-phosphate levels in maternal and cord blood after oral loading with 50 mg. of pyridoxol HCl in 8 normal subjects.

Table I. Levels of B_6 compounds in blood

Subject	Pyridoxal 5′-phosphate (mμg/ml. ± S.D.)	Pyridoxamine 5′-phosphate (mμg/ml. ± S.D.)	Pyridoxal (mμg/ml. ± S.D.)	Pyridoxamine (mμg/ml. ± S.D.)	4-Pyridoxic acid (mμg/ml. ± S.D.)
Man	13.5 ± 2.9(5)	20 ± 5.5(4)	1.3	24.7 ± 19.5(3)	31.9 ± 6.4(4)
Nonpregnant woman	12.1 ± 2.3(23)	9.3 ± 7.4(21)	0.8 ± 0.2(2)	18.0 ± 3.9(21)	31.8 ± 10.1(23)
Pregnant woman	7.7 ± 3.7(5)	9.5 ± 6.4(4)	1.1 ± 0.7(5)	7.6 ± 1.2(4)	30.4 ± 9.2(4)
Fetus	27.5 ± 8.1(5)	27.4 ± 2.6(4)	2.2 ± 0.9(5)	6.6 ± 3.0(4)	25.2 ± 9.8(4)

Table II. Levels of B_6 compounds in urine

Subject	Pyridoxal (μg/24 hr. ± S.D.)	Pyridoxol (μg/24 hr. ± S.D.)	Pyridoxamine (μg/24 hr. ± S.D.)	4-Pyridoxic acid (μg/24 hr. ± S.D.)
Man	45.4 ± 11.2(2)	77.5 ± 31.7(2)	208 ± 66(2)	1324 ± 780(3)
Nonpregnant woman	24.5 ± 12.4(19)	94.1 ± 35.1(19)	121 ± 48(19)	1213 ± 848(26)
Pregnant woman	31.5 ± 8.7(2)	71.9 ± 20.1(2)	132 ± 18(2)	1454 ± 474(10)

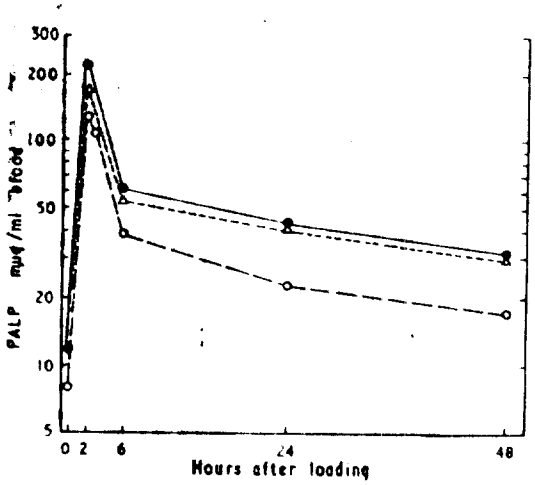

Fig. 1. Levels of pyridoxal 5′-phosphate in blood of different subjects after oral dose of pyridoxol HCl (50 mg.) ●——●, nonpregnant woman; Δ - - - - Δ, man; ○— —○, maternal.

Table III. Pyridoxal 5′-phosphate levels in maternal and cord blood after oral administration of 50 mg. of pyridoxol HCl

Period of gestation	Time after loading (hr.)	Maternal blood (mμg/ml.)	Cord blood (mμg/ml.)
Term	3.5	108	167
Term	5.5	38.7	97.7
Term	6	31.1	150
Term	6.5	32.1	115
Term	9	35.5	109
Term	10	16.0	65.7
21 weeks*	6	6.8	250
25 weeks*	6	43.0	281

*Termination of pregnancy by hysterotomy.

Table IV shows the 24 hour recovery of administered pyridoxol from the urine of nonpregnant and pregnant women.

Pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate were not detected in the urine samples. Pyridoxamine values are excluded as they remained unaltered after loading.

In pregnant subjects, about 60 per cent of the 24 hour urinary 4-pyridoxic acid and 95 per cent of the 24 hour urinary pyridoxol and pyridoxal were excreted in the first 6 hours after oral administration of 50 mg. of pyridoxol HCl.

Comment

During pregnancy, a relative B_6 deficiency is thought to exist in the mother based on the evidence of the tryptophan load test,[3, 16] blood B_6 levels in the mother,[4, 7, 17] and urinary 4-pyridoxic acid excretion.[18] Results of these studies appear to be somewhat contradictory, probably because indirect methods of estimating B_6 com-

Table IV. Urinary recovery of B_6 metabolites in different subjects after administration of 50 mg. pyridoxol HCl (PAM values are not included as they were not affected by the B_6 loading)

Subject	*Method of administration*	*Metabolite (mg./24 hr. ± S.D.)*			*Recovery (per cent of dose ± S.D.)*
		Pyridoxal	*Pyridoxol*	*4-Pyridoxic acid*	
Man	Oral	0.452	0.620	13.2	33
Nonpregnant woman	Oral	0.745 ± 0.107(2)	1.06 ± 0.25(2)	11.7 ± 0.7(2)	31 ± 3
	Intravenous	0.794 ± 0.134(3)	1.77 ± 0.32(3)	14.5 ± 1.2(3)	39 ± 4
Pregnant woman	Oral	0.754 ± 0.353(3)	1.16 ± 0.51(3)	9.74 ± 1.56(5)	27 ± 5
	Intravenous	0.545	3.78	14.2	42

pounds were used in some cases. In order to resolve the existing situation we developed a direct method which relies on the isolation and quantitation of the individual B_6 compounds from body fluids.[11] The method is based on specific fluorometric analysis which has the added advantage of high sensitivity.

Using our method, we have found blood levels of pyridoxal 5′-phosphate, the coenzyme form of the vitamin, to be significantly lower in pregnant women compared to nonpregnant women and men (Table I) indicating a relative B_6 deficiency during pregnancy. This finding is similar to that of Wachstein, Moore, and Graffeo,[4] while Coursin and Brown[7] found no significant decrease in blood pyridoxal 5′-phosphate levels through pregnancy.

The active transport of B_6 across the placenta shown by the high cord blood levels of pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate (Table I) together with the expected increase in metabolic requirements for the vitamin during pregnancy could explain this relative deficiency in the mother.

As no significant differences were found in the urinary output of 4-pyridoxic acid and other B_6 metabolites (Table II), or in the 4-pyridoxic acid blood levels of different subjects (Table I), it could be concluded that the maternal deficiency is not caused by a failure of intestinal absorption of the vitamin. Another possibility is that the phosphorylation of the vitamin to its active form may be impaired or the oxidation to 4-pyridoxic acid may be favored rather than its conversion to pyridoxal 5′-phosphate. This mechanism however seems unlikely as high levels of pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate were found in the fetus (Table I) without any increase in urinary excretion of 4-pyridoxic acid or the nonphosphorylated forms of the vitamin in the mother (Table II).

In an earlier report on blood levels of B_6 metabolites during the menstrual cycle,[11] we found a wide scatter in pyridoxamine 5′-phosphate values while pyridoxal 5′-phosphate values tended to be constant. Our results in this study show a similar scatter (Table I) which is in keeping with the hypothesis of Coursin and Brown[7] that pyridoxamine 5′-phosphate serves as a reservoir from which pyridoxal 5′-phosphate is drawn by a possible oxidation or transamination of the pyridoxamine 5′-phosphate,[19] so that the coenzyme is maintained at a constant concentration. The decrease in blood pyridoxamine levels found during pregnancy (Table I) could be indicative of a maternal deficiency, as pyridoxamine is known to be formed from pyridoxamine 5′-phosphate.[19]

Phosphorylation following oral or intravenous administration of pyridoxol is very rapid, as suggested by our results (Fig. 1). Pyridoxal 5′-phosphate levels are at their maximum 2 hours after oral loading, which is the time taken for absorption from the gut, whereas these levels are reached within 10 minutes if pyridoxal is given by the intravenous route.

Further evidence for a B_6 deficiency dur-

ing pregnancy was demonstrated by differences in blood pyridoxal 5′-phosphate levels in pregnant and nonpregnant subjects at different times after loading (Fig. 1). This is in agreement with the results of Wachstein, Kellner, and Ortiz,[17] and Karlin and associates,[20] who estimated total B_6 in blood after pyridoxol loading. Again, the reason for this "lower pattern" found in pregnancy could also be explained on the basis of the active transport of B_6 to the fetus (Table III) and an increased pyridoxal 5′-phosphate uptake by maternal tissues, rather than by a more rapid metabolism of pyridoxol, as urinary 4-pyridoxic acid excretion remained virtually the same in the different subjects after loading (Table IV). Karlin and associates[20] found that total B_6 levels of blood reached a maximum in the mother 2 hours and in the cord 5 to 7 hours after oral administration of pyridoxol. Our results (Table III) show a similar pattern for pyridoxal 5′-phosphate in blood. This "time lag" between maximum pyridoxal 5′-phosphate levels in maternal and fetal blood has been suggested by us[21] to indicate pyridoxal 5′-phosphate production by the mother followed by its active transport to the fetus, rather than the fetus carrying out its own phosphorylation. We have previously shown[21] that the activity of the pyridoxal 5′-phosphate-forming enzyme, pyridoxal kinase, increases in fetal liver and kidney through gestation. This was interpreted as the fetus preparing for birth and hence becoming ready to form its own coenzyme. An absence of this ability to be independent of the maternal supply could explain the reports describing the early appearance of central nervous system abnormalities in infants who have not received adequate dietary B_6 or have an abnormal requirement for the vitamin.[22-24]

Urinary recoveries of B_6 compounds after administration of pyridoxol (Table IV) showed a decrease in their excretion during pregnancy, but this was not significant. Some workers have found a lowered 4-pyridoxic acid excretion during pregnancy, whereas others have found no difference between pregnant and nonpregnant subjects.[25, 26] It has been suggested that a maternal deficiency of the vitamin could result in a lowered 4-pyridoxic acid excretion before and after pyridoxol loading, but we feel that urinary 4-pyridoxic acid is a bad indicator of the nutritional status of the mother as 4-pyridoxic acid can be formed by nonspecific enzyme action and its production is not likely to be inversely related to the metabolic requirements of the mother. However, the B_6 excretion results (Table IV) demonstrate that there is no failure of intestinal absorption of pyridoxol during uncomplicated pregnancy. The slightly higher urinary excretion of pyridoxol and 4-pyridoxic acid after intravenous loading compared to oral loading was expected, as some of the administered pyridoxol would pass straight to the kidneys while, after oral dosage, all the pyridoxol would travel to the liver first, and presumably be metabolized there.

When results from loading experiments are interpreted, various factors should be borne in mind. Large, nonphysiologic doses are used, which are bound to affect the balance of the different B_6 metabolites. Johansson and associates[27] demonstrated that B_6 metabolism could be visualized as a 2 compartment system consisting of (1) a rapid turnover compartment in which exogenous B_6 is excreted directly into the urine or passed to (2) a slow turnover compartment which could be compared with the body tissues. Large doses of B_6 would disturb the equilibrium of these compartments and one would expect the first compartment to excrete an abnormally high percentage of its B_6 content rather than pass it to the slow turnover "tissue store" compartment. It follows that some of the results obtained from loading experiments might be valid only under nonphysiologic conditions and the results presented will have to await confirmation by further investigations with radioactive tracers, which can be administered in physiologic amounts. It should also be remembered that dietary B_6 from animal sources is usually in the form of pyridoxal

5′-phosphate and pyridoxamine 5′-phosphate so that loading with pyridoxol will not necessarily reflect metabolic differences found in pregnancy. For instance, in preliminary studies on oral loading with pyridoxal and pyridoxal 5′-phosphate, we have found that maternal and cord blood levels of pyridoxal 5′-phosphate were hardly elevated over the preloading values, whereas urinary 4-pyridoxic acid excretion was considerably higher than after pyridoxol administration. However, the interpretation of these results must await further study.

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4-PYRIDOXIC ACID 5'-PHOSPHATE : A METABOLITE OF PYRIDOXOL IN THE RAT

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SUMMARY

An unknown labelled compound found in rat tissues and urine after the administration of ^{14}C-pyridoxol has been identified as 4-pyridoxic acid 5'-phosphate. It was not an artifact caused by the extraction and separation techniques employed. The results suggest that tissue pyridoxal phosphate is oxidised to pyridoxic acid phosphate which is in turn hydrolysed to pyridoxic acid and excreted. The presence of this previously undetected compound in urine could explain the failure of previous investigators to account for vitamin B_6 administered to Man and animals by their urinary excretion of vitamin B_6 metabolites.

In a previous paper[1] describing the stability of the tritium label of ^{3}H-pyridoxol during its metabolism in rats, a hitherto unknown tritiated metabolite was found in urine in appreciable quantity. Studies on the metabolism of ^{14}C-pyridoxol in rats (to be published) have also shown this metabolite to be present in rat tissues. This communication identifies it as pyridoxic acid phosphate and discusses its possible implications in the metabolism of pyridoxol.

Materials and Methods

4-pyridoxic acid 5'-phosphate (PICP) was prepared as described by Wada and Snell[2]. 4-pyridoxic acid (PIC) and 4-pyridoxic acid lactone (PIC lac) were prepared by the method of Heyl[3]. Pyridoxal phosphate (PALP), pyridoxal (PAL), and pyridoxol (POL) were obtained from Koch Light Lab. Ltd., London; pyridoxol phosphate (POLP) from Calbiochem Ltd., London, 2-methyl 3-hydroxypyridine 4.5 dicarboxylic acid (diPIC) from Roche Products Ltd., Welwyn, England.

Male Wistar rats were injected intraperitoneally with a mixture of ^{3}H-POL (Radiochemical Centre, Amersham, Bucks, England) and ^{14}C-POL (Hoffman la Roche, Basle, Switzerland) and 24h urines collected. Urines were lyophilised and separated into fractions on phosphocellulose columns as previously described[1,4]. The electronegative fraction (containing PIC, PALP, POLP and the unknown metabolite) was desalted by adsorbtion and elution from active charcoal and then separated on a DEAE cellulose column as shown on Fig. 1. Organs removed from pregnant Wistar rats injected intraperitoneally with ^{14}C-POL alone were homogenised, deproteinised, and then separated into fractions as described for urine, omitting the desalting step. A typical elution pattern for rat tissue extract is shown in Fig. 2.

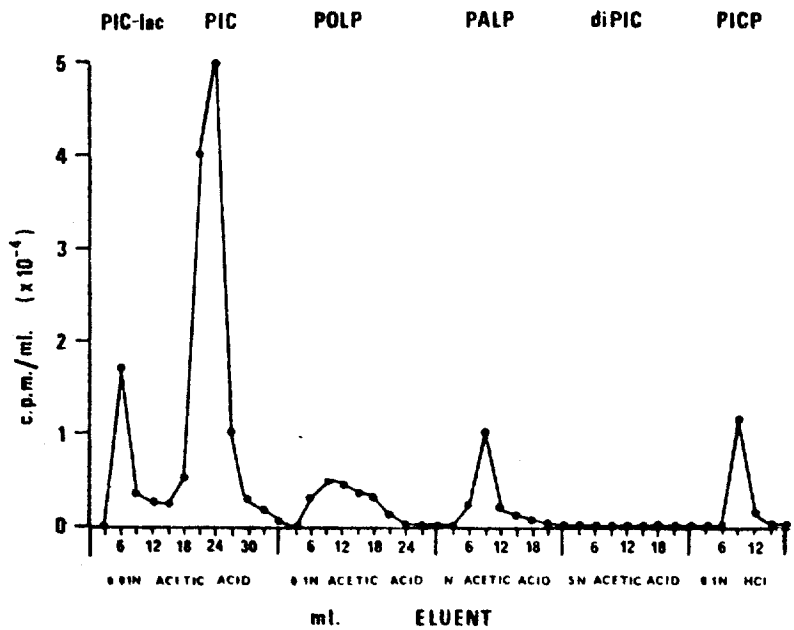

Fig. 1 Elution pattern from DEAE cellulose column of B6 metabolites in desalted electronegative fraction of 24h rat urine after injection of ^{3}H and ^{14}C-pyridoxol. Positions where B_6 standards are eluted are indicated.

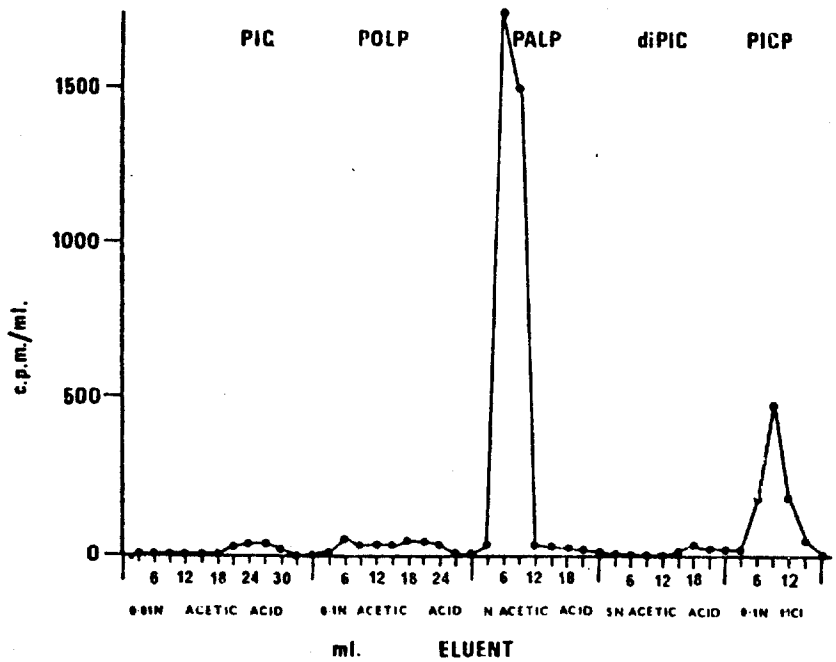

Fig. 2 Elution pattern from DEAE cellulose column of B_6 metabolites in electronegative fraction of rat liver extract 10 min. after intraperitoneal injection of ^{14}C-pyridoxol. Positions where B_6 standards are eluted are indicated.

The unknown metabolite found in the 0.1N HCl eluate from the DEAE cellulose columns was characterised by thin layer chromatography, electrophoresis and fluorescence, before and after HCl hydrolysis.

RESULTS AND DISCUSSION

The metabolite was adsorbed on active charcoal indicating that the pyridine ring of the original POL molecule remained intact. It was not an artifact caused by the desalting technique as it was isolated from tissue extracts which did not involve charcoal adsorption.

Column chromatography on DEAE cellulose (Figs. 1,2) showed the metabolite to be more electronegative than PALP which

suggested an oxidation product such as diPIC or PICP. The possibility of it being diPIC was eliminated as this compound is eluted from the column by 30% acetic acid, whilst the metabolite is eluted by 0.1N HCl in the same position as PICP (Figs 1,2). Fluorescence characteristics of the metabolite were found to be similar to those of authentic PICP (λexcit = 322mμ, λ fluor = 417mμ, pH3.8)[5] When the metabolite was hydrolysed in HCl, the fluorescence peak at 417mμ gradually disappeared and at alkaline pH, a different peak appeared (λ excit = 350mμ, λ fluor = 434mμ), a characteristic which is observed when authentic PICP is hydrolysed to PIC-lac by HCl. The hydrolysed metabolite was chromatographed on a DEAE column with an elution pattern similar to PIC-lac. Thin-layer chromatography on cellulose using (i) tertbutanol : acetone : H_2O-diethylamine (8:7:4:1) and (ii) 1.4 dioxan: H_2O (6.1) as solvent systems, and detection of the compounds by scintillation autography[6], revealed spots with R_f values of 0.75 and 0.92 respectively, which were identical with authentic PIC-lac.

Thin-layer cellulose electrophoresis of the metabolite in 0.05M acetate buffer pH5.1 (30v/cm) followed by radio chromatogram scanning (Packard Inst. Co.) showed a fast moving spot towards the anode (8cm/h) similar to authentic PICP. Electrophoresis of the hydrolysed metabolite showed a spot moving slowly towards the cathode (1cm/h) which was similar to PIC-lac.

Non-radioactive PICP was added to the fraction containing the unknown metabolite and the solution desalted and re-chromatographed as previously described. The eluate obtained was chromatographed on thin-layer cellulose using one bi-dimensional solvent system and three one dimensional solvent systems (Table 1). U.V. light fluorescent areas were removed and eluted with 0.01N acetic acid.

	Solvent System	Specific Activity
	0.1N HCl eluate from DEAE cellulose column	0.342
I	Tert-butanol:acetone: H_2O:diethylamine (8:7:4:1)	0.335
II	1,4-dioxan:H_2O (6:1)	0.335
III	n-propanol:H_2O (5:1)	0.334
	Bi-dimensional:system II/I	0.330

Table 1 Specific activities (cpm/relative fluorescent intensity) of unknown compound plus carrier PICP before and after thin-layer cellulose chromatography. Fluorescence was measured at λexcit = 322mμ, λfluor = 417mμ.

Specific activities of these eluates were determined and expressed as cpm/relative fluorescent intensity. Results (Table 1) show that the specific activity remained constant before and after chromatography in all the solvent systems confirming the identity of the metabolite as PICP. One single fluorescent spot was found in each solvent system and this had fluorescence characteristics of PICP.

To eliminate the possibility of PICP being formed by oxidation of PALP as a result of the extraction and separation procedures, pure PALP was put through the entire procedure and less than 1% was found to be converted to PICP.

Although PICP is known to be a chemical oxidation product of PALP[7], no reports of its presence in animal tissues or urine have appeared in the literature. PIC is the main excretory product of vitamin B_6 found in urine. Based on the evidence of in vitro experiments, the metabolism of PIC has been postulated to be via the hydrolysis of tissue PALP to PAL; which is then oxidised by an aldehyde oxidase to PIC[8].

After administration of ^{14}C-POL to rats, we have found (results to be published) a build up of PICP and very small amounts of PIC following the appearance of PALP in liver, kidney, spleen, brain, uterus, placenta and foetus. However, PIC was more predominant than PICP in blood and urine. PAL built up by hydrolysis of PALP at a later stage. These results suggest PALP is oxidised to PICP which is then hydrolysed to PIC before removal from animal tissues, rather than the accepted metabolic pathway for PIC formation via PAL. Although both pathways probably exist in the metabolism of vitamin B_6 in animal tissues, PIC production with PICP as an intermediary seems to predominate except for instance in blood where there is a large build up of PAL. It is possible that the PIC content in blood could be accounted for by the gradual release of hydrolysed PICP into the blood stream rather than coming from the oxidation of PAL.

Analogues of PALP are known to stabilise enzymes[9] and it is supposed that, in these cases, the co-enzyme is not situated at the active site of the enzyme but in some way stabilises the enzyme structure at a different site. Whilst PIC itself is not known to act as a co-enzyme, it is possible that PICP could play a role in stabilising enzymes which might explain why it is retained by tissues and only released in small quantities into the blood.

Rabinowitz and Snell[10] in their study on the metabolism of vitamin B_6 failed to account for more than 45% of the administered compound in urine.

We have carried out an experiment on the metabolism of ^{14}C-POL on one of us and found as much as 20% of the total radioactivity excreted was in the form of PICP. This figure could not have been included in the balance sheet of Rabinowitz and Snell[10] and may explain some of the large discrepancy in the metabolites which remained unaccounted for in urine. Johansson et al[11] have postulated a two compartmental turnover of body pyridoxine. Administered POL would therefore be retained in the body store, a variable fraction of which would be eliminated in the urine. This would account for a large variation in the total amount of metabolites that is recovered at any given time.

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INSTITUTE OF PEDIATRIC CLINIC OF FLORENCE

DIRECTOR PROF. A. CHIEFFI

EFFECTS OF HIGH DOSAGE OF VITAMINS A - D - B_6 AND PANTOTHENIC ACID ON OSSEOUS DYNAMICS OF THE Sp^{85} IN THE YOUNG RATS.

F. Cordelli, E. Corvaglia, R. Bracci, P. A. Benedetti

The effect of certain vitamins on the osseous metabolism is still only partially known.

As far as the vitamin D is concerned it is known, that if it is administered in high dosages, the effects are very different from moderate dosages. Infact the high amounts of vitamin D have a direct effect on the bone, producing such phenomena as reabsorption, which is similar to effects caused by parathormone. This McLean defined as "calcemic effect". In such circumstances, the calcium is realized from the selection and the vitamin D produces, therefore, a pharmaco-dynamic effect quite opposite to its physiological one.

In 1933 CHIEFFI demonstrated, that while the irradiated ergosterol in small doses caused no effect on the content in bone ashes of young rats, maintained on normal diets, has an effect of a net reduction of mineral content in the bones. Such phenomenon was not observed in aged rats. In addition he demonstrated that young rats maintained on vitamin efficient diets involving different vitamin doses developed a codition of osseous decalcification and an abnormal calcium deposit in the kidneys a veritable and renal calcinosis. READ, on the other hand in 1939 confirmed that other doses of vitamin D produced clear hypercalcemia in man and as

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well as in animals, and thus impoverished the bones of such minerals. Even high dosages of vitamin A would bring about quite different modifications in bones compared to those produced by low doses which at the beginning show an accelerated increase, then rapid disappearance of epiphysial cartilages and the premature accretion of the epiphysis (Wolbach). IRVING in 1949 noticed that high dosages of vitamin A lower the osteoblastic activity. GERBER and COLL in 1954 observed children poisoned by vitamin A calcifications of cartilages, ligaments, tendors and subperiostal structures. Moreover the observation of CAFFEY are well known. He found evidence that children receiving for long periods of time high dosages of vitamin A, develop retardation of bone growth, precocious accretion of the epiphysis, structural and morphological differences between proximal and distal parts of the long bones.

Effects on bones produced by administration of other vitamins are less known. Certain observations are of interest: those regarding the effects of pantothenic acid and of pyrodoxin (NELSON and COLL, SILBELBERY, and LEVY, TESSARY) and their deficiencies which could produce calcification defects, desappearance of spongiosis, alterations of conjunction of cartilages ecc.; but we know very little as to which modifications they bring about on bone metabolism and particularly on the calcification process, if the quantity administrated is in excess.

In previous researches conducted by us in 1963, we showed some modifications induced into osseous metabolism of the Sr^{85} of A and B_6 hypovitaminosis conditions (BRACCI, BENEDETTI, CORDELLI). We maintained that completion of these investigations requires controlling the effect of Sr^{85} on osseous metabolism by administering high dosages of some vitamins D and A, pantothenic acid and pyrodoxine; we have selected these

four vitamins , because, as already stated above, all four seem to have a precise action on minerallization of the skeleton.

MATERIAL AND METHODS

The research was conducted on prepuberal rats of variable weights from 120 to 150 gr. of the Wister's race, selected block ISVT. Animals were subdivided into 5 groups:

1 st group: treated for control with 0.5 cc of physiological solution per day;

2 nd group: treated with D vitamin, hydrosoluble, at a daily dose of U. 2000 in the physiological solution, intramuscular injection for 5 days;

3 rd group: treated with B_6 vitamin at 20 mg daily dose, intramuscular injection in the physiological solution of 0.5 cc for 5 days;

4 th group: treated with A vitamin, in oil, at a daily dose U. 5000, intramuscular injection for 5 days;

5 th group: treates with pantothenic acid in the physiological solution of 1.5 cc (20 mgr), intramuscular injection for 5 days.

Every group consisted of eight elements, control animals and those treated were held in identical ambient conditions. Feeding for all consisted of weighted fodder.

During treatment a dose of Sr^{85} , carrier-free, in physiological solution was introduced to each animal by intraperitonal injection.

Beginning with with 30' from inoculation, the radioactive count on the rat's tail was performed by collimating the slow scintillator on the third and fourth caudal vertebra.

In order to obtain more precise results the equipment was prepared the following way:

a) a large lead cylindrical container, placed on the test bench horizontally; the experimental rat was placed inside of cylinder. The tail of the animal was pulled out through a small window located at one end of the container;

b) scintillation counter with a smooth sodium iodol crystal activated by thallium of 1.5" x 1.5". The scintillator is protected by a lead container and by a collimator of frustrum shape with an external opening of 3 cm in diameter at a distance of 7 cm from the crystal surface. The external opening of the collimator is located in direct contact with the rat's tail at the specific point.

c) one normal stand, for thyroidal captation;

d) one scaler with predetermined timing.

Readings in vivo were repeated at 1st, 2nd, 6th, 9ht, 12th, 24th hours, then at 2nd, 3rd, 4th, 6th, and 7th day.

At the end of the experiment, the animals were put to death by a cardiac blood-letting puncture; then the 3rd caudal vertebre was removed from the carcass.

The calcium content and the radioactivity effect were determined by the blood, separated by the centrifuge and examination of the vertebra.

DATA PROCESSING

The radioactivity relieved each time on the tail external area, detracted from the depth radioactivity is expressed in shocks per minute has been traced on logarithmic paper in a diagram; the time of the relieve is ploted on abscissas and the values of radioactivity on the ordinates.

For better evaluation of the existing differences between various groups, instead of describing the curve, based on radioactivity expressed in shocks per min. at different times for each animal, it is more expedient to proceed with the Bohr's method under similar conditions.

This method consists of determining for each group of animals the average value and to give the value of 100 for the maximum recorded activity, expressing therefore all other values in percentages of this maximum.

Along with this system a curve formed by two phases has been described: one is fast ascending and one fast descending, varying for the duration and depending on one group or other.

RESULTS

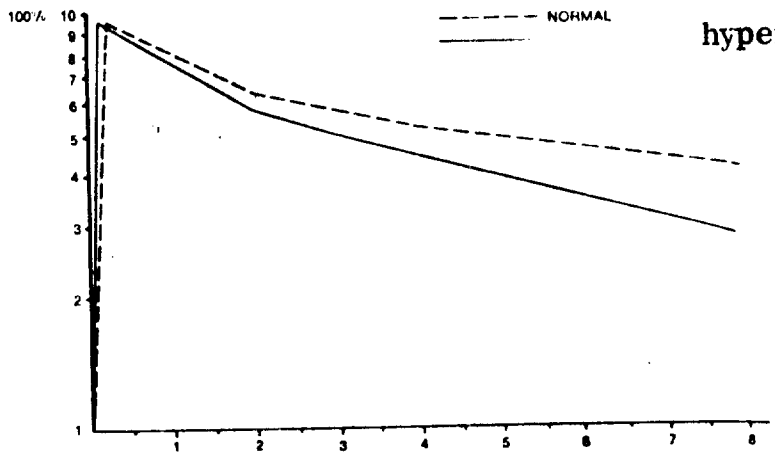
1) Control animals: behavior of the curve (Fig. 1). As shown, the first phase of the curve ascent with maximum to 6th hour with the second phase of slow dismission of the Sr^{85}.

2) Animals with high vitamin D. Behavior of the curve of the curve (Fig.1). First phase of fast ascent reaches maximum values at 2nd hour, next phase of descent for dismission of the Sr^{85} is faster than in the preceeding group.

TABLE 1

	2nd day	4th day	7th day
Control rats	65 ± 8	50 ± 7	42 ± 9
Rats with high vitamin D	59 ± 7	45 ± 5	35 ± 5

The maximum recorded radioactivity in vivo and expressed in percentages of maximum reached values.



hypervitaminosis D

FIG. 1.

The activity at 2nd, 4th and 7th, days refering to table 1 shows, that significant differences between examined groups, can be expressed and confirmed arithmetically the behavior of metabolism due to radium-strontium in bones.

3) Animals with high vitamin A. The form of curve (Fig. 2). First phase of fast ascent; reaches maximum activity in the fourth hour; next phase of descent differing little was recorded in rats of high vitamin D content.

TABLE 2

	2nd day	4th day	7th day
Control rats	65 ± 8	51 ± 7	42 ± 9
Rats with high vitamin A	54 ± 8	49 ± 5	35 ± 5

The maximum recorded radioactivity in vivo expressed in percentage of maximum reached values.

Also in this group, of Table 2, one can see the curve shows, that its numerical percentage values are always lower than those taken from control rats.

4) Animals with hypervitaminosis of the Pyrodoxine. The shape of the curve (Fig. 3). The first phase of the fast ascent reaches the maximum in the fourth hour; the next phase of dismission of the radium-strontium is characterized by an elevated slope up to the fourth day, with a definite slowing in consecutive days.

Osseous Dynamics of the Sr^{85}

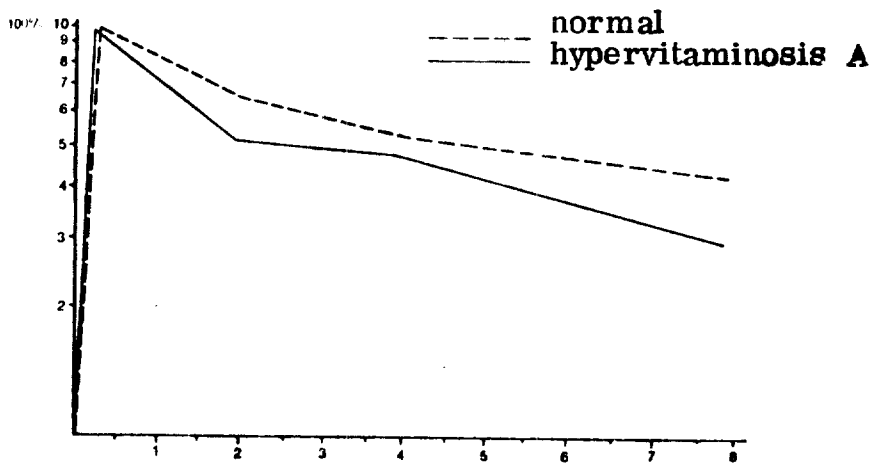


FIG. 2.

Numerical data show net differences between treated and control rats.

TABLE 3

	2nd day	4th day	7th day
Control rats	65 ± 8	51 ± 7	42 ± 9
Rats in hypervitaminosis of Pyrodoxine	61 ± 6	44 ± 3	32 ± 4

The maximum recorded radioactivity in vivo expressed in percentages of the reached values.

5) Animals with hypervitaminosis by pantothenic acid. The shape of the curve (Fig. 4); this group of animals show behavior very different from others, treated with vitamins A, D, B_6. The curve of the disapearance of the isotope from the bone, is very similar to that of animals not treated: (Table 4, Fig. 4).

TABLE 4

	2nd day	4th day	7th day
Control rats	65 ± 8	51 ± 7	42 ± 9
Rats in hypervitaminosis by pantothenic acid	63 ± 9	50 ± 6	34 ± 5

The maximum recorded radioactivity in vivo and expressed in percentages of the maximum values.

CORDELLI & COLL.

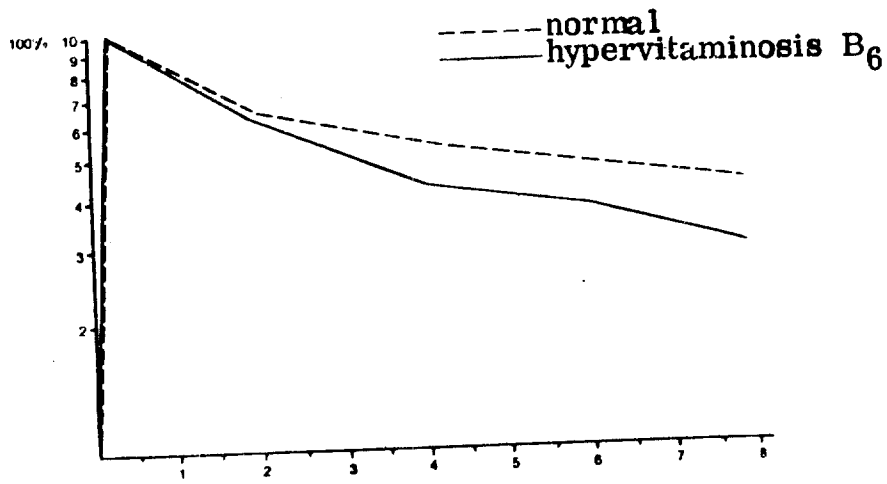


FIG. 3.

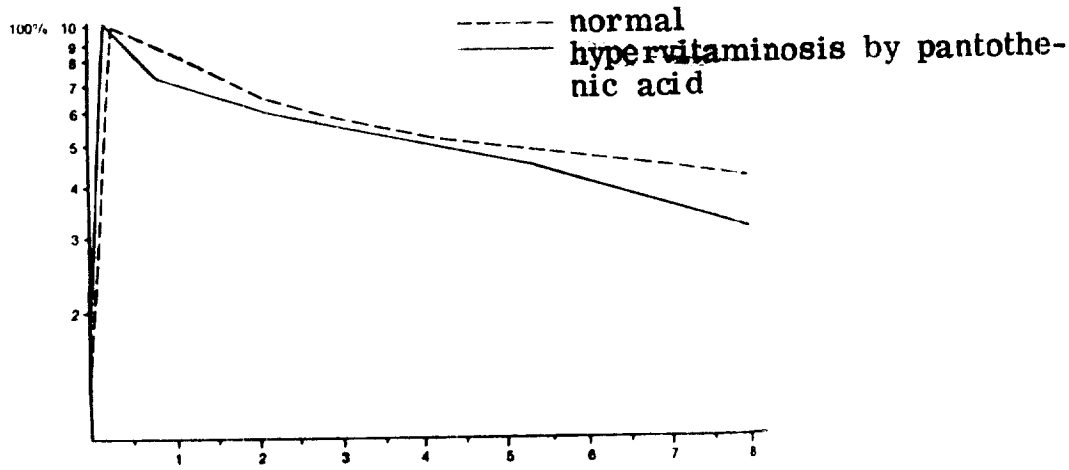


Fig. 4.

CONCLUSIONS

It should be emphasized that, in groups of treated animals with vitamin (A, D, B_6 pantothenic acid) of large doses, the more or less rapid curve modification of the strontium metabolic in the bone, characterized by a osseous captation, according to groups, was recorded, and modifications of the phase of dismission were always faster in non-treated animals. A particular emphasis is placed on modifications in animals treated with vitamin D and A (Fig. 1; Fig. 2); we were able to confirm that the literature refers to a distinct acceleration of osseous calcium metabolism, caused by both of these vitamins (BOHR, NORDIO, CARLSON).

On the other hand we are comparing data with regard to vitamin A, which we have obtained from animals, held on a vitamin A deficient diet, and we note, that the phenomenon of metabolic acceleration appears more distinct, whereas the hypovitaminosis conditions determined a definite slowing of the same metabolism.

An analogous observation can be made as far as B_6 is concerned (Fig. 3); our results show a descending curve slope faster than in the normal animal; our previous data, showed contrary evidence in a specular concealed form of the same curve. As already mentionned there is an insufficient relief in the modifications encountered in animals treated with eccessive pantothenic acid (Fig. 4). On the other hand this gave a confirmation even when it is possible, to deduct from the clinical observations, that do not show particular modificatios from the skeleton due to deficiency or overdosage of this vitamin.

In concluding, we can say that with these investigations, we were able to confirm the action of certain vitamins of B group and of vitamins A and D, which explain not only the formation and organization of the bone's matrix, but the effect also on calcification mechanism.

SUMMARY

The authors studied the turnover of the Sr^{85} in the bone of young rats treated with high dosage of vitamins A-D-B_6 and pantothenic acid. Sr^{85} disappears from the bone more rapidly in treated than in non treated rats. The rate of the disappearence presents some variations among the rats belonging to the various treated groups.

The authors compare the data from treated rats with the data previously obtained from rats in hypovitaminosis A-D-B_6 and pantothenic acid which demonstrated a slower disappearence rate of Sr^{85} comparing with normal rats.

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ISTITUTO DI CLINICA PEDIATRICA DELL'UNIVERSITÀ DI FIRENZE
DIRETTORE: PROF. A. CHIEFFI

EFFETTI DELL'IPERDOSAGGIO VITAMINICO A.D.B_6 E ACIDO PANTOTENICO SULLA DINAMICA OSSEA DELLO Sr^{85} NEL RATTO IN ACCRESCIMENTO

F. CORDELLI E. CORVAGLIA R. BRACCI P. A. BENEDETTI

L'azione esercitata da alcune vitamine sul metabolismo osseo è tuttora solo parzialmente nota.

Per quel che riguarda la vitamina D si sa che se essa è somministrata in dosaggi elevati produce effetti assai differenti da quelli determinati da dosaggi modesti. Infatti la somministrazione di grandi quantità di vitamina D ha un effetto diretto sull'osso, determinando quei fenomeni di riassorbimento analoghi a quelli prodotti dal paratormone, che MC LEAN definisce come « calcemic effect ». In tali circostanze il calcio è mobilizzato dallo scheletro e la vitamina D esercita pertanto un effetto farmaco dinamico del tutto opposto a quello fisiologico.

Nel 1933 CHIEFFI dimostrò che mentre l'ergosterina irradiata somministrata a piccole dosi non esercitava alcuna influenza sul contenuto in ceneri delle ossa di ratti giovani tenuti a dieta normale, la stessa ergosterina somministrata ad alte dosi in giovani ratti tenuti alla stessa dieta normale aveva per effetto una netta riduzione del contenuto minerale delle ossa. Tale fenomeno non si verificava in ratti in età più avanzata. Inoltre egli dimostrò che in ratti giovani tenuti a dieta carenzata si otteneva con l'uso di alte dosi di vitamina una condizione di decalcificazione ossea ed un deposito abnorme di calcio nel rene; una vera e propria condizione di calcinosi renale. READ d'altra parte confermava nel 1939 come alte dosi di vitamina D determinassero netta ipercalcemia sia nell'uomo che nell'animale ed impoverissero di tali minerali l'osso. Anche gli iperdosaggi di vitamina A possono causare a carico dell'osso modificazioni ben differenti da quelle prodotte da basse dosi che si manifestano in iniziale accelerazione dell'accrescimento, rapida scomparsa delle cartilagini epifisarie e prematura saldatura delle epifisi (Wolbach). IRVING nel 1949 ha notato che gli iperdosaggi di vitamina A deprimono l'attività osteoblastica. GERBER e Coll. nel 1954 hanno notato in bambini e giovani intossicati di vitamina A calcificazioni delle cartilagini, dei legamenti, dei tendini e delle strutture sottoperiostee. Sono ben note inoltre le osservazioni di CAFFEY il quale in bambini che avevano ricevuto per lungo tempo iperdosaggi di vitamina A ha potuto mettere in evidenza, tra l'altro, ritardo della crescita ossea, saldatura precoce delle epifisi, differenze strutturali e morfologiche tra le parti prossimali e distali delle ossa lunghe.

Molto meno noti sono gli effetti prodotti sulle ossa da altre vitamine. Interessanti sembrano alcune osservazioni relative agli effetti dell'acido pantotenico e della piridossina (NELSON e Coll., SILBELBERY e LEVY, TESSARI) la cui deficienza produrrebbe difetti di calcificazione, scomparsa della spongiosa, alterazioni delle cartilagini di coniugazione ecc.; ma poco sappiamo quali modificazioni esse

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possano produrre sul metabolismo dell'osso e in particolare sul processo di calcificazione se somministrate in quantità eccessiva.

In precedenti ricerche da noi condotte nel 1963, abbiamo dimostrato alcune modificazioni indotte sul metabolismo osseo dello Sr^{85} da condizioni di ipovitaminosi A e B_6 (Bracci, Benedetti, Cordelli). Abbiamo ritenuto opportuno completare queste indagini controllando l'effetto che sul metabolismo osseo dello Sr^{85} esercitano gli iperdosaggi di alcune vitamine, e precisamente di vitamina D e A, acido pantoterico e piridossina; abbiamo scelto queste quattro vitamine perché, come brevemente è stato accennato dianzi, tutte e quattro sembrano possedere una ben precisa azione sulla mineralizzazione dello scheletro.

Materiali e metodi.

La ricerca è stata condotta su ratti prepuberi del peso variabile da g 120 a g 150, di razza Wister, ceppo ISVT selezionato. Gli animali sono stati suddivisi in 5 gruppi:

1° gruppo: controllo trattato con cc 0,5 di soluzione fisiologica al dì;

2° gruppo: trattato con vitamina D idrosolubile, alla dose giornaliera di U. 2000, in soluzione fisiologica, per via intramuscolare per giorni 5;

3° gruppo: trattato con vitamina B_6 alla dose giornaliera di mg 20 per via intramuscolare in soluzione fisiologica cc 0,5 per giorni 5;

4° gruppo: trattato con vitamina A, in olio alla dose giornaliera di U. 5000 per via intramuscolare per giorni 5;

5° gruppo: trattato con ac. pantotenico in soluzione fisiologica cc 1,5 (mgr 20), per via intramuscolare per giorni 5.

Ciascun gruppo era costituito da otto elementi, gli animali controllo e quelli trattati sono stati tenuti in condizioni ambientali identiche. L'alimentazione era costituita per tutti da mangime bilanciato.

Al termine del trattamento a ciascun animale è stata introdotta per via intraperitoneale una dose di Sr^{85}, carier free, in soluzione fisiologica cc 0,3.

A cominciare da 30' dall'inoculazione, si è proceduto al conteggio della radioattività sulla coda del ratto, collimando lo scintillatore piano sulla terza, e quarta vertebra caudale.

Allo scopo di ottenere i risultati più precisi possibili l'apparecchiatura era stata così preparata:

a) un grosso contenitore cilindrico di piombo, posto orizzontalmente sul tavolo di lavoro, entro il quale veniva posto il ratto in esame. La coda dell'animale veniva fatta fuoriuscire da una piccola finestra posta ad una estremità del contenitore;

b) un contatore a scintillazione, con cristallo piano allo ioduro di sodio, attivato al tallio, di 1,5" × 1,5". Lo scintillatore era protetto da una contenitore in piombo e da un collimatore tronco-conico con foro esterno del diametro di cm 3 distante cm 7 dalla superficie del cristallo. Il foro esterno del collimatore veniva posto a diretto contatto con la coda del ratto, nel punto stabilito;

c) uno stativo normale, da captazione tiroidea;

d) uno scaler, con tempo prefissato.

I rilevamenti in vivo sono stati ripetuti alla 1ª, 2ª, 6ª, 9ª, 12ª, 24ª, ora e poi alla 2ª, 3ª, 4ª, 6ª, 7ª, giornata.

Al termine di questa esperienza gli animali sono stati sacrificati per puntura-salasso cardiaco; è stata quindi prelevata dalla carcassa la 3ª vertebra caudale.

Sul sangue, separato per centrifugazione e sulle vertebre sono state praticate le determinazioni del contenuto in calcio e della radioattività presente.

Elaborazione dei dati.

La radioattività rilevata all'esterno sulla coda, a ciascun tempo, detratta dalla radioattività di fondo ed espressa in colpi per minuto (cpm) è stata riportata su carta similogaritmica in un diagramma avente sulle ascisse i tempi di rilievo e sulle ordinate i valori di radioattività.

Allo scopo però di meglio valutare le differenze esistenti tra i vari gruppi, piuttosto che descrivere per ciascun animale una curva in base alla radioattività espressa in cpm ai vari tempi, si è ritenuto opportuno procedere secondo il metodo usato da Bohr in condizioni analoghe.

Esso consiste nel determinare per ogni gruppo di animali il valore medio e nel dare il valore 100 alla attività massima registrata, esprimendo quindi tutti gli altri valori in percentuale di quello massimo.

Con tale sistema è stata descritta una curva formata da due fasi, una di rapida ascesa ed una di discesa rapida, variante, per la durata e pendenza tra un gruppo o l'altro.

Risultati

1) Animali controllo: comportamento della curva (Fig. 1). Si nota una prima fase di ascesa con massimo alla 6ª ora, con una seconda fase di lenta dismissione dello Sr^{85}.

2) Animali in ipervitaminosi D. Comportamento della curva (Fig. 1). Prima fase di rapida ascesa, raggiungente i valori massimi alla 2ª ora, successiva fase di discesa per dismissione dello Sr^{85} più rapida che nel gruppo precedente.

TABELLA 1.

	2° gg	4° gg	7° gg
Ratti controllo	65 ± 8	51 ± 7	42 ± 9
Ratti in pervitaminosi D	59 ± 7	45 ± 5	35 ± 5

Radioattività massima registrata in vivo ed espressa in percentuale del valore massimo raggiunto.

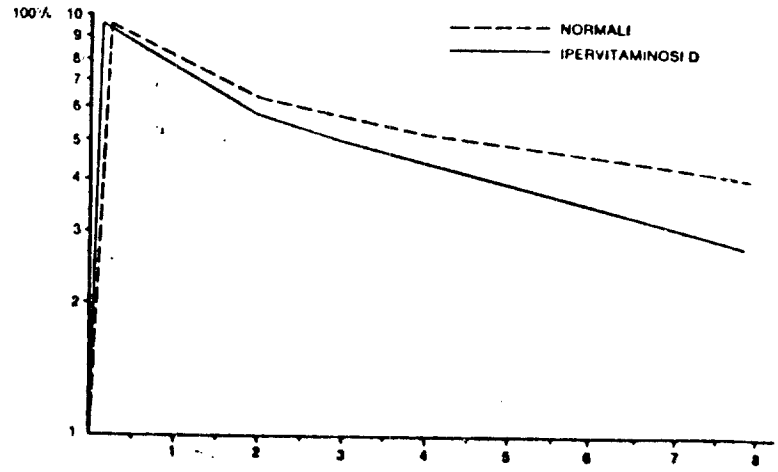


Fig. 1.

Le attività al 2°, 4°, e 7°, giorno riferito nella Tabella 1, ci sembra possano esprimere significativamente le differenze fra i due gruppi esaminati, e confermano in maniera aritmetica il comportamento del metabolismo del radiostronzio nell'osso.

3) Animali in ipervitaminosi A. Comportamento della curva (Fig. 2). Prima fase di rapida ascesa; raggiungimento della attività massima alla quarta ora; successiva fase di discesa di poco differente da quella registrata nei ratti in ipervitaminosi D.

Tabella 2.

	2° gg	4° gg	7° gg
Ratti controllo	65 ± 8	51 ± 7	42 ± 9
Ratti in ipervitaminosi A	54 ± 8	49 ± 5	35 ± 5

Radioattività massima registrata in vivo ed espressa in percentuale del valore massimo raggiunto.

Anche in questo gruppo, dalla Tabella 2 si rileva come la tendenza della curva, abbia la sua espressione in valori percentuali numerici, sempre inferiori a quelli rilevati nei ratti controllo.

4) Animali in ipervitaminosi da Piridossina. Comportamento della curva (Fig. 3). La prima fase di rapida ascesa raggiunge il massimo alla quarta ora; la successiva fase di dismissione del radiostronzio è caratterizzata da una pendenza elevata fino alla quarta giornata, con netto rallentamento nei giorni successivi.

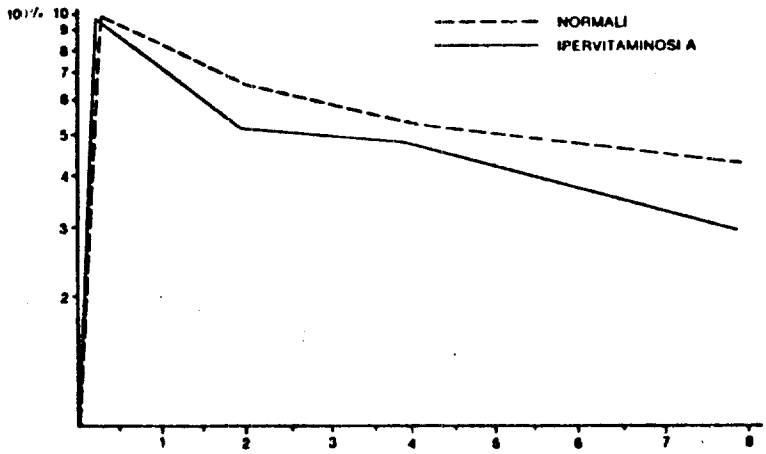


Fig. 2.

I dati numerici denotano che le differenze tra ratti trattati e ratti controllo sono molto nette.

Tabella 3.

	2° gg	4° gg	7° gg
Ratti controllo	65 ± 8	51 ± 7	42 ± 9
Ratti in ipervitaminosi da piridossina	61 ± 6	44 ± 3	32 ± 4

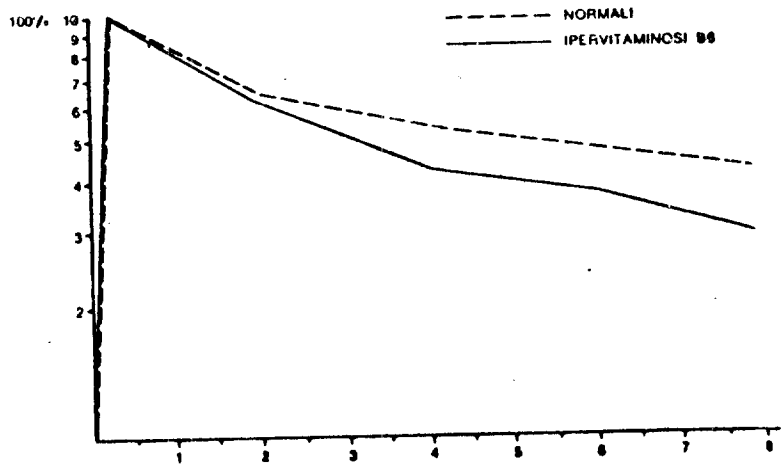
Radioattività massima registrata in vivo ed espressa in percentuale del valore raggiunto.

5) Animali in ipervitaminosi da ac. pantotenico. Comportamento della curva (Fig. 4): questo gruppo di animali ha mostrato un comportamento nettamente diverso da quello degli altri, trattati con vitamina A, D, B_6. La curva di scomparsa dell'isotopo dall'osso, è molto simile a quella degli animali non trattati: (Tabella 4; Fig. 4).

Tabella 4.

	2° gg	4° gg	7° gg
Ratti controllo	65 ± 8	51 ± 7	42 ± 9
Ratti in ipervitaminosi da ac. pantotenico	63 ± 9	50 ± 6	34 ± 5

Radioattività massima registrata in vivo ed espressa in percentuale del valore massimo raggiunto.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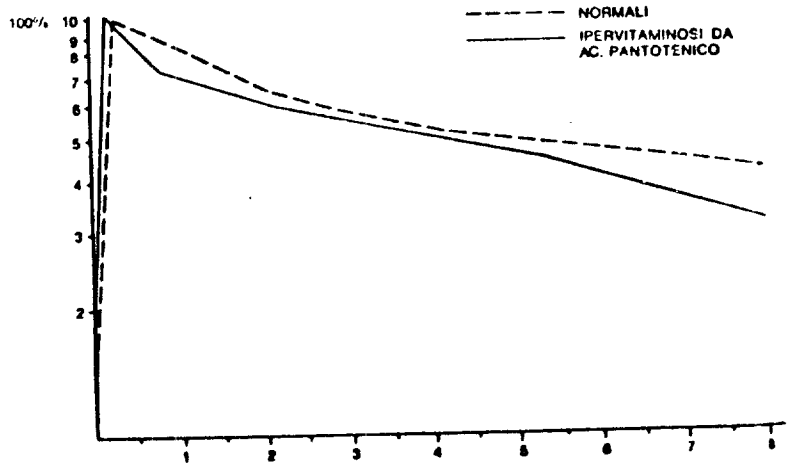
NORMALI
IPERVITAMINOSI B6

Fig. 3.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IPERVITAMINOSI DA
AC. PANTOTENICO

Fig. 4.

CONCLUSIONI.

È da sottolineare come in tutti i gruppi di animali trattati con larghe dosi di vitamine (A, D, B_6, ac. pantotenico), si è registrata una modificazione dell'andamento della curva metabolica dello stronzio nell'osso, caratterizzata da una captazione ossea, più o meno rapida a seconda dei gruppi, e da una fase di dismissione, sempre più veloce di quella riscontrata negli animali non trattati. Di particolare rilievo le modificazioni rilevate in tal senso negli animali trattati con vitamina D e vitamina A (Fig. 1; Fig. 2); in essi abbiamo potuto confermare quel che la letteratura riferisce circa uno spiccato acceleramento del metabolismo osseo del calcio, che ambedue queste vitamine inducono (BOHR, NORDIO, CARLSON).

D'altra parte, se confrontiamo i dati che riguardano la vitamina A con quelli da noi ottenuti in animali tenuti a dieta carente di tale vitamina, notiamo come il fenomeno dell'accellerazione metabolica appaia ancora più netto, laddove le condizioni di ipovitaminosi avevano determinato un netto rallentamento dello stesso metabolismo.

Una analoga osservazione può essere fatta per quel che riguarda la vitamina B_6 (Fig. 3); i nostri risultati fanno rilevare una curva a discesa più rapida che nell'individuo normale; i nostri dati precedenti mettevano invece in evidenza uno speculare appiattimento della stessa curva. Di scarso rilievo, come abbiamo già detto, sono le modificazioni riscontrate negli animali trattati con ac. pantotenico in eccesso (Fig. 4). D'altra parte, questo dato conferma anche quanto è possibile rilevare dall'osservazione clinica, che non mostra particolari modificazioni dello scheletro per deficienza o sovraccarico di questa vitamina.

Concludendo possiamo dire che con queste nostre indagini abbiamo potuto confermare come l'azione di alcune vitamine del gruppo B e della vitamina A e della vitamina D, si esplichino non solo sulla formazione e sull'organizzazione della matrice organica dell'osso, ma agiscano anche sul meccanismo della calcificazione.

RIASSUNTO

Effetti dell'Iperdosaggio vitaminico A-D-B_6 ed acido pantotenico sulla dinamica ossea dello Sr^{85} nel ratto in accrescimento. – F. CORDELLI, E. CORVAGLIA, R. BRACCI, P. A. BENEDETTI.

Gli AA. hanno studiato il ritmo di rinnovamento dello Sr^{85} in ratti trattati con alte dosi di vitamina A-D-B_6 ed cido pantotenico. È stata riscontrata una alterazione costante della velocità di dismissione ossea dello Sr^{85} che nei diversi gruppi di animali trattati con le varie vitamine assume un andamento generalmente opposto a quello osservato in precedenti esperienze in animali sottoposti a diete carenti delle stesse vitamine. Si osserva in particolare una curva di dismissione più o meno rapida a seconda dei gruppi ma sempre più veloce di quella riscontrata negli animali non trattati.

SUMMARY

Effects of the high dosage of vitamins A-D-B_6 and pantothenic acid on the metabolism of Sr^{85} in the young rats. – F. CORDELLI, E. CORVAGLIA, R. BRACCI, P. A. BENEDETTI.

The authors studied the turnover of the Sr^{85} in the bone of young rats treated with high dosage of vitamins A-D-B_6 and pantothenic acid. Sr^{85} disappears from the bone more rapidly in treated than in not treated rats. The rate of the disappearence presents some variations among the rats belonging to the various treatep groups.

The authors compare the data from treated rats with the data previously from rats in hypovitaminosis A-D-B_6 and pantothenic acid which demonstrated a slower disappearence rate of Sr^{85} comparing with normal rats.

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Bib No. 539

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Metabolism of Tritium-Labeled Pyridoxine in Rats.* (27166)

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Earlier investigations on the metabolism of pyridoxine in animals have involved the study of degradation products by chemical methods and microbiological assay procedures (1-5). The present knowledge of the metabolism of Vit. B_6 (pyridoxine, pyridoxamine, and pyridoxal) in man and other animals, obtained by using these procedures, has been summarized by Snell(6). Low recoveries of ingested or injected pyridoxine in previous metabolic studies in animals have led to the speculation that a portion of the "missing" pyridoxine is excreted in conjugated forms as yet unidentified. An alternate hypothesis for the low recoveries would be that the ingested drug was stored in the tissues. This latter explanation was discounted by Rabinowitz and Snell(5) on the basis that no great storage of the vitamin would be expected in normal animals, and on the fact that excretion levels rapidly returned to base levels in human subjects. The present study of the turnover times and urinary and fecal excretion of H^3-pyridoxine was undertaken to investigate these hypotheses further.

Methods. Tritium- (H^3) labeled pyridoxine hydrochloride was obtained by exchange of pyridoxine borate in tritium water over platinum catalyst(7). Chemical and radioactive purity was established by paper chromatography. Specific activity of the H^3-pyridoxine hydrochloride was 308 μc/mg. The solution used for injection contained 2 mg (616 μc) of labeled and 18 mg of unlabeled pyridoxine hydrochloride/ml. All injections were intravenous *via* the jugular sinus route. The rats weighed between 250 and 350 g. Rat A received 184.8 μc H^3-pyridoxine · HCl. Rats B and C received 369.6 μc, while rats D through K received 616 μc H^3-pyridoxine · HCl. Following injection of the drug, the rats were placed individually in standard metabolism cages.

Rats A, B, and C had blood and urine samples taken at various time intervals post injection. Urine and fecal samples were collected from rats D to K for detailed urinary and fecal excretion and chromatographic studies.

Blood and urine specimens were measured for tritium using the liquid scintillation-dioxane system as described previously by Richmond *et al.*(8). The feces were homogenized in distilled water, decolorized with charcoal, and counted as above. The counting efficiency of each sample was determined with the use of an internal standard. All calculations were in absolute counting rates (dpm) and results expressed as % of injected dose.

All urine specimens obtained up to 5½ hours after injection were chromatographed by the descending method(9). One-tenth ml of urine was placed directly on No. 1 Whatman filter paper strips. In all instances, this was at least 5×10^6 dpm of H^3 activity. Eighty per cent propanol was the solvent system used. It was necessary to expose the chromatogram strips to X-ray film for at least 4 weeks to get satisfactory exposures on the film.

Results. Concentrations of H^3 activity in blood as a function of time after administration of labeled pyridoxine are shown in Fig. 1. The log of the % injected dose is plotted

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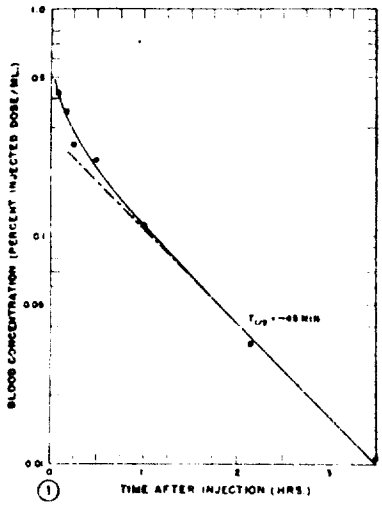

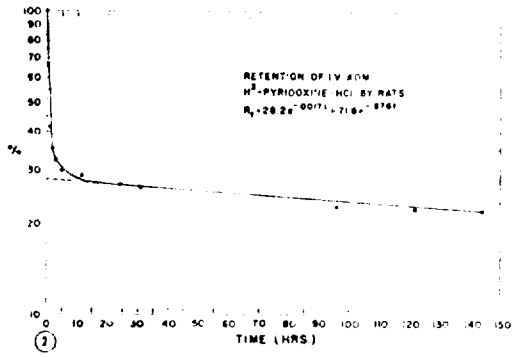

FIG. 1. Tritium activity in blood following intrav. administration of H^3-pyridoxine hydrochloride in rats.

FIG. 2. Retention of H^3-pyridoxine hydrochloride by rats following intrav. administration.

against time in hours. Excluding the early component of the blood clearance curve (*i.e.*, the first 15 minutes after injection), the turnover halftime of the activity in the blood was approximately 45 minutes during the first 3 hours after drug injection. Activity in the red blood cells was less than 1% of total activity in the blood.

The data in Tables I and II show accumulated urinary and fecal excretion, respectively. Seventy-five to 80% of the activity was excreted in the urine the first 24 hours. Average fecal excretion was negligible, being less than 0.03% in 96 hours.

Fig. 2 shows average retention of pyridoxine as a function of time after injection, assuming excretion was essentially all *via* the urine. The retention curve was fit by the following 2-component rate equation:

$$R_t = 28.2\ e^{-0.0017\ t} + 71.8\ e^{-0.876\ t}$$

in which R_t is retention in % of the injected dose, and t is time after injection in hours. Approximately 28% was retained with a half-time of 17 days and 72% with a half-time of only 47 minutes, corresponding to the blood clearance half-time of 45 minutes shown in Fig. 1.

One predominant band with R_f value of 0.65 ± 0.03 was present in all urine samples chromatographed. This R_f value corresponded to that of unchanged pyridoxine hydrochloride. A very faint trace of only one other band was present on all strips, R_f value of this unidentified band being 0.53 ± 0.03. The only degradation product of Vit. B_6 so far found in animals is 4-pyridoxic acid, a product which may be represented by this band.

Discussion. The urinary excretion results are in agreement with an earlier report(1) that 50-70% of pyridoxine was excreted essentially unchanged by rats within 24 hours, regardless of route of administration. Only one degradation product was found in very

TABLE I. Cumulative Urinary Excretion of Tritium Activity Following Intravenous Administration of H^3-Pyridoxine · HCl in Rats.

Time after inj. (hr)	Rat No. & cumulative excretion (%)				
	D	E	F	G	Avg
1.5	64.3	54.5	58.0	57.2	58.5
2.5	69.3	62.4	65.1	62.3	64.8
3.5	—	68.1	—	67.5	67.8
4.5	—	—	68.4	—	—
5.5	71.9	—	—	—	—
12	73.3	73.5	69.7	69.1	71.4
24	75.6	75.7	71.5	70.5	73.3
31	76.3	76.2	71.7	70.8	73.7
96	79.8	78.1	78.3	73.8	77.5
122	80.2	78.1	78.4	73.9	77.7
144	80.4	78.2	78.4	75.0	78.0

TABLE II. Cumulative Fecal Excretion of Tritium Activity Following Intravenous Administration of H^3-Pyridoxine · HCl in Rats.

Time after inj. (hr)	Rat No. & cumulative excretion (%)				
	H	I	J	K	Avg
12	<.001	<.001	—	<.001	<.001
24	.011	.020	.018	.008	.014
48	.016	.022	.023	.019	.020
72	.019	.027	.025	.022	.024
96	.028	.027	.026	.024	.026

small quantities. This was so for at least 5 hours after injection of the labeled material.

The majority of the pyridoxine (about 70%) was excreted unchanged with a half-time of about 45 minutes. There may be 2 reasons for rapid excretion of the unchanged drug: *in vivo* substrate concentrations of pyridoxine exceed the levels in which the tissue enzyme systems responsible for its oxidation can operate, and it may be a reflection of the inability of tissue fixation or conjugation. Retention of about 28% of the label with a half-time of 17 days may represent the portion which is fixed and stored in the tissue. It is possible that previous colorimetric and microbiological assay methods are not quantitatively refined to detect this slow turnover rate.

Only one degraded metabolite of pyridoxine could be detected in small amounts in the urine excreted within 5 hours after injection. At later times, the label was so dilute it was difficult to detect the tritium beta activity (18 kv).

It would appear from the present study that pyridoxine is excreted primarily as unchanged drug in the rat, and in small amounts as 4-pyridoxic acid. Also, the very slow excretion half-time of 17 days of about 28% of the drug would tend to support the hypothesis that the "missing" pyridoxine, referred to by Snell(6) as possibly being excreted in an unidentified conjugated form, may be stored in the tissue and excreted with a rather long half-time.

Summary. Urine, blood, and fecal samples from rats injected intravenously with tritium-labeled pyridoxine hydrochloride were collected at various time intervals post injection for excretion and chromatographic study. Approximately 70% of the administered label was excreted in the urine in 5 hours. Fecal excretion was less than 0.03% in 96 hours post injection. There were 2 rates of excretion: the initial rapid rate with a half-time of about 45 minutes, followed by a slower rate with a half-time of 17 days. Chromatographic studies of urine showed that the pyridoxine was excreted primarily unchanged with a small amount of only one metabolite, most likely 4-pyridoxic acid.

The authors are grateful to Dr. John E. Furchner, who assisted in mathematical analysis of the data.

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Studies on the Distribution and Elimination of [$^3H_8$] Pyridoxine in Mice

By

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Abstract

DAHLKVIST, G., S. LINDSTEDT and H.-G. TISELIUS. *Studies on the distribution and elimination of [$^3H_8$] pyridoxine in mice.* Acta physiol. scand. 1969. *75.* 427—432.

[$^3H_8$] Pyridoxine has been administered by i.v. injection to mice, and the concentration of isotope has been determined in different tissues up to 30 days after the administration. An initial high uptake was found in all tissues except brain. The concentration decreased rapidly during the first day after administration and then at a slow rate. The decrease of isotope concentration was more rapid in liver than in muscle or carcass. The elimination of the vitamin from the body reservoir was estimated to be 7.9 per cent per day from day 1 to 30 compared to 6.5 per cent per day as calculated from the rate of excretion of isotope in the urine. One week after the administration of the isotope the concentration was approximately the same in heart, skeletal muscle, brain and kidney, and 20 % higher in liver. Between day 1 and 30 after the administration [$^3H_8$] pyridoxine 75 % of the recovered isotope was found in carcass, 10—15 % in liver and the remainder in other tissues.

Few studies have appeared concerning the metabolism of vitamin B_6. Cox *et al.* in 1966 reported that 70 % of an injected amount of pyridoxine was rapidly eliminated from rats. Johansson and co-workers could follow the excretion of labeled metabolites for more than 3 weeks after the administration of a small amount of labeled pyridoxine to rats (Johansson *et al.* 1966 a) and humans (Johansson *et al.* 1966 b) and they concluded that the rate of elimination of the vitamin from the body pool was of the order of 1—5 % per day. In order to get further information on the fate of administered pyridoxine we have now determined the distribution of isotope in various tissues at different times after an i.v. injection of [$^3H_8$] pyridoxine.

Material and methods

Labeled pyridoxine

[$^3H_8$] Pyridoxine which had been labeled by exposure to tritium gas was obtained from the Radiochemical Centre, Amersham, Bucks., England. It had a specific radioactivity of about [illegible] C per mg and gave one single isotope-containing peak when chromatographed on a column (diameter 0.9 cm; height 40 cm) of Dowex 50-X8 (200—400 mesh) in formate form.

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Administration of labeled pyridoxine and preparation of tissues

Mice of the N.M.R.I.—strain weighing about 20 g were kept in metabolism cages on a commercial mouse diet (Harald Forss, Stockholm, Sweden). They were injected in the tail vein with 200 μl of a solution of 22 μg of [^{3}H$_8$] pyridoxine in water. After 2, 15 and 120 min, and after 1, 7 and 30 days two mice were killed each time by cutting off their heads. As much blood as possible was allowed to escape and the organs were then dissected out of the animal and rinsed in distilled water. They were blotted dry with a filter paper and weighed. The intestines were rinsed with water. A small piece of the gastrocnemius muscle was cut out and used as an example of skeletal muscle. Each organ was cut into small pieces which were weighed into small glass vials. These were placed in a desiccator over P_2O_5. The skin was removed from the carcass, which was weighed and then homogenized in a Vir Tis 23 homogenizer (The Vir Tis Co. Inc. Gardiner. New York, N.Y.) in about 50 ml of water. The homogenate was then freeze-dried to a fluffy powder.

Isotope determination

Weighed pieces of the different dried tissues (30—50 mg) or corresponding amounts of freeze-dried carcass were placed in a basket of platinum net and combusted in an oxygen atmosphere (Kelly *et al.* 1961). The water of combustion was pumped into a spiral glass-trap which was cooled in liquid nitrogen. The pumping rate was adjusted so that the pressure remained below 2 mm of mercury in the trap. With a mercury manometer connected to the system it could be ascertained that all vapor had been completely collected. The condensed carbon dioxide was allowed to escape by placing the trap in a dry ice-acetone bath, and the trap rinsed out with 8 ml portions of the fluid used for scintillation counting. This was of the following composition: PPO (2,5-diphenyloxazol) 10 g, dimethyl POPOP (1,4-bis-2-(4-methyl-5-phenyloxazylol) benzene) 0,3 g, toluene 1,000 ml, and methyl cellosolve 600 ml. The samples were counted in a liquid scintillation spectrometer (Packard Instrument Co. La Grange Ill.) to give a statistical error of less than 5 per cent. The counts for each sample were corrected for quenching by the addition of an internal standard of tritium-labeled pyridoxine in water. For each tissue and animal triplicate samples were carried through the procedure. For isotope determination in urine 0.3 ml was added to 16 ml of the counting fluid.

Results

Evaluation of the method for isotope determination

To test the completeness of combustion and the recovery of water in the spiral trap 50 to 200 μl volumes of a standard solution of pyridoxine containing 2,000 cpm per ml were either directly counted in the liquid scintillation counter or added to 50 mg of a freeze-dried homogenate of mouse carcass, which was then dried and carried through the combustion procedure. As seen in Fig. 1, a good agreement was obtained between the two methods for isotope assay.

Retention of isotope

Fig. 2 shows the per cent of the injected amount of isotope which remained in the body at different times after the administration as calculated (i) from the actual determinations in the different tissues and (ii) as the difference between the administered amount of isotope and the isotope recovered in the urine in a series of 4 mice. There was a rapid initial loss of isotope from the body during the first day after injection which was followed by a phase with a slow rate of elimination.

The isotope content per mg of tissue in the different organs of the mouse at varying times after the administration of [^{3}H$_8$] pyridoxine is shown in Fig. 3. An initially high concentration fell off rapidly within the first day after the injection, whereafter the isotope concentration decreased slowly. In the brain the initial high uptake of isotope was not seen and the maximal isotope concentration was found 1 day after the administration.

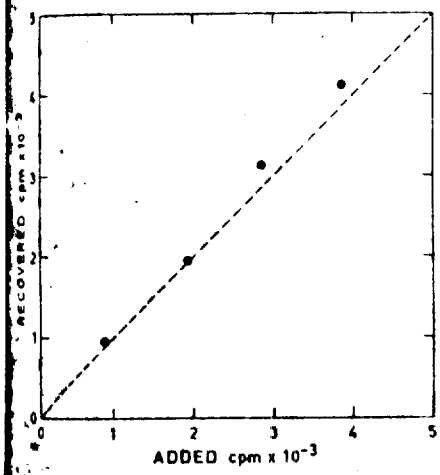

Fig. 1

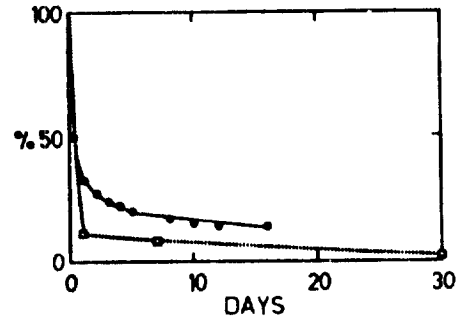

Fig. 2

Fig. 1. Recovery of isotope in the combustion method. Different amounts of [^{3}H8] pyridoxine in water solution were either counted directly in a liquid scintillation spectrometer *(abscissa)* or added to 50 mg of freeze-dried tissue. After combustion the isotope in the water was determined by the same method *(ordinate)*.

Fig. 2. Per cent of isotope remaining in the body at different times after the administration of [^{3}H8] pyridoxine. The solid line has been calculated from the excretion of isotope in the urine of a series of 4 mice, and the dotted line from measurements of isotope in the tissues.

Distribution of isotope between different tissues

A comparison of the concentrations of isotope in the different tissues 1 and 7 days after the administration is shown in Fig. 4. At both times the concentration was highest in the liver followed by heart and skeletal muscle. One day after injection a high concentration was also found in the kidney. After 1 week the concentration of isotope per mg of tissue was the same in heart and skeletal muscle, brain and kidney and lower in lung, intestine and spleen. On an absolute basis carcass and liver accounted for 80—90 per cent of the isotope recovered. Initially, equal amounts of isotope were found in liver and carcass but the relative amount in the carcass then increased and between day 7 and 30 after the injection about 75 % of the recovered isotope was located in the carcass, 10 to 15 % in the liver, and the remainder in other tissues as shown in Fig. 5 and 6.

Discussion

Previously, studies on the in vivo turnover of vitamin B_6 have been performed in rats (Cox *et al.* 1966, Johansson *et al.* 1966 a), monkeys (Greenberg and Peng 1965) and humans (Johansson *et al.* 1966 b, Gentz *et al.* 1967). Isotope-retention curves have been calculated from the excretion of isotope in the urine after the administration of tritium- or ^{14}C-labeled pyridoxine and used in a metabolic model to

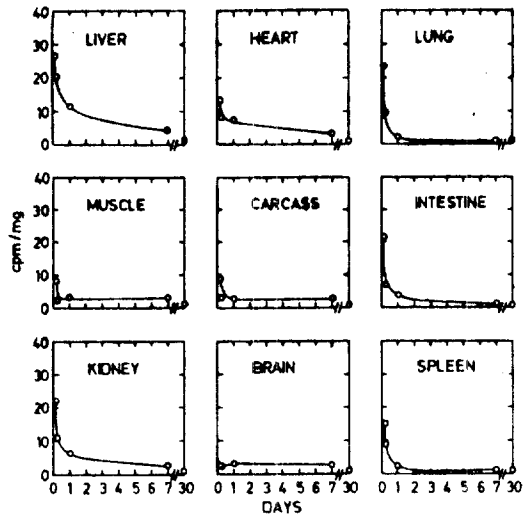

Fig. 3. Variation with time of isotope concentration in different tissues of mice after the injection of [^{3}H] pyridoxine.

estimate the rate of elimination of vitamin B_6 from the body (Johansson *et al.* 1966 a, b, Gentz *et al.* 1967). Necessarily, conclusions drawn from experiments of this type are indirect and have to be based on assumptions on the fate of the injected pyridoxine in the body. They should therefore be supported by direct measurements in the tissues. We have now chosen mice as experimental animals to make it easy to determine the isotope in organs and tissues. Chromatographic separations have established that following the administration of [^{3}H$_5$] pyridoxine, the isotope which is recovered in perchloric-acid extracts of tissues is distributed between the six different forms of vitamin B_6, i.e. pyridoxine, pyridoxal, pyridoxamine and their respective phosphorylated forms (Dahlkvist *et al.* 1967). A measure of the total vitamin derived from the injected pyridoxine was therefore obtained by direct combustion of the respective tissue, losses by extraction and purification procedures were avoided and it was possible to follow the disappearance of labeled derivatives of vitamin B_6 over a long period of time.

The course of the excretion of isotope in the urine of mice after the administration

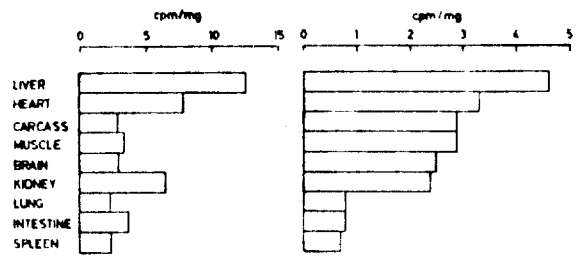

Fig. 4. Isotope concentration in different tissues of mice at different times after the injection of [^{3}H$_5$] pyridoxine at 1 day *(left)* and 7 days *(right)* after the injection.

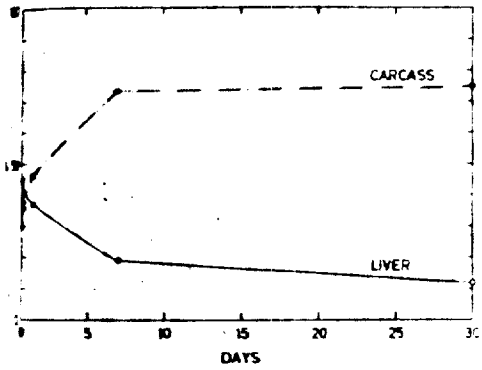

Fig. 5

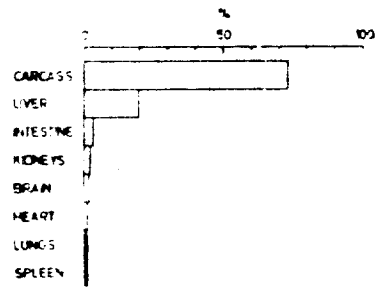

Fig. 6

Fig. 5. Per cent of total amount of recovered isotope in liver and carcass 1 to 30 days after the injection of [^{3}H$_8$] pyridoxine.

Fig. 6. Per cent of total amount of recovered isotope in different organs of mice 7 days after the injection of [^{3}H$_8$] pyridoxine.

labeled pyridoxine resembled that found in rats and humans, i.e. an intial rapid excretion followed by a slow elimination (Fig. 2). The slope of the second phase of the isotope-retention curve calculated from excretion data was 0.065 day^{-1}, i.e. slightly higher than found previously in rats, but in accordance with the generally high metabolic rate in mice. The loss of isotope from the body during the first 2 days after administration was high. Previous studies in rats have clearly demonstrated that the magnitude of this initial excretion is dependent on the amount of pyridoxine injected; in this series 2 μg by the intravenous route. The isotope-retention curve obtained by subtracting the excreted amount of isotope from the administered amount should be compared with that drawn from results of direct measurements of isotope remaining in the body at different times after injection of [^{3}H$_8$] pyridoxine. The two curves run almost parallel; the slope of the second phase of the curve drawn from direct measurements is 0.079 day^{-1}. After 1 week about 10 % of the injected amount of isotope was recovered in the body by direct measurements, but 40 % calculated to remain on the basis of excretion values. It is, however, technically difficult to obtain quantitative collection of mouse urine and even a small absolute loss during the first collection period would tend to displace the isotope-retention curve upwards, whereas incomplete recovery of isotope from the whole animal would have the opposite effect.

The variation of isotope concentration with time was similar in the different tissues. All, with the exception of brain, showed initially a high concentration which rapidly decreased and then declined more slowly between day 1 and 30. The absence of the initial high uptake in brain may possible be explained by a low permeability of the blood-brain barrier for the injected pyridoxine. From Fig. 3 it is apparent that there were some variations in the rate of elimination from the different

tissues. Thus, the loss of isotope from liver was faster than from muscle or carcass. An approximate estimate of the slopes of the elimination curves for carcass and liver gave the values 0.053 day^{-1} and 0.087 day^{-1}, which should be compared with the above mentioned figure of 0.079 day^{-1} for the whole animal. The slope of this part of the isotope-retention curve may be taken as an approximate measure of the rate of elimination of the vitamin from the storage compartment of a two-compartment system (Johansson *et al.* 1966 a, b).

The highest initial concentration was found in the liver, which after 15 minutes contained 40 % of the total recovered isotope. Between day 5 and 30 about 75 % of the recovered activity was located in the carcass, 10—15 % in the liver and the remainder in other tissues. It is of interest in this connection that Krebs and Fischer (1964) have estimated that over 50 % of the total amount of vitamin B_6 in the body occurs as pyridoxal-5'-phosphate bound to phosphorylase. In a study on the distribution of vitamin B_6 in mice Lyon *et al.* (1962) have reported concentrations of vitamin B_6 of about 400 μg per g of nitrogen in liver and 150 to 200 μg per g of nitrogen in muscle in two strains of white mice in agreement with the higher concentration of isotope in liver observed in this study.

The present study has thus verified that part of an administered amount of pyridoxine is retained for a long period of time in the body and is eliminated with a half-life of about 10 days. It has also shown that the concentration in the tissues reaches approximately the same value after an initial period of equilibration. The rate at which the administered pyridoxine is converted to the active forms of the vitamin in the different tissues remains to be studied.

This work has been supported by grants from the Swedish Medical Research Council (project 13X-585) and the Swedish Nutrition Foundation.

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28 (5): 1030-1032; 1952.

Ipardosaggio di riboflavina o di piridossina o di acido pantotenico e sindrome generale di adattamento nel ratto albino

Hyperdosage of Riboflavin, of Pyridoxine, or of Pantothenic Acid and the General Syndrome of Adaptation in Albino Rat

by

G. De Bastiani and P. Zatti

(From the Institute of Human Physiology of the University of Padua)
(Padua Section - May 20, 1952 meeting)

It has been observed (De Bastiani and Zatti) that hyperdosage of vitamin B_1[1] or of the B complex vitamins[2] provokes in rat a general syndrome of adaptation that arises (Dean and collab.[3], Skelton[4]) also in the course of the acute deficiency of these vitamins. Since, on the other hand, such a syndrome develops during the lack of pantothenic acid, but not during that of riboflavin or of pyridoxine (Dean and collab.[3,5]), we have extended our researches to the effects of the hyperdosage of the vitamin B_2, B_6, or of pantothenic acid.

The research, carried out with the modalities indicated in the

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preceding articles[1,2], was made on white male rats, 70 g. in weight, divided into 4 groups of 14 animals, which were subjected to a daily intramuscular injection of 10 mg. of riboflavin, 50 mg. of pyridoxine, or 50 mg. of pantothenic acid, respectively. One group of animals was held for control.

The experiment lasted a total of 28 days, two rats of each group having been sacrificed every 4 days.

We assumed as indices of the activity of the adrenal glands their weight and histological picture, the condition of the thymus, and the variations of the leukocyte formula.

Experiment results. 1) Rats treated with vitamin B_2. The relative weight of the adrenal glands of these animals underwent no greater variations than those of the controls. The relative weight of the thymus, on the contrary, intensely diminished from the first, then it increased moderately, and finally it was reduced again.

At histological examination the adrenal glands show no marked differences from the controls, with the exception of a moderate increase of the besprinkling of the gland especially in the first days of treatment and of a diminution, although rather moderate, of the sudanophil lipids in the fasciculated portion in preparations made after 4 and 8 days of experiment.

The thymus shows the usual structural changes testifying to progressive atrophy of the gland (diffuse nuclear pyknosis in the cortical part and increase of the number of Hassall's corpuscles in the reticulum).

The leukocyte formula was never changed.

2) Rats treated with vitamin B_6. In these animals the weight of the adrenal glands is moderately higher than that of the controls in the first half of the treatment and within the limits of the norm in the second half.

The relative weight of the thymus progressively diminishes in the first 15 days of observdion, then increases, although remaining below normal levels.

Histological preparations of the adrenal glands show in rats sacrificed after 8,12, and 16 days of experimentation a moderate hypertrophy of the fasciculated portion which, in its more external part, has a spongy appearance through the presence of numerous intracellular vacuoles and through an increase of the spaces between the cellular trabeculae in which numerous red corpuscles are frequently found. The sudanophil lipid granules are reduced in the first 12 days of therapy.

The thymus shows the common signs of atrophy.

The leukocyte formula shows 3-4 hours after injection of the

vitamin and in the first 10 days of observation a moderate neutrophil leukocytosis with relative lymphopenia.

3) Rats treated with pantothenic acid. The relative weight of the adrenal glands increases progressively in these animals up to the 16th day of the experiment and diminishes, as time goes on, beyond the values of the controls in a rapid, intense manner.

The weight of the thymus, on the contrary, is progressively reduced during the whole period of the observations.

The adrenal glands show in the cortical zone and during the first half of the treatment distinct signs of hypertrophy (especially marked in the fasciculated portion) with accentuated besprinkling and progressive disappearance of the distinctive limits between the various portions and of the sudanophil lipids.

In the preparations made after 20, 24, and 28 days of therapy no differences are noted in regard to the controls.

In these animals too the thymus shows the common histological signs of atrophy.

The leukocyte formula shows during the first 12-14 days a neutrophil leukocytosis with relative lymphopenia that reaches the maximum 4-5 hours after administration of the vitamin.

From the data referred to above it follows that the administration of strong doses of vitamin B_2 alters in no appreciable way the structure

and activity of the adrenal cortex and that such therapy, therefore, does not constitute "stress" for the albino rat.

Strong doses of vitamin B_6, the administration of which stimulates the adrenal cortex (the fasciculated portion) between the eighth and the sixteenth day, show, on the contrary, an action of this (the "stress") type, however moderate.

Finally, treatment with strong doses of pantothenic acid provokes a marked hyperfunction of the fasciculated portion of the adrenal cortex during the first 16 days.

Atrophy of the thymus, found in rats treated with vitamin B_6, or with pantothenic acid, which begins simultaneously with increase of the adrenal-cortex activity, in our opinion is embodied in the general syndrome of adaptation that develops in such experiment conditions. A like interpretation seems little likely for the atrophy of the thymus that is observed during hyperdosage of riboflavin, which does not determine, as said above, an increase of adrenal-cortex activity. In this condition of experiment atrophy of the thymus might be due to a direct action of the vitamin.

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We are grateful to Prof. G. Stella for his interest and suggestions in the course of the research.

We are grateful to Ditta Roche, which kindly furnished the vitamins for us.

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De Bastiani-'52
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IPERDOSAGGIO DI RIBOFLAVINA O DI PIRIDOSSINA O DI ACIDO PANTOTENICO E SINDROME GENERALE DI ADATTAMENTO NEL RATTO ALBINO.

— G. De Bastiani e P. Zatti.
(Dall'Istituto di Fisiologia umana dell'Università di Padova).

Sezione di Padova — Seduta del 20 maggio 1952.

E' stato osservato (De Bastiani e Zatti) che l'iperdosaggio della vitamina B_1 (1) o delle vitamine del complesso B (2) provoca, nel ratto, una sindrome generale di adattamento, che insorge (Dean e Coll., 3; Skelton, 4) anche nel corso della deficienza acuta di queste vitamine. Poichè, d'altra parte, tale sindrome si svolge durante la carenza di ac. pantotenico, ma non durante quella di riboflavina o di piridossina (Dean

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e coll., 3, 5), abbiamo esteso le nostre ricerche agli effetti dell'iperdosaggio della vitamina B_2, B_6 o dell'ac. pantotenico.

La ricerca, eseguita con le modalità indicate nelle note precedenti (1, 2), è stata compiuta su ratti bianchi maschi di 70 g. divisi in 4 gruppi di 14 animali, che sono stati sottoposti ad una iniezione intramuscolare quotidiana di 10 mg di riboflavina o, rispettivamente, di 50 mg di piridossina o di 50 mg di ac. pantotenico; un gruppo di animali è stato tenuto di controllo.

L'esperimento è durato complessivamente 28 giorni, essendo stati sacrificati ogni 4 giorni due ratti di ciascun gruppo.

Quali indici dell'attività dei surreni abbiamo assunto il loro peso e quadro istologico, lo stato del timo e le variazioni della formula leucocitaria.

Risultati sperimentali. — 1) Ratti trattati con la vitamina B_2. — Il peso relativo dei surreni di questi animali non ha subito variazioni maggiori di quelle dei controlli. Il peso relativo del timo, invece, è diminuito da prima in maniera intensa, poi è modicamente aumentato ed infine si è nuovamente ridotto.

All'esame istologico i surreni non presentano, rispetto ai controlli, differenze marcate ad eccezione di un modico aumento della irrorazione della ghiandola specie nei primi giorni di trattamento e di una diminuzione, pure assai modica, dei lipidi sudanofili nella porzione fascicolata, nei preparati allestiti dopo 4 e 8 giorni di esperimento.

Il timo presenta le consuete modificazioni strutturali comprovanti la progressiva atrofia della ghiandola (picnosi nucleare diffusa nella parte corticale, aumento del numero dei corpi di Hassal nel reticolo).

La formula leucocitaria non risultò mai modificata.

2) Ratti trattati con la vitamina B_6. — In questi animali il peso dei surreni è modicamente superiore a quello dei controlli nella prima metà del trattamento, compreso nei limiti della norma nella seconda.

Il peso relativo del timo diminuisce progressivamente nei primi 15 giorni di osservazione, quindi aumenta, pur mantenendosi al di sotto dei valori normali.

I preparati istologici dei surreni mostrano, nei ratti sacrificati dopo 8, 12, 16 giorni di esperimento, una modica ipertrofia della porzione fascicolata che, nella sua parte più esterna, ha un aspetto spugnoso per la presenza di numerosi vacuoli intracellulari e per un aumento degli spazi tra le travate cellulari dove si repertano di frequente numerosi globuli rossi. I granuli lipoidei sudanofili sono diminuiti nei primi 12 giorni di trattamento.

Il timo presenta i comuni segni di atrofia.

La formula leucocitaria mostra, 3-4 h dopo la iniezione della vitamina e nei primi 10 giorni di osservazione, una modica leucocitosi neutrofila con linfopenia relativa.

3) Ratti trattati con ac. pantotenico. — Il peso relativo dei surreni aumenta progressivamente, in questi animali, fino al 16° giorno di esperimento e diminuisce, in proseguo di tempo, oltre i valori dei controlli, in maniera rapida ed intensa.

Il peso del timo, invece, si riduce progressivamente durante tutto il periodo delle osservazioni.

I surreni mostrano nella zona corticale e durante la prima metà del trattamento, netti segni di ipertrofia (soprattutto marcati nella porzione fascicolata), con accentuata irrorazione e progressiva scomparsa dei limiti distintivi tra le diverse porzioni e dei lipidi sudanofili.

Nei preparati allestiti dopo 20, 24, 28 giorni di trattamento non si notano invece differenze rispetto ai controlli.

Anche in questi animali il timo presenta i comuni segni istologici di atrofia.

La formula leucocitaria mostra durante i primi 12-14 giorni una leucocitosi neutrofila con relativa linfopenia, che raggiunge la massima entità 4-5 h dopo la somministrazione della vitamina.

Dai dati surriferiti risulta che la somministrazione di forti dosi della vit. B_2 non modifica in maniera apprezzabile la struttura e l'attività della corteccia surrenalica e che pertanto tale trattamento non rappresenta uno «stress» per il ratto albino.

Un'azione, seppure modica, di questo tipo esplicano, invece, le forti dosi di vitamina B_6 la cui somministrazione stimola la corteccia surrenalica (porzione fascicolata) tra l'8° ed il 16° giorno.

Il trattamento, infine, con forti dosi di ac. pantotenico provoca una marcata iperfunzione della porzione fascicolata della corteccia surrenalica durante i primi 16 giorni.

L'atrofia del timo, riscontrata nei ratti trattati con la vit. B_6 o con l'ac. pantotenico, che inizia contemporaneamente all'aumento dell'attività cortico-surrenalica, si inquadra, a nostro avviso, nella sindrome generale di adattamento, che si svolge in tali condizioni sperimentali. Eguale interpretazione sembra poco probabile per l'atrofia del timo che si rileva durante l'iperdosaggio della riboflavina, il quale non determina, come sopra detto, un aumento della attività della corteccia surrenalica; in questa condizione sperimentale l'atrofia del timo potrebbe essere dovuta ad una azione diretta della vitamina.

Siamo grati al Prof. G. Stella per il suo interessamento e per suggerimenti nel corso della ricerca.

Siamo grati alla Ditta Roche che gentilmente ci ha fornito le vitamine.

(1) Questo *Bollettino*, 1950, 26, 896, 899. — (2) *Ibidem*, 1951, 27, 1565. — (3) *J. Nutr.*, 1947, 34, 1. — (4) *Amer. J. Phys.*, 1950, 161, 515. — (5) *Endoc.*, 1946, 38, 385.

Driskel-'71
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Alanine Aminotransferase Activity in Liver and Erythrocytes of Pregnant and Nonpregnant Rats Fed Different Levels of Pyridoxine [1,2]

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ABSTRACT The use of alanine aminotransferase activity in the assessment of pyridoxine requirements of pregnant and nonpregnant rats was studied. A 25% casein diet containing 0, 30, 60, 90, 120, 180, 240, 800 or 8000 μg pyridoxine per 10 or 15 g diet for nonpregnant and pregnant rats, respectively, was fed ad libitum for 4 weeks beginning 1 week prior to mating. In a second experiment 5, 10, 15, 20, 25 and 30 μg of vitamin were incorporated similarly into the diets which were fed from weaning to 4 months of age. The levels of pyridoxine per 10 and 15 g diet approximated daily intake of vitamin per rat Successful reproductive performance was supported by an intake of 15 μg pyridoxine per day or more. No adverse effects of the high levels of intake were evident. An intake of 20 μg or more of the vitamin daily was needed for maximal alanine aminotransferase activity in liver and erythrocytes of pregnant animals. Activity in erythrocytes was less affected by the pregnancy state than that in liver. The failure of liver or erythrocyte activities to change when the level of pyridoxine intake was greater than 20 μg daily suggested that the regulation of activity was operative when intakes ranged from 20 to 8000 μg. J. Nutr. *101:* 85–92, 1971.

Among the methods employed to determine pyridoxine nutriture, the tryptophan load test has been widely used; however, several limitations have been cited in its use (1). Since there is little evidence that the vitamin levels required to produce a normal tryptophan load test are beneficial or harmful in normal pregnancy, other methods of assessing pyridoxine nutriture in pregnancy have been sought. Determination of the amount of vitamin required to produce a plateau in the activity of certain pyridoxine-dependent enzymes is one method which has received attention. On this basis erythrocyte alanine aminotransferase activity in nonpregnant animals appeared to be as sensitive to pyridoxine deficiency as the tryptophan load (2) In pregnant rats erythrocyte alanine aminotransferase activity was observed to increase up to 400 μg/day, the highest pyridoxine level employed (3). The investigators concluded that the dose required to produce a plateau in enzyme activity should be determined in order to define the pyridoxine requirement in terms of this enzyme. Basic to such recommendations is the assumption that alanine aminotransferase activity is not increased by high intakes due to cofactor-induced apoenzyme synthesis. The validity of this assumption is challenged by the fact that pyridoxine-mediated enzyme induction has been demonstrated for at least one pyridoxine-dependent enzyme and has been shown to result in increased enzyme activity (4, 5).

Studies to determine the effects of high levels of dietary pyridoxine on reproductive performance have shown no adverse

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[2] Part of these data was presented at the meeting of the American Institute of Nutrition, Atlantic City, New Jersey, April 1970, Federation Proc. 29: 823 (abstr.).

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gross effects (6). However, the finding that pyridoxine can induce synthesis of at least one apoenzyme and possibly others (7) indicates that metabolism could be altered by large daily intakes of the vitamin. There is evidence that the fetus draws pyridoxine from maternal blood (8) and therefore may not be protected from adverse effects resulting from the large amounts of vitamin ingested by the mother.

The purpose of the present study was to better define the relation between dietary pyridoxine and alanine aminotransferase [L-alanine:2 oxoglutarate aminotransferase (EC 2.6.1.2)] activity in nonpregnant, pregnant and fetal tissues, and to study the effects of the ingestion of large amounts of dietary pyridoxine on reproductive performance.

MATERIALS AND METHODS

Animals and diets: Experiment 1. Female rats[5] weighing approximately 250 g were randomly assigned to nine groups of 12 animals each. Animals were fed ad libitum a basal diet containing: (in %) vitamin-free casein, 25; sucrose, 19; cornstarch and vitamin mix, 34.85; cellulose, 2.0; Jones and Foster (9) salt mixture, 4; hydrogenated fat, 10; L-cystine, 0.15; and corn oil with vitamins A, D and E, 5. The vitamin mix contained: (in mg/kg) thiamin, 2.5; riboflavin, 4.1; niacin, 10; pantothenic acid, 20; biotin, 0.1; *p*-aminobenzoic acid, 20; folic acid, 2; triturate of crystalline B_{12} in mannitol, 3; menadione, 10; and choline, 3000. Corn oil with vitamins A, D and E contained: (in mg) retinyl acetate, 1.03; vitamin D_2, 0.019; and α-tocopherol, 6. The diet was supplemented with 0, 30, 60, 90, 120, 180, 240, 800 or 8000 μg pyridoxine per 10 g diet for nonpregnant rats and per 15 g for pregnant rats. Actual food intakes were close to these expected rates, so the daily intake of pyridoxine differed only slightly from that planned. Animals were housed in individual wire-bottomed suspended cages in a room maintained at 21°, 60% humidity, and alternate 12-hour periods of light and dark. Six animals in each dietary treatment group were mated following a 1-week adjustment period to the diet and the remaining animals in each group served as nonpregnant controls. Mating was confirmed by the presence of sperm in vaginal smears and/or vaginal plugs. Nonpregnant animals were started on the experiment at the same time as pregnant animals in order to minimize age differences.

Experiment 2. Fourteen weanling female rats[6] were randomly assigned to each of six groups. Animals were fed ad libitum the basal diet described above supplemented to contain 5, 10, 15, 20, 25 or 30 μg of pyridoxine per 10 g diet for nonpregnant rats and per 15 g for pregnant rats. The pyridoxine levels represent the approximate quantity of vitamin consumed daily per rat. Seven rats receiving each treatment were mated at 80 to 110 days of age. At the time of mating, the average body weights of rats assigned to the 5, 10, 15, 20, 25 and 30 μg level of vitamin were 197, 212, 227, 227, 231 and 226 g, respectively. Other procedures were as previously stated for experiment 1.

Tissue samples. Animals were killed by electrical shock on day 21 of gestation or after an equivalent time period for nonpregnant rats. Blood was obtained by cardiac puncture with a heparinized syringe and transferred to a tube packed in ice. A liver slice was taken from the left lateral lobe and immediately placed on dry ice. The entire liver was then removed and weighed. In pregnant animals, the uterine sac was opened and the fetuses were removed; numbers of implantation sites and resorptions and fetal weights were recorded. Fetal livers were removed and placed on dry ice. Liver tissues were stored at − 20° until analyzed.

Enzyme analysis. Blood was assayed the same day the samples were drawn. Samples were centrifuged for 10 minutes at 1600 × *g* and 5°, and the plasma supernatant was discarded. Cells were washed by mixing with twice their volume of physiological saline solution followed by centrifuging and discarding the supernatant; the procedure was repeated. The volume of red cells was recorded. The method of Tonhazy et al. (10) was used in preparing liver homogenates for assay. Alanine aminotransferase activity of erythrocytes and liver was determined by the

[5] Obtained from Sprague-Dawley, Inc., Madison, Wis.
[6] See footnote 5.

ethod of Tonhazy et al. as modified by Reddle et al. (11).

Statistical treatment. All data were analyzed by the CDC 6500 computer using the Duncan (12) multiple range test. Means and standard errors of the means were included in output from this test.

RESULTS

Food intake, weight gain, liver weight, and reproductive data are given in table 1. As would be expected, food intake, weight gain and liver weight were significantly decreased by pregnancy. Food intake was not significantly affected by the level of vitamin intake in experiment 1. Body weight gain was decreased in pregnant rats fed the pyridoxine-deficient diet; liver weights were depressed in rats consuming this diet compared with other rats in experiment 1. The high levels of vitamin intake did not influence body or organ weights. In experiment 2, pregnant animals consuming 5 and 10 μg pyridoxine gained significantly less weight than those fed higher levels. Food intake was slightly depressed in pregnant rats receiving the 5 μg level. Fetal weights and fetal liver weights were lower in animals consuming the 5 μg level of pyridoxine. Reproductive performance did not appear to be influenced by feeding the pyridoxine-free diet a week prior to and during gestation in spite of lowered maternal gains in this group (exp. 1). However, animals receiving the 5 μg level of pyridoxine from weaning had a significant increase in resorptions and a decrease in number of live young compared with data for animals receiving higher vitamin levels (exp. 2).

Alanine aminotransferase activities of liver are shown in figure 1. Activities were higher in nonpregnant than pregnant animals, and the lowest activity was found in fetal liver. Hepatic enzyme activities in nonpregnant and pregnant animals and in fetuses were significantly lower in groups receiving no pyridoxine than in those receiving other vitamin levels in experiment 1. The activities did not increase as the level of pyridoxine in the diet was increased from 30 to 8000 μg 15 g diet. Hepatic activities of both nonpregnant and pregnant animals receiving 20 μg pyridoxine or more daily from weaning were significantly higher than in those fed lower levels when expressed either as pyruvate formed per gram wet tissue/hour or per whole liver/hour. Liver enzyme activities in fetuses of dams fed 10 μg or less of pyridoxine daily from weaning were significantly lower than in offspring whose mothers received higher levels.

Erythrocyte enzyme activities of nonpregnant animals receiving 10 μg or less of pyridoxine daily from weaning were significantly lower than those of animals fed higher levels (fig. 2). In pregnant animals activities were significantly lower in animals consuming a level of 15 μg or less of pyridoxine than in rats fed higher vitamin levels. The higher vitamin intakes in experiment 1 did not increase the activity.

DISCUSSION

Numerous investigations have shown that pyridoxine-deficient diets depress weight gain if provided for a sufficient length of time. The 1- to 2-week adjustment period provided prior to mating combined with the 21-day gestation period was sufficient time for producing these changes in the pregnant animals; nonpregnant rats receiving no pyridoxine were able to gain weight at a rate comparable to that of animals receiving pyridoxine. This difference between pregnant and nonpregnant animals presumably reflects the increased need for pyridoxine during gestation.

The quantity of pyridoxine required to maintain a satisfactory growth rate from weaning until sexual maturity was between 10 and 15 μg daily; this is in agreement with the recommendation of 12 μg by the National Research Council (13). Beaton and Cheney (14) have reported that the vitamin requirement was between 40 and 80 μg daily for maximal weight gain in male albino rats. Brin and Thiele (15) reported that the greatest weight gain in female albino rats was evident at an intake of 20 μg daily. A decrease in fetal weight in pyridoxine deficiency has been reported by several investigators (16–18). In this study the numbers and weights of fetuses of dams receiving 10 μg or more of the vitamin daily agreed with values reported by the National Research Council (13) as a satisfactory standard of reproductive performance. Normal reproductive

TABLE 1

Food intake, body weight gain, liver weight and reproductive performance

Experiment	Dietary pyridoxine	Food intake [1]		Weight gain [1]		Liver weight			No. resorptions	No. live young	Fetal weight
		Non-pregnant	Pregnant	Non-pregnant	Pregnant	Non-pregnant	Pregnant	Fetal			
	μg [2]	*g/day*		*g*		*g*					*g*
1 [3]	0	11.0 [4] ± 0.7	15.4 ± 0.8	17 ± 2	96** ± 2	7.4* ±0.1	10.6** ± 0.3	0.30* ±0.01	0.8 ±0.2	12.2 ± 0.5	4.8 ±0.1
	30	10.4 ± 0.5	15.0 ± 0.5	29 ± 3	149 ± 3	9.2 ±0.1	15.2 ± 0.1	0.33 ±0.01	1.0 ±0.3	12.8 ± 0.4	4.6 ±0.6
	60 to 8000 [5]	10.2 ± 0.4	15.1 ± 0.5	20 ± 2	149 ± 4	8.7 ±0.1	13.9 ± 0.3	0.34 ±0.01	0.5 ±0.3	12.0 ± 0.4	4.9 ±0.1
2 [6]	5	10.1 ± 0.8	13.8* ± 0.9	15 ± 2	72** ± 6	7.0 ±0.1	9.0** ± 0.3	0.29* ±0.01	1.9** ±0.5	7.8** ± 0.8	4.2** ±0.2
	10	10.2 ± 0.6	14.8 ± 0.6	14 ± 2	93* ± 4	7.1 ±0.3	9.7** ± 0.5	0.32 ±0.01	0.4 ±0.2	9.9 ± 0.5	4.8 ±0.1
	15	10.5 ± 0.5	15.1 ± 0.6	18 ± 2	117 ± 6	7.5 ±0.2	11.4 ± 1.4	0.35 ±0.01	0.9 ±0.6	11.0 ± 1.0	5.0 ±0.1
	20	10.1 ± 0.4	15.3 ± 0.5	19 ± 3	123 ± 3	7.7 ±0.1	12.3 ± 0.5	0.34 ±0.01	0.4 ±0.2	11.3 ± 0.4	4.9 ±0.1
	25	10.4 ± 0.5	15.5 ± 0.6	15 ± 2	127 ± 4	7.5 ±0.3	13.2 ± 0.5	0.33 ±0.01	0.4 ±0.2	11.9 ± 0.5	4.9 ±0.1
	30	10.1 ± 0.4	15.0 ± 0.5	20 ± 2	120 ± 6	7.8 ±0.4	11.8 ± 0.5	0.32 ±0.01	0.7 ±0.2	11.1 ± 0.7	5.0 ±0.1

[1] Twenty-one day experimental period.
[2] Per 10 g and 15 g diet for nonpregnant and pregnant groups, respectively.
[3] Diets fed 1 week prior to and during the 21-day experimental period to 12 rats per group; six became pregnant, six remained nonpregnant.
[4] Mean ± SEM.
[5] Values for groups fed the 60, 90, 120, 180, 240, 800 and 8000 μg levels of pyridoxine were similar and have been combined into one set of data.
[6] Diets fed from weaning to 14 rats per group excepting the 15 μg groups which consisted of 12 rats each. One half of the rats in each group were mated and became pregnant at 80–110 days of age.
*, ** Significantly different at $P < 0.05$ and $P < 0.01$, respectively, within each column within each experiment.

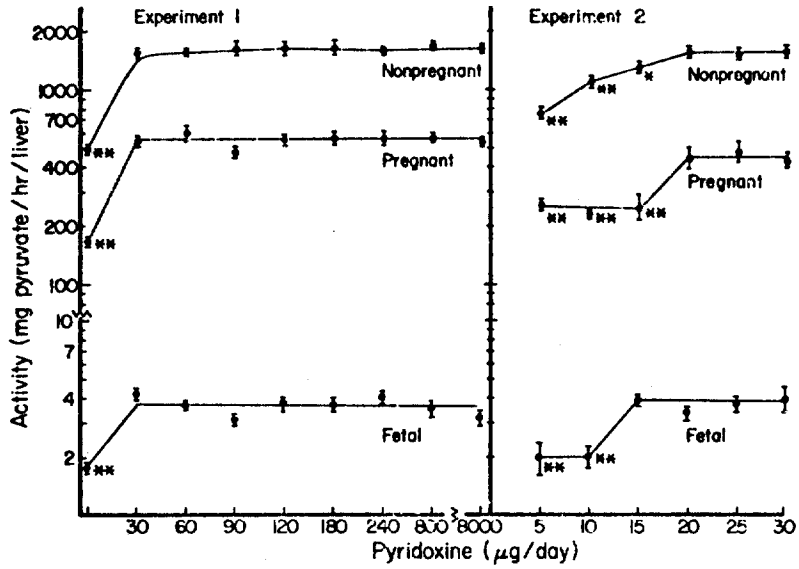

Fig. 1 Alanine aminotransferase (ALAT) activity in liver. Standard errors of the means are indicated by vertical bars. $*P < 0.05$; $**P < 0.01$.

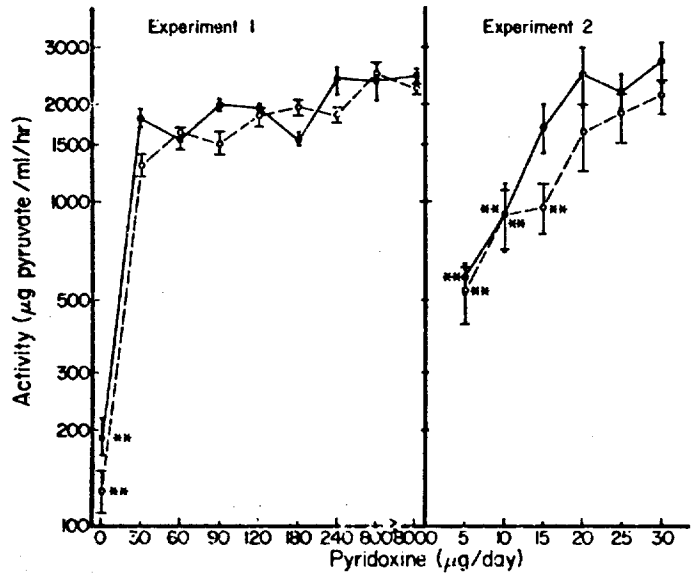

Fig. 2 Alanine aminotransferase (ALAT) activity in erythrocytes. Standard errors of the means are indicated by vertical bars; —— nonpregnant; - - - - pregnant. $**P < 0.01$.

performance of animals fed the pyridoxine-free diet in experiment 1 is attributed to the short dietary adjustment period of 1 week prior to mating. The lack of adverse effects on weight gain and reproductive performance in animals receiving levels as high as 8000 μg pyridoxine daily may also be related to the short period of dietary adjustment. Morrison and Sarett (6) found that litter size and growth of progeny until weaning were not affected by intakes as high as 1.125 mg pyridoxine daily.

A decrease in hepatic alanine aminotransferase activity has been observed in pregnancy by Harding et al. (19). Beaton et al. (20) observed that this decrease in alanine aminotransferase activity coincided with increased protein deposition and that both changes occurred at or near day 15 of gestation. Decreased alanine aminotransferase activity has been associated with conditions of increased protein deposition including growth, growth hormone administration and tumor growth, whereas increased activity has been noted during starvation and cortisone treatment and in conditions associated with increased protein catabolism. The concept of an inverse relation between alanine aminotransferase activity and protein synthesis (20) is consistent with the findings in this study that alanine aminotransferase activity in maternal and fetal livers was lower than in livers of nonpregnant animals. Harding et al. (19) proposed that the increased requirement for protein precursors for fetal growth resulted in a relative amino acid deficiency in the liver which limited the synthesis of enzymes induced by cortisol.

Several investigators (2, 21–23) have observed low alanine aminotransferase activities in livers of rats consuming pyridoxine-deficient diets. The hepatic alanine aminotransferase activities reported by Cheney and Beaton (24) suggested that the maximum response to pyridoxine was attained with a dosage of approximately 20 μg/day. This quantity of pyridoxine was also needed for maximal concentration of the vitamin in the liver (15, 19, 25). No significant differences were found in alanine aminotransferase activities in rats fed 20 and 100 μg pyridoxine daily, but these activities were significantly higher than those found in animals fed 10 μg or less (15). Activities in livers of pregnant and nonpregnant rats receiving 16, 80 and 400 μg pyridoxine hydrochloride orally/day were similar. In this study the failure of adult, maternal, and fetal liver enzyme activities to increase as intake was increased above 20 μg pyridoxine daily suggested that the cofactor did not interfere with the regulation of the enzyme activity in liver when vitamin intakes ranged from 20 to 8000 μg.

The suggestion has been made that pyridoxine requirement in pregnant rats could be defined by determining the dose required to produce a plateau in erythrocyte enzyme response. The results of Beaton and Cheney (14) have indicated that the pyridoxine requirement for maximal erythrocyte alanine aminotransferase activity for nonpregnant rats was at least 80 μg daily. However, Brin and Thiele (15) reported that erythrocyte activity for rats receiving 20 μg pyridoxine daily was as high as for those fed 100 μg. Dirige and Beaton (26) fed rats varying levels of casein and pyridoxine; they hypothesized that the requirement for the vitamin is directly correlated with the level of dietary protein, provided that the protein is complete. Cheney and Beaton (3) found that the activity in pregnant rats increased with each increment in pyridoxine dosage up to the highest dose employed (120 and 40[illegible] μg).

The amount of pyridoxine required to produce maximal weight gains in this study was 5 to 10 μg less than the amount required to produce a plateau in erythrocyte or liver enzyme activity. Weight gain and alanine aminotransferase activity do not, therefore, appear to be comparable parameters in nutritional assessments. The amounts of vitamin required for a plateau in erythrocyte and liver enzyme activities were similar in pregnant animals, but approximately 5 μg less was needed for a plateau in activity in erythrocytes compared with liver in nonpregnant animals. Liver alanine aminotransferase activity is known to be more affected by adrenocorticoids and the pregnancy state than that of the erythrocyte. For these reasons and that of convenience, erythrocyte activity may be preferable to that of liver in assessments of vitamin nutriture. Erythrocyte activities have been used in assessing the pyridoxine nutriture of humans (27, 28). In the present investigation, erythrocyte data suggested that the pyridoxine requirement for nonpregnant rats was between 10 and 15 μg daily and between 15 and 20 μg for pregnant rats.

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Effect in Man of Large Doses of Pyridoxine on Serum Cholesterol

By Robert B. Failey, Jr., M.D.

Pyridoxine (400 mg./day) was given to 21 individuals for periods up to a maximum of 36 days. A slight but significant fall in serum cholesterol levels was observed, the effect being more marked in diabetic than in nondiabetic subjects.

ATHEROSCLEROTIC plaques observed in man and several species of animals characteristically contain large amounts of cholesterol, and numerous experimental and epidemiologic studies have demonstrated a relationship between serum cholesterol levels and the incidence of atherosclerosis. Pyridoxine (vitamin B_6) has been among the several agents considered effective in inhibiting the development of atherosclerosis. Rinehart and Greenberg[1] reported the finding of atherosclerotic lesions in pyridoxine-deficient monkeys. Witten and Holman[2] presented evidence indicating that pyridoxine in the rat enhanced the conversion of linoleic to arachidonic acid. Their work indicated that pyridoxine and linoleic acid together were more effective in controlling the deficiency manifestations of these two substances in rats than either did separately. Kinsell et al.[3] showed that linoleic acid, as a constituent of dietary fat, had a marked effect in lowering serum cholesterol levels. At the present time the substitution of vegetable fats high in linoleic acid content for the highly saturated animal fats of many human diets represents the most effective if not necessarily the most practical means of lowering serum cholesterol levels. Pyridoxine has been shown necessary for the conversion of tryptophane to niacin, and nicotinic acid (although not the amide) has been shown to lower the serum cholesterol level of man and animals.[4, 5]

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Method

The purpose of the present study was to assay the effect of large doses of pyridoxine upon the human serum cholesterol level. Pyridoxine hydrochloride up to 400 mg. day was administered orally. Twenty-one patients hospitalized at the Indiana University Medical Center and at the Indianapolis Veterans Hospital were studied. Five were female diabetics ranging in age from 50 to 71 years, who were hospitalized for periods of from 21 to 35 days because of occlusive arterial disease of the toes and feet. Some of these subjects had undergone surgical removal of small areas of gangrene during their hospitalizations; none showed signs of acidosis, coma, febrile disease, or any marked variation in insulin requirement while in the hospital. None required more than 50 units of insulin per day to control their diabetes. The remaining 16 patients were hospitalized essentially for domiciliary care. These were males, ranging in age from 41 to 80 years, who were hospitalized for a variety of chronic pulmonary and cardiac diseases. None of these subjects showed any evidence of nutritional impairment, and there were no significant fluctuations in weight during the period of the study. No special dietary program was employed beyond that used routinely for the diabetics. Cholesterol determinations were done by the method of Zak et al.[6] as modified by Herrmann.[7]

Results

Results of the study are presented in table 1. In this table the diabetic patients are presented as two groups. Although 5 diabetics were studied initially, 3 left the hospital before follow-up controls could be obtained. Similarly, the results for the combined group of diabetic and nondiabetic patients are presented twice because of lack of follow-up con-

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TABLE 1.—*Serum Cholesterol Levels Before, During and After Oral Administration of Pyridoxine*

	Controls* (mg. %)	Pyridoxine (200 mg. deg.)	Pyridoxine (400 mg. deg.)	Controls
5 diabetic subjects	20—5 days 253.2	—	28—7 days 234.0 mg. %	—
2 diabetic subjects	8—5 days 264.0	—	9—5 days 259.5 mg. %	10—5 days 275.5 mg. %
6 nondiabetic subjects	24—10 days 256.7	24—20 days 247.0 mg. %	24—16 days 235.8 mg. %	24—19 days 242.2 mg. %
10 nondiabetic subjects	50—7 days 221.8	—	50—7 days 225.9 mg. %	50—7 days 228.4 mg. %
21 subjects, combined group	94—5-10 days 239.2	—	102—5-16 days 230.7 mg. %	—
18 subjects, combined group	82—5-10 days 238.1	—	83—5-16 days 232.9 mg. %	84—5-19 days 238.2 mg. %

* The figures above each serum cholesterol level indicate the number of determinations and the period of observation.

trols in the three above-mentioned individuals. The two groups of nondiabetics are composed of different individuals and do not represent any duplication. Figures for serum cholesterol are group averages obtained by combining the mean figures obtained for each individual, so that in obtaining the group averages each individual rather than each separate determination is weighed as one unit. Four of the 5 diabetic patients exhibited a fall in mean serum cholesterol levels while on the medication. In this group the highest single individual value obtained was 310 mg. per cent, the lowest 200 mg. per cent. In the long term study of nondiabetic patients, 5 of 6 individuals exhibited a fall in mean serum cholesterol levels. In this group the highest single individual value was 339 mg. per cent, the lowest 170. In the short-term nondiabetic group, 6 of 10 individuals showed lower cholesterol levels. In this group the highest single individual value was 300 mg. per cent, the lowest 150.

Statistical analysis of data obtained from the 6 nondiabetic patients in the long term study (line 3, table 1) showed that the control levels prior to medication were significantly higher than the follow-up controls after cessation of medication. The first control period values were significantly higher than those from any of the other groups. The mean control values of both control periods were significantly higher than the mean of the periods of medication, but there was not a significant difference between follow-up controls and either of the periods of medication.

Probability values of less than 0.05 are considered as significant.

Data from the short term study of nondiabetic patients (line 4, table 1) revealed no fall in mean cholesterol values either during medication or in the follow-up control period. In terms of the planned experiment, these values are obviously without significance.

Because of difference in the numbers of tests, two methods of analysis were used to determine significance of results obtained from the diabetic patients (lines 1 and 2, table 1). By the first method, the t values for the difference between the means during the control period and during medication were determined for each patient. Values of p were then determined, and then combined according to the method of Fisher. Significant difference ($p < .05$) between values from control periods and medication periods was ob-

served. By the second method of analysis, the mean serum levels for each patient were determined for the control and medication periods and were subjected to analysis of variance. Again a significant difference was noted.

In this study no placebo medication was employed during the control periods; however, the individuals serving as subjects for the study were not informed as to its purpose, and none inquired as to the results.

Discussion

The average human requirement of pyridoxine sufficient to prevent deficiency signs has been stated to be 2 to 3 mg./day,[8] and the dosage used in the present study was grossly in excess of this amount. It was also much larger than that employed in any of the presently recommended programs by which pyridoxine alone or in combination is administered in an attempt to lower serum cholesterol levels or inhibit formation of arterial atherosclerosis. Results do not indicate that these large doses of pyridoxine had any striking effect on serum cholesterol levels. The evidence does suggest that prolongation of the experiment over several more weeks' time might have led to a greater effect. The reduction in serum cholesterol is, however, not nearly as great as could be anticipated from starvation, dietary substitution of unsaturated fat, nicotinic acid, sitosterol, or certain of the substituted butyric acids. The combination of pyridoxine in small doses with linoleic acid has been proposed as an agent to lower serum cholesterol levels, and it is entirely possible that pyridoxine in combination with other agents may be even more effective. The reaction by which pyridoxine enhances the conversion of tryptophane to niacin could possibly be accelerated by excess pyridoxine. In the present study diets appeared to be normal, adequate, and essentially unvarying throughout the experiment. Again the possibility exists that variations in quantity and composition of protein as well as fat in the diet might, in combination with the pyridoxine, prove more effective than would either the drug or the diet separately. The significant and early fall in cholesterol levels seen in the diabetic patients in the present study when compared with that of the nondiabetic patients suggests that pyridoxine in large doses may be more effective in this group than among others. The mean serum cholesterol levels of the controls in this study approximated those given as normal for the present day population of the United States. Of this entire group only two individuals had control levels that were consistently over 300 mg. per cent, and it is possible that had the mean values of the controls been greater, the observed fall under medication would have been greater. We have observed that serum cholesterol levels of ambulatory people seem to be higher than those of hospitalized patients, even in instances in which debility and disease do not seem significant. It may well be that repetition of this study on ambulatory individuals would lead to a different result. Data reporting the effect of nicotinic acid upon serum cholesterol levels[4, 5] typically show a fall to around 250 mg. per cent, at which point values tend to stabilize in spite of increase in dosage of the drug. If, as has been suggested, pyridoxine acts somewhat in the manner of nicotinic acid, greater effect would be observed at higher initial cholesterol levels, and cholesterol levels would not be expected to go much below the 250 mg. per cent range. The failure to observe any fall in mean serum cholesterol levels in the short term, nondiabetic group may be attributable to the low (221.8 mg. per cent) mean control level, and it seems quite possible that a higher control level would have been followed by a fall during the period of medication. By contrast, the long term study of the nondiabetic patients does show a significant fall in serum cholesterol levels during the period of medication. In this group the failure of follow-up controls to attain the levels of the initial controls suggests a prolonged and cumulative effect of the drug.

Summary

Pyridoxine in the amount of 400 mg./day

was administered orally to 21 individuals for periods of observation ranging from 5 to 36 days. Five diabetics who received medication for a maximum of 7 days showed a significant ($p < .05$) fall in serum cholesterol levels. Ten nondiabetics who received medication for a maximum of 7 days did not show a significant fall in serum cholesterol levels. Six nondiabetics who received medication for a maximum of 36 days showed a significant fall in serum cholesterol levels with a delayed rise during a follow-up control period.

ACKNOWLEDGMENT

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SUMMARIO IN INTERLINGUA

Pyridoxina in quantitates de 400 mg per die esseva administrate per via oral a 21 individuos durante periodos de observation de inter 5 e 36 dies. Cinque diabeticos, qui recipeva le medication durante 7 dies o minus, monstrava un reduction significative del nivellos de cholesterol seral ($p < 0.05$). Dece non-diabeticos, qui etiam recipeva le medication durante 7 dies o minus, non monstrava un reduction significative del nivellos de cholesterol in lor seros. Sex non-diabeticos, qui recipeva le medication durante 36 dies o minus, monstrava un reduction significative in le nivellos del cholesterol seral sequite per un retardo del re-ascendita durante le periodo de observation consecutori.

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CLINICAL STUDIES ON PYRIDOXINE (VITAMIN B_6)

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The isolation (1), synthesis (2) and physiological significance (3 to 9) of pyridoxine[1] in animal nutrition have recently been established. Clinical studies have not been very extensive. Spies and his collaborators (10) have noted that certain residual symptoms (*i.e.*, insomnia, irritability, weakness, difficulty in walking and abdominal pain) in treated pellagrins responded to pyridoxine therapy. In a case of arsenical neuritis, Vilter (11) commented on the rapid remission when pyridoxine was added to the already instituted therapy. He also observed an exacerbation when pyridoxine was discontinued. Smith and Martin (12) successfully treated three out of four patients suffering with cheilosis. In the fourth patient the addition of liver extract brought relief. These investigators state that deficiency of either riboflavin or pyridoxine may be the etiological factor primarily responsible for cheilosis or that both together are necessary in maintaining the integrity of the mucocutaneous junction. Antopol (13) noted improvement in a group of patients with pseudohypertrophic muscular dystrophy when pyridoxine was administered. Lessening in rigidity and improvement in muscle strength were noted in a group of patients suffering with Parkinsonism after the use of pyridoxine (14, 15). This improvement was observed particularly in early cases of senile Parkinson's disease.

There have been isolated reports indicating favorable response to pyridoxine therapy in both macrocytic (16) and microcytic (12) anemias. Kark and his associates (17), however, after studying the responses of six anemic patients following pyridoxine administration, did not observe any improvement in the hematological picture.

Recently, Scudi and his co-workers (18) reported a colorimetric test for the determination of pyridoxine. They (19) found that normal rats excreted a higher percentage of the ingested vitamin when compared to rats maintained on a diet deficient in pyridoxine. Applying this test to dogs and normal humans, Scudi and his collaborators (20) found that pyridoxine is rapidly absorbed from the gastro-intestinal tract and excreted by the kidneys. In dogs, 18 per cent of the vitamin was excreted in one hour when it was administered intravenously, while 20 per cent was excreted in six hours when given orally. In thirteen healthy adults receiving 50 mgm. of pyridoxine intravenously, an average of 8.7 per cent was excreted in the urine in one hour, while 7.6 per cent was recovered in the urine in four hours after 100 mgm. were given orally.

The investigations of Scudi have provided clinicians with a test which may indicate the degree of saturation or ability of the body to utilize pyridoxine. Spies (21) recently reported clinical data which confirm the usefulness of Scudi's test. This paper is an attempt at further evaluation of the method for clinical use. An effort has also been made to determine the influence of various pathological factors on the pyridoxine status in humans.

MATERIALS AND METHODS

Patients studied in this series were from the wards and dispensary of the New York Post-Graduate Hospital and the First Medical Division of the Welfare Hospital for Chronic Diseases. The patients were on standard balanced hospital diets. No special therapy was employed except where noted. The tests were carried out by obtaining a control specimen of urine following which 50 mgm. of pyridoxine were administered intravenously and approximately 400 ml. of water were then given orally. One hour after the administration of the vitamin another urine specimen was obtained. Patients with Parkinson's disease required catheterization in order to obtain accurately timed urine specimens. The pyridoxine determinations and calculations were carried out according to the method outlined by Scudi (19, 20).

[1] The term pyridoxine is used interchangeably to signify pyridoxine or pyridoxine hydrochloride.

RESULTS

Ninety-eight tests were performed on eighty-four patients. For purposes of analysis, the patients have been grouped first, according to age and pyridoxine output; and secondly, according to specific ailment, namely: Parkinson's disease and renal insufficiency.

TABLE I

One hour urinary output of pyridoxine in patients with normal renal function from 5 to 15 years of age after the administration of 50 mgm. of pyridoxine intravenously

Patient	Age	Sex	Pyridoxine output	Diagnosis
			per cent	
Var...	9	M	24.6	Pseudohypertrophic muscular dystrophy.
Den...	6	M	20.1	Reticular cell sarcoma.
Rol....	10	M	18.5	Migraine.
Gri....	12	M	18.2	Pleuritis.
Spa...	8	F	22.2	Rheumatic heart disease.
Dan...	8	F	27.3	Chorea (Sydenham).
Fum...	5	M	22.3	Chronic tonsillitis.
Fra....	15	M	15.3	Primary muscular atrophy (juvenile type).
D'ag..	6	F	19.4	Common cold.
Pie....	6	M	25.4	Common cold.

Ten patients between 5 and 15 years of age had an average urinary output of pyridoxine of 21.3 per cent (range 18.2 to 27.3 per cent). A case of pseudohypertrophic muscular dystrophy and one of primary muscular atrophy (juvenile type) were included in this group (Table I). In thirteen of fifteen patients between the ages of 16 and 50 years, the excretion of pyridoxine ranged from 5.2 to 11.4 per cent, with an average output of 8.4 per cent. This approximates closely the normal figure obtained by previous investigators (20, 21) (Table II). Two patients excreted 1.6 and 3.8 per cent, respectively. In forty-five patients over 50 years of age, thirty-two were within the normal range (4.1 to 11 per cent), with an average output of 7.2 per cent. Thirteen patients were below normal, with an average output of 2.3 per cent (range 1.0 to 3.1 per cent) (Table III). Table IV summarizes the influence of age on pyridoxine output; standard deviations for each group are also indicated.

TABLE II

One hour urinary output of pyridoxine in patients with normal renal function from 16 to 50 years of age after the administration of 50 mgm. of pyridoxine intravenously

Patient	Age	Sex	Pyridoxine output	Diagnosis
			per cent	
Til....	38	M	9.4	Exfoliative dermatitis.
Bar....	43	M	9.2	Psoriasis.
Sil....	33	M	8.8	Diabetes mellitus.
Met..	47	F	6.4	Menopause.
Por....	16	F	5.2	Rheumatic heart disease.
Bor...	39	F	10.8	Essential hypertension.
McM..	46	F	8.0	Essential hypertension.
Tem...	32	M	5.8	Diabetes mellitus.
Bar...	47	M	6.4	Hodgkin's disease.
Ros...	43	M	10.4	Rheumatic heart disease.
Wol...	30	F	8.6	Spastic colon.
Smi...	33	M	11.4	Peptic ulcer.
Bre....	32	M	6.5	Spinal injury.
Fis....	34	M	3.8	Exfoliative dermatitis.
Com...	24	F	1.6	Essential hypertension.

TABLE III

One hour urinary output of pyridoxine in patients with normal renal function over 50 years of age after the administration of 50 mgm. of pyridoxine intravenously

Patient	Age	Sex	Pyridoxine output	Diagnosis	Remarks
			per cent		
O'R....	74	M	7.4	Generalized arteriosclerosis.	
Fou....	58	M	4.1	Exfoliative dermatitis, renal calculus.	
Bau....	55	M	6.6	Exfoliative dermatitis.	Liver extract.
Bec....	65	M	5.2	Arteriosclerotic heart disease.	
Wal....	76	M	6.4	Chronic dermatitis.	
Dia....	63	M	11.0	Generalized arteriosclerosis.	
Coh....	53	F	8.2	Diabetes mellitus.	
Mos....	65	F	10.9	Rheumatoid arthritis.	
Pow....	68	F	4.1	Generalized arteriosclerosis.	
Sch....	70	M	6.2	Diabetes mellitus.	
Bar....	75	M	5.0	Generalized arteriosclerosis.	
Smi....	68	M	7.6	Generalized arteriosclerosis.	
Sal....	71	M	8.8	Generalized arteriosclerosis.	
Col....	63	M	7.8	Generalized arteriosclerosis.	
McC....	70	M	5.8	Alcoholism.	Brewer's yeast, Vitamin B.
Spi....	62	M	8.8	Generalized arteriosclerosis.	
Fre....	74	M	7.0	Fibroid tuberculosis.	
Spo....	79	M	8.0	Arteriosclerotic heart disease.	
She....	70	M	4.4	Generalized arteriosclerosis.	
Bey....	64	M	6.8	Chronic dermatitis.	
Con....	64	M	9.1	Generalized arteriosclerosis.	
Din....	80	M	10.0	Pernicious anemia. Generalized arteriosclerosis.	
Rog....	74	F	6.0	Arteriosclerotic heart disease.	
Dia....	64	F	6.4	Generalized arteriosclerosis.	
D'am...	53	M	8.6	Toxic adenoma of the thyroid.	
Sci....	51	M	8.4	Peptic ulcer.	
Coh....	59	M	4.8	Diabetes mellitus.	
Min....	63	M	4.6	Diabetes mellitus.	
Wal....	63	M	9.4	Exfoliative dermatitis.	
Hel....	64	M	5.2	Hypertensive cardiovascular disease.	Repeat test 24 hours, 5.4 per cent.
Tom....	62	M	9.3	Pulmonary fibrosis.	Repeat test 24 hours, 9.4 per cent.
Mat....	74	M	1.2	Arteriosclerotic heart disease with chronic cardiac decompensation.	Repeat test at weekly intervals, 1.5 per cent, 1.4 per cent.
Cus....	65	M	2.9	Chronic bronchitis.	
Doh....	66	M	2.8	Tuberculosis of cecum.	
Rot....	63	M	2.8	Hypochondriasis.	
Boe....	67	M	1.3	Diabetes mellitus.	
Mon....	67	M	1.2	Osteoarthritis.	
Lut....	69	F	2.6	Generalized arteriosclerosis.	
Whi....	66	F	1.3	Generalized arteriosclerosis and syphilis.	
Tos....	56	F	1.0	Bronchial asthma.	
McG....	80	F	3.6	Generalized arteriosclerosis.	
Fer....	61	M	2.6	Tabes dorsalis.	
Kal....	61	M	3.5	Generalized arteriosclerosis.	
Mat....	62	F	3.4	Diabetes mellitus.	Repeat test, one week later, 6.4 per cent.

TABLE IV

Influence of age on pyridoxine output

Group	Number of patients	Urinary output average	Deviation	
			Standard	Maximum
		per cent	*per cent*	
5–15	10	21.3	±3.68	6.0
16–50	13	8.4	±2.04	3.1
	2	2.7		
Over 50	32	7.2	±1.87	3.8
	13	2.3		

In seven patients with Parkinson's syndrome, five had a definitely low pyridoxine urinary output, and a sixth was low normal. Six of these showed an average output of 2.5 per cent (range 0.5 to 4.6 per cent), while one had a normal output of 10.7 per cent. Tests repeated one month later showed an increase to normal in two previously low patients, while the borderline normal now showed a diminished excretion. The complaints of these patients were considered to be sequelae of previous encephalitis (Table V).

TABLE V

Influence of post-encephalitic Parkinson's syndrome on pyridoxine output

Patient	Age	Sex	Pyridoxine output	Renal impairment	Remarks
			per cent		
Pin.....	55	F	10.7*	0	
Fei.....	60	F	0.6 6.6	0	Repeat test, one month later.
McG.....	51	M	2.5	0	
Gol.....	51	M	0.5	0	
Psy.....	42	F	4.6 1.6	0	Repeat test, one month later.
Bor.....	34	F	3.8 5.3	0	Repeat test, one month later.
Eng.....	30	F	3.2	0	

Average output 6 cases—2.5 per cent.
* Not included in average.

Fourteen patients with varying degrees of renal insufficiency showed an average output of only 2.2 per cent (range 0.0 to 5.4 per cent). Twelve of these (88 per cent) gave definitely low response, while the remaining two had a low normal output (Table VI).

TABLE VI

Influence of renal impairment on pyridoxine output

Patient	Age	Sex	Pyridoxine output	Diagnosis	Renal impairment	Remarks
			per cent			
Din	83	M	3.1 2.0	Osteoarthritis, generalized arteriosclerosis.	+	Repeat tests, two months.
Blo	74	M	1.2 1.6	Diabetes mellitus, generalized arteriosclerosis.	±	Repeat tests, one week.
Sim	71	M	3.0 2.2	Diabetes mellitus, generalized arteriosclerosis.	±	Repeat tests, one week.
Bro	70	M	1.9	Generalized arteriosclerosis.	±	
Bro	70	M	1.2	Spinal degeneration with generalized arteriosclerosis.	++	
O'Da	67	M	0.6 0.4	Primary contracted kidney.	+++	Repeat tests, two months.
Wei	64	M	1.6	Generalized arteriosclerosis.	++	
Exe	59	F	1.6	Hypertensive cardiovascular disease.	++	
Cre	55	M	3.2	Hypertensive cardiovascular disease.	+++	
Bra	51	F	0.0	Chronic diffuse glomerular nephritis.	++++	Uremia.
Ric	47	M	1.4	Chronic diffuse glomerular nephritis.	+++	
Fle	42	F	4.8 3.2	Chronic diffuse glomerular nephritis.	++	Repeat tests, three months.
Ros	25	M	2.0 0.7	Chronic diffuse glomerular nephritis.	++++	Repeat tests, three months.
Kra	20	M	5.4	Chronic diffuse glomerular nephritis.	+	

Average output 2.2 per cent.

DISCUSSION

It is interesting to note that when cases of renal insufficiency are excluded, thirteen of fifteen adult patients under 50 years of age have an average pyridoxine output of 8.4 per cent; ± 2.04 per cent, which corresponds very closely to the normal values previously reported. In individuals over 50 years of age, however, only thirty-two of forty-five patients showed an excretion of pyridoxine in the urine which approximated the normal range. The remaining thirteen patients had an average output of 2.3 per cent which is well below the accepted normal.

The increased urinary output in the patients from 5 to 15 years of age probably implies that the 50 mgm. dose is excessive in relation to body weight. Investigations to determine the optimum dose per unit of body weight will be undertaken.

While the effect of age on the urinary excretion of pyridoxine is of great interest, it apparently is not a factor influencing the low average output found in six of seven patients with Parkinson's disease. It is worthy of note that the patients with parkinsonism in this series were post-encephalitic in type. Jolliffe (14), however, found pyridoxine to be a more effective therapeutic agent in the senile type of parkinsonism than in the post-encephalitic variety.

The low output encountered in patients with renal insufficiency is not surprising, and serves to emphasize the importance of knowing the renal status of all patients.

SUMMARY

Following the intravenous administration of a test dose of pyridoxine, the urinary excretion of this vitamin was studied 98 times in eighty-four patients. Twelve of fourteen individuals (88 per cent) under 50 years of age excreted in one hour an average of 8.4 per cent of the amount injected. Thirty-two of forty-five patients (71 per cent) over 50 years of age showed an average output of 7.2 per cent; the remaining thirteen subjects (29 per cent) excreted an average of only 2.3 per cent.

Ten patients between 5 and 15 years of age eliminated an average of 21.3 per cent of the amount of pyridoxine injected. This observation suggests the advisability of using a test dose based on body weight.

Six of seven patients with post-encephalitic parkinsonism showed a diminished output of pyridoxine averaging 2.5 per cent. Twelve of fourteen patients with varying degrees of renal insufficiency demonstrated a definite impairment in the excretion of pyridoxine, while the remaining two had a low normal output.

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AN ANTILACTOGENIC EFFECT OF PYRIDOXINE

BY

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Summary

The efficacy of pyridoxine in the suppression of lactation has been tested in a placebo-controlled, double-blind trial of 254 puerperal patients. Ninety-five per cent of the women treated with pyridoxine had their lactation successfully suppressed within one week, compared to 83 per cent for stilboestrol and 17 per cent for the placebo.

ŒSTROGEN has been used for many years in the suppression of lactation, but has been criticized because of endometrial withdrawal bleeding, rebound filling of the breasts, and an increased likelihood of puerperal thromboembolism (Daniel *et al.*, 1967; Jeffcoate *et al.*, 1968).

Since Kamberi *et al.* (1970) observed that prolactin, the hormone primarily responsible for milk secretion, is markedly inhibited by increased hypothalamic concentrations of dopamine, and since Wurtman (1966 and 1970) demonstrated in animals that brain concentrations of dopamine can be increased by the administration of its precursor L-DOPA, it has been suggested that a similar effect might be obtained in man. Indeed, Friesen *et al.* (1972) and Turkington (1972) succeeded in reducing prolactin secretion by the administration of L-DOPA, in cases where elevated prolactin levels were accompanied by non-puerperal galactorrhea.

In order to avoid the risk of side-effects of this drug for the patient, it has been suggested that an increase in the hypothalamic content of dopamine could be achieved in lactating women by promoting the natural conversion of DOPA to dopamine through the administration of pyridoxine, which in the form of pyridoxal phosphate serves as a coenzyme of DOPA decarboxylase.

MATERIAL AND METHODS

Three identical tablets were used containing either a placebo, 5 mg. of stilboestrol or 200 mg. of pyridoxine. A course of treatment consisted of one tablet three times a day for six days, beginning on the second or third day after delivery so that women with deficiency or failure of lactation were excluded. The code was not

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TABLE I
Time taken for lactation to cease

Drug	Number of patients	Per cent of patients in whom lactation ceased within:			
		1 week	2 weeks	3 weeks	4 or more weeks
Placebo	86	17	45	28	10
Stilboestrol	68	83	12	5	—
Pyridoxine (blind)	75	93	7	—	—
Pyridoxine (known)	25	100	—	—	—

broken until the trial was complete. A form was filled in by the nurse, which required observations for six days on the state of the breasts and the amount of milk secretion. The nurse was instructed to administer analgesics as needed so that no patient suffered unnecessary pain and if the breasts remained tender and congested, a further course of treatment was to be given at home. All decisions were taken by the nurse in charge in association with the medical staff of the ward responsible for the follow-up of the patients.

Of 375 questionnaires issued 70 were not completed and 51 were inadequately filled in. The 254 patients for whom full information was available were divided into four groups: 86 patients were given the placebo; 68 were given stilboestrol; and 75 were given pyridoxine. A fourth group of 25 patients were knowingly given pyridoxine in the same dosage as those in the blind trial. Each patient was asked to return another form about one month after delivery, providing information about further treatment by the family doctor and about the time when the breasts ceased to secrete milk.

Results

Table I shows that, judged in terms of cessation of lactation within one week, pyridoxine (5 per cent failures) was more effective than stilboestrol (17 per cent failures) and markedly more effective than the placebo (83 per cent failures). The speed with which symptoms were relieved also differed conspicuously: this period was 10 to 12 hours for the patients given pyridoxine, over 24 hours for the patients given stilboestrol, several days for those given placebo. The relief of painful engorgement was confirmed by clinical examination of the breasts.

Patients given stilboestrol experienced rebound filling three times as often as those given pyridoxine. The Table shows that in most women lactation ceased during the second or third week postpartum whatever the treatment, probably because of diminishing prolactin secretion during this time, but with pyridoxine only a few women continued to secrete milk during the second week and none thereafter. Of 100 patients treated with pyridoxine only three needed a further course of treatment. With two others milk secretion persisted, although without any congestion. After their release from the clinic and contrary to our advice these patients had continued to empty their breasts by expression. After the second course of pyridoxine lactation ceased completely in all five patients.

In order to reduce the congestion and the attendant discomfort to a minimum we now begin the pyridoxine course within 24 hours of delivery and the results confirm the experience of the trial. There has been no excess vaginal bleeding or delay in the return of menstruation. With the lessening of engorgement the daily pyridoxine dosage can be reduced to 300 mg.

Discussion

These results indicate that the oral administration of pyridoxine was associated with a rapid and trouble-free suppression of milk secretion in 95 per cent of lactating women. The exact mechanism by which pyridoxine inhibits milk secretion is not known. In its form of pyridoxal phosphate it can promote, as co-enzyme of DOPA decarboxylase, the conversion of the intraneuronally preformed DOPA to dopamine. Pyridoxine can be taken up by the hypothalamic neurones either by the same potent amine-concentrating mechanism adherent to the cell membrane (Fuxe and Ungerstedt, 1966; Fuxe and Hökfelt, 1969) or by diffusion. A rise of pyridoxine may therefore increase the formation of dopamine in neurones and their major dendrites. The experimental findings of Kamberi *et al.* (1970) would suggest that such an increase of the dopamine content in the hypothalamus might lead to the inhibition of prolactin, probably through stimulation of prolactin inhibiting factor.

From the therapeutic results in the present series further experience in the use of pyridoxine would appear warranted and may lead to a better understanding of the mechanism for the regulation and release of prolactin.

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Isoniazid: A Review with Emphasis on Adverse Effects

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A review of the adverse effects of isoniazid (INH) was undertaken and it was found that these effects could be divided into: (1) toxic, (2) idiosyncratic and (3) hypersensitivity reactions. Major toxic reactions are peripheral neuropathies at conventional dosage and seizures from overdosage. Pyridoxine is of benefit in these disorders. Other presumed toxic reactions are autonomic neuropathy, encephalopathy, psychosis, optic atrophy, and pyridoxine responsive anemia. Idiosyncratic reactions are INH-induced lupus erythematosus, rheumatic-like syndromes and various hematologic disorders. These respond promptly to withdrawal of the drug. Hypersensitivity reactions to INH include hepatitis, dermatitis, fever, angiitis and hemolytic anemia. These reactions promptly subside with withdrawal of the drug although in mild reactions this may not be necessary. Corticosteroid therapy or desensitization can be used if INH needs to be readministered.

Isoniazid (INH) is one of the primary drugs used in the treatment of tuberculosis and is of definite value in preventive therapy (chemoprophylaxis). There has been no comprehensive review of the multisystem adverse effects of this drug in recent years. It is with this in mind that the following is presented, as we can expect to see more of these effects and perhaps new ones in the future.

Pharmacology

Isonicotinic acid hydrazide (isoniazid) is rapidly absorbed both orally and parenterally so that the peak plasma level occurs one to two hours after an oral dose. The drug is distributed to all organs and can be recovered from ascitic, pleural, and cerebrospinal fluid as well as from caseous material. INH is both bacteriostatic and bactericidal to suceptible strains of *Mycobacterium tuberculosis*, but the exact mode of action is unknown.

INH is metabolized in the liver by an acetylation process which is under genetic control. Plasma levels of isoniazid six hours after an oral dose are bimodal. "Slow" inactivation of isoniazid is probably an autosomal recessive trait and people with this have higher blood levels of the free compound than autosomal dominant "rapid" inactivators. Para-aminosalicylic acid (PAS) and severe liver disease also reduce the acetylation of INH. INH is excreted primarily in the urine in both the free and acetylated form. Consequently, the dosage of INH may need to be reduced in uremia.

Untoward effects of INH are dose related: about 1 percent to 2 percent occurring at conventional low dose therapy (3 to 5 mg/kg/day) and 15 percent at 10 mg/kg/day. The adverse effects of INH can be roughly divided into:

1. *Side Effect*—an undesirable pharmacologic action experienced by some people, such as epigastric distress.

2. *Idiosyncracy*—an individual genetic defect that causes a qualitative abnormal response, such as the lupus phenomenon.

3. *Toxicity*—an effect which is an expected pharmacologic action that will occur in a majority of patients at a given dose, such as the neurologic effects of INH.

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4. *Allergic Reaction*—an immunologic response such as drug fever, dermatitis, and hepatitis.

Other untoward effects, such as some hematologic abnormalities and diphenylhydantoin intoxication do not conveniently fit into the above schema.

INH-Induced Neurologic Syndromes

The earliest known and most widely recognized untoward effects of INH are the neurologic syndromes. Foremost among these is peripheral neuropathy. In the fully developed syndrome, the patient first complains of numbness or tingling of the feet. If the drug is withdrawn at this point, the symptoms will abate. However, with continuation of the drug, these paresthesias will spread up the legs to the knees and possibly even develop in the hands or fingers in a typical bilateral stocking-glove distribution. Frequently these symptoms are accompanied by muscle aching, which is made worse by activity. While sensory complaints dominate, weakness and rarely ataxia can be seen. The physical examination reveals sensory and possibly motor involvement primarily of the lower extremities. The modalities of pain, touch, and temperature are most frequently affected, while position and vibratory sense are rarely impaired. Other findings can include a decrease or absence of the achilles reflex and a hyper- or hypoactive patellar reflex. Muscle weakness is a minor component, but muscle atrophy and fasciculation can be observed late in the course. Unless the syndrome has progressed to obvious muscle weakness, all findings are reversible within a few weeks after withdrawal of the drug. If despite the appearance of symptoms, therapy is continued for more than a few weeks, residual difficulties may persist for a year or more.

This syndrome is dose related and susceptibility is highest in chronic alcoholics, individuals with malnutrition and "slow" inactivators. With the commonly employed dose of 300 mg/day (3 to 5 mg/kg), an incidence of up to 2 percent of patients should be expected. The time of onset of symptoms after the institution of INH therapy varies roughly with the dosage. With high doses of INH, symptoms often appear within three to five weeks. In patients receiving conventional low dose INH therapy, symptoms usually do not appear until six months. Pyridoxine, 50 mg daily, can prevent the occurrence of peripheral neuropathy in the high susceptibility groups.

Symptoms of pellagra, including skin lesions, cheilosis, watery stools, and mental changes may occur following the administration of INH and are responsive to the administration of nicotinamide.

There was early recognition of autonomic dysfunction following therapy with INH. Symptoms of dry mouth, difficulties in visual accommodation, constipation, frequent erection, nocturnal emission, frequency of urination, difficulty in micturition, and frank urinary obstruction were noted with low-dosage INH therapy. Reversal of symptoms on withdrawal of the drug is to be expected. Symptoms of vasomotor instability such as flushing of the face and Raynaud's phenomenon rarely occur.

Central nervous system stimulation due to INH can cause irritability, euphoria, restlessness, insomnia, and headache. Central nervous system depression causing lethargy and drowsiness also occurs. Psychological effects from INH are not uncommon. Patients may complain of depression, unbalanced state of mind, or impairment of memory. In a prospective study, special memory tests showed a statistically significant impairment of memory after several months of ambulatory treatment. Symptoms disappeared a few months after treatment had been stopped.[1]

In a recent report, seven alcoholic patients who were treated with conventional low dosage INH for 30 days without complications developed changes in their effect or behavior when begun on disulfiram (antabuse) therapy.[2] These changes were not seen in comparable patients on disulfiram who were not put on INH therapy. It was felt that both disulfiram and INH interfered with the metabolism of biologic amines in the central nervous system and in combination caused mental changes. With time, other drugs will probably be shown to interact with the biologic effects of INH.

The appearance of acute psychosis following administration of INH is most often seen in those who have an antecedent history of unstable personality, although it can occur in otherwise normal people. Weirdorn and Ervin[3] reported on five cases of INH-induced acute psychosis. They noted increased psychomotor activity about one month prior to the onset of the acute psychosis. These changes consisted of restlessness, irritability, and a heightened awareness of surrounding visual and auditory stimuli. None of the patients had any awareness of these new symptoms. During the acute phase, purposeless, poorly coordinated muscle activity and gross muscle jerking were frequently noted. In addition, disorientation to time, place and person, and hallucinatory experiences, usually persecutory in content, were noted. Withdrawal of INH will usually lead to a reversal of psychosis; however, this is not invariable.

Although INH has no definite effect on EEG tracings, it should be used with caution in those

with known seizure history or with previous head trauma. Death or prolonged coma from persistent seizure activity can follow the administration of conventional low dosage INH to patients with known seizure history. Anticonvulsants, especially phenobarbital, usually are effective in preventing these seizures.

It has long been recognized that diphenylhydantoin (Dilantin, DPH) intoxication occurs at the commonly prescribed dose of 300 mg per day when INH is given concomitantly. Kutt and coworkers[4] described 11 percent DPH intolerance in 72 patients taking concomitant INH. There was no correlation with hepatic function tests, nor any age or sex predilection. They confirmed that this occurred only in slow inactivators, and in vitro rat studies revealed INH to be a strong noncompetitive inhibitor of DPH metabolism. PAS potentiated this inhibition of DPH metabolism by INH.

Treatment of DPH intoxication in a patient receiving INH consists of discontinuing DPH for five to ten days and then restarting it at 100 to 200 mg per day.

Other rare but serious neurologic complications of INH therapy are toxic myelopathy, encephalopathy, and optic neuritis. These may or may not respond to withdrawal of INH. Pyridoxine and steroids may be beneficial.

Overdosage

With widespread use of INH, it was inevitable that both accidental and intentional overdosages would occur.

Terman and Teitelbaum[5] reported four patients treated for INH "self poisoning." The clinical syndrome consisted of:

1. Coma developing one to three hours after ingestion often with respiratory depression.
2. Status epilepticus
3. Severe metabolic acidosis
4. Hyperglycemia and acetonuria
5. Occasionally hyperpyrexia

Treatment of INH overdosage is similar to other intoxications. In four severely ill patients, Terman successfully treated two with hemodialysis, one with peritoneal dialysis, and one with forced diuresis. The rates of recovery of INH were not cited.

Treatment of seizures associated with INH overdosage has been controversial. Standard anticonvulsants have been used. Katz and Jobin[6] at the Alaska Native Medical Center treated 22 INH ingestions with up to 10 gm of intravenous pyridoxine with prompt termination of seizures and without any evidence of pyridoxine toxicity. Among Terman's patients, some took INH-pyridoxine combination tablets and they did not find additional intravenous pyridoxine useful. They found the seizures unresponsive to anticonvulsants until the metabolic acidosis was corrected by bicarbonate.

INH-Induced Rheumatic Syndromes

The association of INH and rheumatic syndromes has recently been discussed by Good.[7] Typically, the patients experienced sudden onset of joint pain, tenderness, and morning stiffness in the hands, especially the proximal interphalangeal joints. This occurred from five days to 20 weeks after institution of INH. Other joints affected include the metacarpal-phalangeals, elbows, wrists, shoulders, hips and spine. Within a few days, some developed marked limitation of motion leading to complications such as frozen shoulder and tendon contractures in the hands. There was no evidence of synovial effusions although diffuse swelling of the fingers was sometimes seen. No evidence for an underlying rheumatic disease could be detected. Negative findings included antistreptolysin 0 titers, LE cell preparations, serum uric acid levels, rheumatoid factors, and roentgenograms of involved joints. Ethionamide, a drug similar in structure to INH, has also been thought to produce rheumatic symptoms in tuberculosis patients.

The association of INH and this rheumatic syndrome is based purely on the temporal relation of the appearance of the symptoms and the administration of the drug. This syndrome in well-nourished, mobile individuals on conventional low dosage INH should not be a frequent finding. Although some patients in whom INH is not discontinued will demonstrate slow improvement with salicylates and physical therapy, the rapid improvement on withdrawal of the drug and severe disability that could result from long-term administration of INH make it mandatory that this syndrome be recognized.

INH-Induced Lupus Syndromes

Since the advent of the lupus erythematosus cell phenomenon, a growing list of drugs known to induce the lupus erythematosus syndrome (LE) has been compiled. These drugs fall into two categories:[8] (1) those that bring about the lupus syndrome by eliciting an allergic reaction such as penicillin, sulfonamides, tetracycline, oral contraceptives, propylthiouracil, reserpine, and methyldopa and (2) those that induce LE by having some specific pharmalogic property such as hydralazine, anticonvulsants, procainamide hydrochloride, and INH.

The drug-induced syndromes closely resemble

those of the spontaneously occurring types. Drug-induced lupus shows a predominance of cases in women as does the idiopathic form. Although there is a predominance of Negroes in spontaneous LE, no racial predilection was found for the drug-induced forms.

Although over 20 cases of suspected INH-induced LE have been reported in the literature, there has not been enough clinical information given about most of the cases to determine whether any distinct clinical pattern will appear. A few distinct differences have been apparent with the other drug-induced forms, such as the predominance of pleuropulmonary symptoms in procainamide lupus, the paucity of anemia and leukopenia in both hydralazine and procainamide lupus, and the lower incidence of renal manifestations in all of the drug-induced forms.

The first case of INH-induced lupus syndrome was reported in 1966 by Zingale and co-workers[9] in a patient who had received approximately two months of antituberculosis therapy including 400 mg of INH daily. The patient developed fever, arthritis, bilateral pleural effusions and electrocardiographic evidence of evolving pericarditis. Laboratory findings included a relative leukopenia and many strongly positive LE cells. Serum protein electrophoresis demonstrated elevated alpha 2 and beta globulin fractions, and a positive antinuclear antibody (ANA) was found. The patient clinically improved after INH was discontinued and prednisone begun. Several months after discontinuing INH, LE cell tests and ANA tests were negative.

The drug-induced form of LE is usually reversible on withdrawal of the precipitating agent. However in some cases, clinical manifestations and positive ANA tests have persisted for years.

Strong evidence supporting the LE cell inducing ability of INH has been obtained from serologic studies in tuberculosis patients. Cannat and Seligmann[10] found the incidence of ANA significantly higher in tuberculosis patients after prolonged therapy with INH (19 percent) than in untreated patients or healthy controls (2 percent) matched for sex and age. Some patients were followed from a pretreatment ANA negative status to an ANA positive status three to six months after treatment was begun, although no clinical manifestations of LE appeared. Mice studies have emphasized the importance of age, sex and strain on the experimental induction of ANA, suggesting that genetic factors play a major role in addition to environmental factors in the drug-induced lupus cell phenomenon. The genetic influences may be weak, requiring environmental influences to bring about either clinically silent or overt LE. A genetically predisposed individual who takes INH, a relatively weak LE-inducer, may reach a clinically silent stage manifested only by serologic abnormality; or, the same individual if exposed to hydralazine or procainamide, two stronger LE inducers, may develop the full-blown clinical syndrome. An individual with strong genetic predisposition may reach the second or symptomatic-stage with little or no environmental stimulation. Alarcón-Segovia[8] likens these two stages to those of prediabetes and diabetes. The clinical spectrum results from the interplay between genetic predisposition and the strength of environmental factors.

The mechanism by which any drug may activate the LE syndrome is to date unknown. Recently, however, there has been some interesting experimental work that may shed light on a possible mechanism for INH, hydralazine and possibly other drugs. Both INH and hydralazine are acetylated in the liver by the hepatic enzyme acetyl transferase. Perry and associates[11] found that as with INH, there were fast and slow acetylators of hydralazine and that the induction of ANA by this drug was related to both the rate of acetylation and the total intake of drug. Few fast acetylators had a positive ANA after 400 to 1200 gm of hydralazine. Of those who developed the LE syndrome, all were slow acetylators. Whether only slow acetylators of INH will develop the lupus syndrome remains to be determined.

The evidence for the relationship between INH and overt LE or the prelupus state manifested only by serologic abnormalities seems quite strong at this time. Clinical recognition of the syndrome followed by the quick withdrawal of the drug should lead to prompt remission in most cases. What the far-reaching clinical implications are of such variables as rapid and slow INH acetylation, family predisposition to LE, and high and low dose regimen, remains to be worked out.

Hematology

Isoniazid has been associated with a variety of hematologic abnormalities; however, in most cases, other antituberculosis drugs were given concomitantly and a cause and effect relationship is hard to establish. Those abnormalities with which INH has been associated are: hemolytic anemia, pyridoxine-responsive anemia, agranulocytosis and red cell aplasia.

Hemolytic anemia due to INH has recently been reported.[12] A three and one-half year-old Negro boy was treated with INH 15 mg/kg per day for

one year at which time he developed fever, abdominal pain, and hemoglobinuria. Laboratory examination revealed a Coombs'-positive hemolytic anemia. Discontinuation of INH and insitution of prednisone reversed the hemolysis.

The pathogenesis of isoniazid induced pyridoxine-responsive anemia is unclear. The anemia is usually microcytic and hypochromic and generally develops 4 to 16 weeks after institution of INH. Serum iron is usually normal to increased with a normal iron binding capacity and plentiful iron stores in the bone marrow. Bone marrow examinations have usually shown normoblastic hyperplasia with maturation arrest of red cells. McCurdy and Donohoe[13] described three patients who also had target cells and two demonstrated Howell-Jolly bodies. Two of these also demonstrated low erythrocyte GOT levels. In a few cases of pyridoxine-responsive anemia, the anemia has been macrocytic with megalobastic bone marrow.

Treatment consists of parenteral pyridoxine 10 to 100 mg per day. INH may be continued. Response is manifested by reticulocytosis and a rise in hemoglobin, but the response is usually incomplete. Red cell morphology often does not return to normal. Some of the cases that are megaloblastic appear to respond partially to folic acid and revert to a normoblastic bone marrow and then respond more completely to pyridoxine. McCurdy and Donohoe noted central nervous system depression in two of their three cases following parenteral pyridoxine and this should be watched for. In addition, they treated two of their patients with Nupa-sal (0-hydro-benzal isonicotinyl hydrazone) after which the hematocrit remained stable inspite of the discontinuation of pyridoxine. Nupa-sal is apparently metabolized to INH and is not felt to be a pyridoxine antagonist.

Goodman and Block[14] in 1964 reported a case of red cell aplasia in a 32-year-old diabetic man treated for tuberculosis with INH 250 mg/day, pridoxine 100 mg/day and PAS 8 mg/day. This patient's hemoglobin fell from 18 gm percent to 4.9 gm percent after seven months therapy at which time his direct Coombs' test was 3+ and his Cr 51 red blood cell survival was 18 days. Bone marrow revealed aplasia of red blood cells. The medications were discontinued after which his hemoglobin and bone marrow rapidly returned to normal. The investigators thought that INH was the culprit, but the possibility of PAS induced aplasia or drug induced hemolytic crisis cannot be discarded.

Isoniazid has also been incriminated as a possible cause of agranulocytosis.[15] A four and one-half-year-old child with tuberculous meningitis was treated initially with INH, PAS, and streptomycin for 18 days, then INH alone at a dose of 8 mg/kg per day. After ten weeks, she developed a skin infection treated with penicillin. Two weeks later the skin infection recurred at which time a complete blood count (CBC) was normal. The infection cleared spontaneously but two weeks later recurred and a CBC revealed white blood cell count of 4900 with 1 percent polymorphonuclear leukocytes (PMN's), normal hematocrit, and normal platelet count. Penicillin and aureomycin were administered and INH was discontinued. Two days later a bone marrow examination revealed active granulopoiesis and three days later the CBC revealed 6698 PMN's per mm³.

Hypersensitivity

Isoniazid, like other antituberculosis drugs, causes a significant number of hypersensitivity (allergic) reactions. This reaction may be manifested by fever, rash, arthralgias, angiitis, hepatitis, adenopathy, eosinophilia, or leukopenia.

The incidence of this response is hard to determine because in most studies INH was given with other drugs which can elicit hypersensitivity, and once hypersensitivity develops the patient often will become hypersensitive to all drugs given.

In a retrospective study[16] of 1744 patients hospitalized for treatment of tuberculosis, "drug intolerance" developed in 11.1 percent; streptomycin intolerance occurred in 10.3 per cent of 400 patients, PAS intolerance in 8.8 percent of 1698 patients, and INH intolerance in 1.3 percent of 1724 patients. Of 792 patients taking high dose INH (usually 16 mg/kg), 20 developed intolerance, whereas only two of 932 patients who received 100 mg tid developed intolerance. Of the reactions attributed to INH, fever accounted for 13, dermatologic manifestations for nine, hepatitis for six (four of the six had fever and rash concomitantly), leukopenia for two and arthralgia for one. These reactions generally occurred during the first 60 days of treatment. Desensitization was successful in all 12 in whom it was used.

Liver

Liver abnormalities may be caused by tuberculous granulomatous hepatitis or by a hypersensitivity response to one or several drugs. Tuberculous granulomas are commonly found in liver biopsy, in both acute and chronic tuberculosis, presumably from hematogenous seeding. They are most common in miliary tuberculosis, but rarely cause clinical jaundice. In patients with chronic pulmonary tuberculosis without clinical evidence of extrapulmonary disease, hepatic function tests including bilirubin,

SGOT and SGPT are usually normal although liver biopsy is often abnormal.

Drug-induced hepatic injury may be caused by 1) a direct hepatotoxin, *ie* CCL_4 which is a protoplasmic poison, its toxicity is dose related, and after a brief interval produces a characteristic lesion on liver biopsy, 2) an indirect hepatotoxin which inhibits essential metabolites, and 3) a hypersensitivity reaction which has a low incidence, is not dose related, and follows a sensitization period (usually one to four weeks). This hypersensitivity reaction tends to recur promptly on readministration of small doses and is frequently accompanied by fever, rash, urticaria, eosinophilia, and sometimes blood dyscrasias. The liver involvement may be hepatocellular or cholestatic.

Several antituberculosis drugs are known to cause hepatotoxicity: INH, PAS, pyrazinamide and ethionamide. INH causes a hepatocellular type of hypersensitivity reaction and histologically may be indistinguishable from viral hepatitis. Cohen and coworkers[17] in 1961, reviewed all six previous cases of supposed INH induced jaundice and found that five of them had also received PAS. Jaundice occurred nine days to seven weeks after starting treatment. In addition, they reported a case of a 50-year-old man with sarcoidosis who received INH, NPH insulin, and methylprednisolone (Medrol). He developed a rise in SGOT and INH was withheld. SGOT returned to normal in two weeks, but later INH was restarted by mistake and he developed "hepatitis" and lapsed into hepatic coma and died. Autopsy revealed "acute massive necrosis of the liver," and no evidence of hepatic sarcoid was found.

Sharer and Smith,[18] in part of a prospective study, noted that in 90 employees given chemoprophylaxis with INH, 11 (12.2 percent) developed SGOT and SGPT levels greater than 50 in the first two months, nine of whom were asymptomatic. This occurred from 14 to 60 days after beginning INH (average of 37 days) and the SGOT ranged from 65 to 160, SGPT from 76 to 500. Bilirubin and alkaline phosphatase levels when measured were normal. In two of nine asymptomatic patients, INH was discontinued because of marked enzyme elevations and the SGOT returned to normal in 14 and 60 days. In the other asymptomatic cases, INH was continued and the SGOT returned to normal in 14 to 120 days (average 50 days). In part of the same study among 77 hospitalized patients being treated for tuberculosis, INH was felt to be the cause of transaminase elevations in seven, and was discontinued. In one of those cases, the SGOT was 600 and the SGPT 900. Desensitization with small progressing doses, was no more successful in preventing enzyme changes than readministration of full dosage. INH was restarted without further enzyme rises after as many as four trials.

After an outbreak of tuberculosis early in 1970 on Capitol Hill, 2,321 people with positive tuberculin tests were placed on INH chemoprophylaxis.[19] On November 19, 1970, the deaths of two INH recipients from liver disease were reported. Further studies identified 17 others who had a history of jaundice or dark urine without known exposure to needles, blood products, raw clams, or raw oysters. These cases were noted throughout the nine-month period of therapy. Six cases had negative tests for Australian antigen. Whether or not INH can be incriminated is conjectural at this point, but this report raises the question whether INH induced hepatitis can occur after the three month interval commonly cited.

A very interesting recent report[20] describes a 42-year-old man being treated with INH, PAS, and streptomycin who, after one month, developed fever, rash, eosinophilia and adenopathy, and subsequently jaundice shortly after the drugs were discontinued. Skin tests with INH and PAS were unreactive as were antigen induced leukocyte histamine release and serum reagins: INH however caused a positive lymphocyte transformation test with increased incorporation of radioactive thymidine. Streptomycin caused less transformation and PAS caused an insignificant amount. After desensitization, there was no transformation with INH. The patient's son who also had INH allergy manifested by fever and rash without hepatitis had a negative lymphocyte stimulation test. This report supports the view that INH induced hepatitis in the full blown allergic syndrome is due to a delayed type of hypersensitivity. Further studies need to be carried out with patients who develop asymptomatic SGOT elevations to see if the same mechanism is operative.

Conclusions

Isoniazid is presently in widespread usage. The adverse effects may be generally divided into 1) toxic effects which are dose related and 2) idiosyncratic and allergic reactions which are not dose related.

Toxic effects are predisposed to by liver disease, PAS and genetically "slow" acetylation which by reducing the metabolism of INH raise the serum level. Uremia, by reducing excretion of INH, raises the serum level. Alcoholism, malnutrition and pyridoxine deficiency also seem to predispose to INH toxicity.

Foremost among the toxic effects of INH are the neurologic syndromes. Peripheral neuropathy is definitely dose related: rare in conventional low dosage INH therapy except in alcoholic or severely malnourished patients and of considerably higher frequency in high dosage INH therapy. Although the mechanism of this neuropathy is unknown, pyridoxine administration will prevent or treat it. Consequently, patients on high dosage INH and alcoholic or malnourished patients on low dosage INH should receive pyridoxine 10 mg per 100 mg INH.

Seizures also are dose related. This is dramatically seen in INH overdosage. Known epileptics should be maintained on anticonvulsant medications especially phenobarbital even when given low dosage INH therapy. One must be alert to the possibility of diphenylhydantoin intoxication and reduce the dosage accordingly.

Encephalopathy, psychosis, memory impairment, central stimulation and depression, autonomic dysfunction and optic atrophy are believed to be toxicities of INH although there is no evidence to show they are definitely dose related. Pyridoxine has been used in the treatment of optic neuritis. Pyridoxine seems to have no effect in preventing psychosis, encephalopathy, dizziness, or ataxia.

Pyridoxine-responsive anemia, although not proved to be dose related, would seem to be a toxic effect. The interactions of INH and pyridoxine remain to be fully discovered. The anemia simulates iron deficiency, but serum iron is normal and marrow iron plentiful. Treatment consists of adding pyridoxine 10 to 100 mg per day and, in those few cases that are megaloblastic, folic acid appears to be beneficial.

Idiosyncratic and hypersensitivity reactions are not dose related. Those that seem to be idiosyncratic reactions are the lupus syndrome, rheumatologic abnormalities, agranulocytosis, and red cell aplasia. Because of the severity of these reactions and the availability of other antituberculosis drugs, INH should be discontinued in these circumstances.

Hypersensitivity reactions include fever, rash, eosinophilia, hemolytic anemia, angiitis, and hepatitis. It is important to recognize that these may occur singly or in combination, and treatment depends on the severity of the reaction. In some cases, the drug may be continued and the reaction may spontaneously subside as in INH induced asymptomatic hepatitis. In more severe reactions, such as florid hepatitis, hemolysis, angiitis or severe skin reactions, the drug should be discontinued and antihistamines or steroids may be given. Depending on the indications for INH, the severity of the reaction and the availability of other effective antituberculous agents, a decision should be reached whether to desensitize the patient or administer other agents. After discontinuation of INH so that fever, rash, etc have subsided, desensitization consists of administering small progressive amounts of INH until the therapeutic dose is reached or the reaction recurs. Densensitization may require several attempts before being successful. Steroids may be given concomitantly for rapid desensitization.

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MARKED AND SUSTAINED BLOOD CHOLESTEROL LOWERING EFFECT BY MEDICATION WITH NIACIN AND PYRIDOXINE

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Altschul, Hoffer and Stephen[1] reported in 1955 the original finding of hypocholesterolemic effect of large doses of nicotinic acid in man. Their observation found immediate clinical application by Parsons, Jr. and his associates at the Mayo Clinic[5], who administered daily doses of 3 to 6 gm. nicotinic acid to 20 patients with hypercholesterolemias of various types and obtained marked and sustained decreases in blood cholesterol levels in all instances.

The great clinical implications of these findings are obvious. Any agent which promises to induce a lowering of the blood cholesterol level deserves thorough investigation, in view of the role which hypercholesterolemia is likely to play in the development of atherosclerosis. Attempts to elucidate the basic mechanism of this hitherto unknown action of nicotinic acid are needed as much as further extension of the clinical application.

The present study was undertaken to add such observations. While it was in progress, a second report was published by Parsons and Flinn[6], extending and confirming their previous observations. Our own findings are in full agreement with these reports and give further evidence of the efficacy of nicotinic acid medication in lowering elevated blood cholesterol levels. They also add information about the effect of these large doses of niacin on liver function and carbohydrate metabolism, and about an apparent potentiation of the effect of niacin by combining it with pyridoxine.

Materials and Methods. The patient material. Fifty-three patients were studied. 45 were inpatients of the Jewish Chronic Disease Hospital and 8 were outpatients. Thirty-five of the patients were females and 18 males. Their ages varied between 50 and 96 years with an average age for the female group of 63 years and for the males of 56 years. All patients had clinical evidence of advanced degenerative vascular diseases, most had hypertension, 8 had in addition arteriosclerotic Parkinsonism, 10 had diabetes mellitus, 12 were hemiplegics, 3 were hypothyroids and one suffered from familial hypercholesterolemia and hyperlipemia. All patients were well known to us for at least one year and their serum cholesterol level had been established and checked by repeated examinations. In all but 5 patients the serum cholesterol level was above 250 mg. per 100 ml. Twenty of these patients have been under continuous treatment for more than 10 weeks. Others have been either under interrupted treatment, or have been on medication for less than 10 weeks.

The medication. In a preliminary group nicotinic acid in capsules of 50, 100, and 500 mg. each was used, 1 to 3 t.i.d. Many patients objected to this treatment, not only because of the attendant flushing, but also because it involved the ingestion of a considerable number of capsules. Since most of our geriatric patients are on medication with Rauwolfia or pentylenetetrazole, or both, and receive regularly vitamin supplementation as is common in the management of elderly depressed patients with chronic degenerative vascular dis-

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TABLE 1.—SERUM CHOLESTEROL LEVELS DURING CONTINUOUS TREATMENT FOR 10 WEEKS OR MORE WITH LARGE DOSES OF NIACIN SUPPLEMENTED WITH PYRIDOXINE IN A GROUP OF 20 PATIENTS WITH HYPERCHOLESTEROLEMIA

NO.	CASE	SEX	AGE	DIAGNOSIS	FASTING SERUM CHOLESTEROL LEVEL							DOSAGE GM. PER DAY	SIDE EFFECTS	
					INITIAL	WEEKS 2	4	6	8	10	12		FLUSHING	OTHER
1	B.C.	F	69	CVA, Hypertension, Arteriosclerotic cardiovascular disease.	289	224	177	189	203	182		3	Mild	
2	B.S.	F	79	Hypertensive arteriosclerotic cardiovascular disease.	329	325	288	303	254	285		2	Marked	
3	D.L.	F	82	Generalized atherosclerosis.	378	198	236	179	187	165		3	Mild	
4	R.M.	F	57	Rheumatic heart disease, Atrial fibrillation, Cerebral embolus.	306	221	265	227	241	215		3	Mild	Soft stool.
5	H.G.	M	57	CVA, Arteriosclerotic cardiovascular disease.	347	275	260	307	288	156		2 until 8th wk. 3 thereafter.	Minimal	
6	M.L.	M	64	Generalized atherosclerosis.	346	251	244	300	297	222		3	Moderate	Soft stool.
7	M.G.	M	46	Multiple sclerosis.	236	187	205	219	216	148		3	—	—
8	S.F.	M	30	Cerebral Palsy	259	218	246	247	251	201		3	—	—
9	M.T.	M	43	Cerebral Palsy	238	211	-	-	203	181		3	—	-
10	M.B.	F	80	Generalized Atherosclerosis	279		247	211	250	218		[illegible]	M[illegible]	[illegible]

11		F	68	Arteriosclerotic Parkinsonism	314	196	166	235	184	215		3	Minimal	
12	M.L.	M	69	CVA. Diabetes, Atherosclerotic cardiovascular disease.	271	175	187	151	173	148	129	2	—	—
13	I.P.	M	75	CVA. Hypertensive arteriosclerotic cardiovascular disease.	240	-	136	-	85	118	125	2	Moderate	
14	J.K.	M	71	CVA. Diabetes, Generalized atherosclerosis.	423	390	423	318	365	268		3	-	—
15	H.A.	M	72	CVA. Generalized atherosclerosis.	324	280	279	178	290	220		3	Marked	
16	F.F.	F	52	CVA. Hypertensive atherosclerotic cardiovascular disease	388	225	-	280	225	180		3	Moderate	
17	M.R.	M	57	Arteriosclerotic Parkinsonism.	293	260	-	-	217	229		3	Minimal	Poor sleep
18	B.H.	F	78	Arteriosclerotic cardiovascular disease. Hypothyroid?	444	281	296	295	291	263		3	Minimal	
19	R.H.	F	68	CVA. Generalized atherosis. Obesity. Hypothyroid.	454	289		275	261	263		3	Minimal	Nausea. Occas. diarrhea
20	E.F.	F	32	Myxedema	453		251	280	277	182		3	Mild	
				AVERAGE TOTAL	338	251	244	246	236	203				
				Average of 16 patients on 3 gm. per day	346					209				

cases, an attempt was made to combine these drugs with the nicotinic acid in one tablet. In an effort to increase the efficiency and cut down side effects of the medication, we added to the tablet a small dose of Vitamin B_6 and Vitamin C. Pyridoxine was added for the specific reason that it plays a role in the synthesis of unsaturated fatty acids (Sinclair[8] and Whitton and Holman[9]), and thus might potentiate the hypocholesterolemic effect of niacin. None of the other components appears to be implicated in cholesterol or fat metabolism. We thus arrived at a formula* which combined in each tablet:

Niacin	330.0	mg.
Pyridoxine HCl	0.5	mg.
Thiamine mononitrate	1.0	mg.
Riboflavin	2.0	mg.
Ascorbic acid	20.0	mg.
Cyanocobalamine (B_{12} activ.)	.001	mg.
Reserpine	.025	mg.
Pentylenetetrazole	20.0	mg.

DOSAGE. With the exception of the very first cases in which niacin alone was used, these tablets, designated as DC 710, were used. One tablet t.i.d. was administered during the first 2 or 3 days. The dosage was then increased to 2 tablets t.i.d. for the remainder of the first week and increased further to 3 tablets t.i.d. after the second week or later, as seen fit. Larger doses were neither used nor required. The tablets were given together with meals.

THE CONTROLS. During the treatment periods, the patients were kept as closely as possible under the same environmental conditions. This applied particularly to their nutrition. For the inpatients who represent the great majority of our group, this was easily achieved. The hospital diet is a rather constant one. All inpatients had been on a low fat diet consisting of 100 gm. protein, 50 gm. fat and 250 gm. carbohydrate and supplying about 2000 calories. Wherever hypocholesterolemic agents had been used previously, these were discontinued and a control period of sufficient length was provided to establish base levels of the cholesterolemia. The blind test was applied inasmuch as neither the patients nor the ward personnel were informed about the purpose of the medication, which was said to be a new vitamin preparation. Placebos, however, were not used. This appeared unnecessary for 2 reasons. 1) The expected marked objective changes in the serum cholesterol level did hardly [illegible] placebo-controls which are in plac[illegible] subjective errors must be avoided. [illegible] chronically ill institutionalized patie[illegible] easily affected emotionally by a n[illegible] He usually has adjusted and resigne[illegible] invalidism and harbors little hope [illegible] provement. He reluctantly enters [illegible] treatment trial, particularly since th[illegible] involves not only the discomfort of [illegible] drugs, but also repeated tests of [illegible] urine and other infringements of h[illegible] limited freedom.

Serum cholesterol determinatio[illegible] done in all patients at frequent [illegible] once weekly or once biweekly. Only [illegible] serum cholesterol level was estimated [illegible] the method by Zak, Dickenman, White, Burnett and Cherney[10].

A complete physical checkup was carried out at least weekly. All patients had complete blood counts at about 2-week intervals and weekly urinalyses. Serial liver function tests, blood sugar determinations, and other special examinations including EKG were done in selected patients or where warranted. The liver function tests included cephalin flocculation, thymol turbidity, and the total serum protein with the A/G ratio.

Results. A. CONTINUOUS PROLONGED MEDICATION WITH LARGE DOSES. The most impressive result was the regularity with which a hypocholesteremic effect of some degree could be observed in each of the patients under study. This occurred within the first 2 weeks. With continued therapy, the level usually decreased further but less precipitously and a plateau was reached gradually. Occasionally increases in the serum cholesterol level occurred, but these rarely reached the initial hypercholesterolemic values, and usually were soon followed by a new fall. Representative serum cholesterol curves are demonstrated in Fig. 1 which shows the course of Cases 1, 40, 18 and 41.

The pertinent data of the 20 patients on continuous medication for 10 weeks are shown in Table 1. It will be noted

*This formula was kindly prepared for us by Dr. B. J. Brent, Clinical Research Director of the Wampole Laboratories, Stamford, Conn. It has received the trade name Sin-Sterol.

TABLE 2.—SERUM CHOLESTEROL LEVELS AND LIPOPROTEIN FRACTIONS IN PATIENTS WITH HYPERCHOLESTEROLEMIA UNDER TREATMENT WITH RELATIVELY SMALL DOSES OF NICOTINIC ACID

No.	Case	Sex	Age	Diagnosis	Nicotinic Acid Medication: Duration	Nicotinic Acid Medication: Dosage mg.	Serum Cholesterol mg./100 ml.	SF 12-20 mg./100 ml.	SF 20-100 mg./100 ml.
1	B. Te.	M	44	Xanthelasma	--	--	270	60	110
					6 wks.	200	210	45	87
2	B. Nov.	F	42	Obesity, hypertension	--	--	222	47	62
					6 wks.	200	196	38	53
3	A. Sch.	M	53	Coronary artery disease, obesity, old myocardial infarct.	--	--	200	47	62
					8 wks.	200	214	40	51
4	A. Be.	M	45	Normal	--	--	240	—	—
					4 wks.	200	210	—	
5	T. G.	M	47	Normal	--		210	—	—
					4 wks.	100	200		—
6	M. Ges.	F	52	Diabetes mellitus (controlled)			250		
					6 wks.	200	205		
7	H. B.	M	46	Normal			258		
					4 wks.	200	214		

that 4 patients of this group were on only 2 gm. of nicotinic acid per day throughout this period, and that they too showed rather marked decreases of their serum cholesterol level. In 9 patients the total serum cholesterol fell to levels below 200 mg. per 100 ml., in 7 below 250 mg. and in only 4 the 10 week level was higher than 250 mg., but still significantly lower than the initial hypercholesterolemia. The decrease in the serum cholesterol occurred in the arteriosclerotic patients as well as in the hypothyroid and diabetic subjects. The table demonstrates, moreover, that the mean cholesterol level

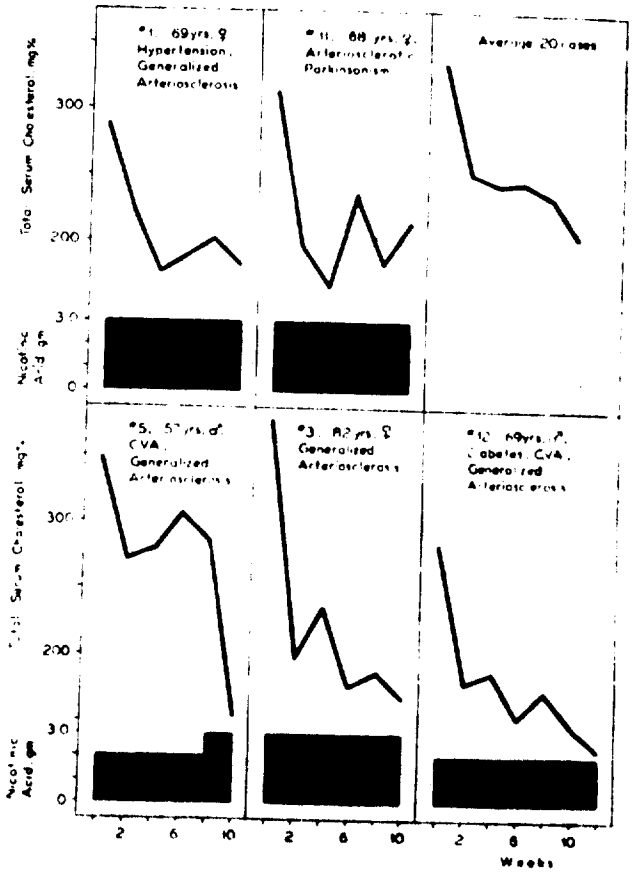

Fig. 1.—The course of the serum cholesterol level during continuous treatment with large doses of niacin supplemented with pyridoxine in 5 individual patients with hypercholesterolemia and the average fall of the serum cholesterol in the group of 20 patients under continuous treatment for 10 weeks or more.

decrease in the 16 patients receiving 3 gm. of niacin in the form of DC 710 for a period of 10 weeks was highly significant. It fell during this period from an initial mean of 345 mg. per 100 ml. to a mean of 209 mg., a drop

of 137 mg. per 100 ml., or 39%. A reduction occurred in every one of the patients and the mean drop is highly significant ($p < .001$). The course of Case 51 is illustrative and of special interest:

Case Report. K.S. is a 57-year-old white man with idiopathic (familial) hypercholesterolemia and hyperlipemia, mild hyperglycemia and glycosuria and long standing complaints of precordial pain and intermittent claudication. His mother suffered from similar vascular symptoms and one brother died of coronary thrombosis at the age of 41. The patient is not obese, has a blood pressure within normal range and an electrocardiogram showing nothing more than left ventricular strain. His heart is not enlarged and there are no abnormal sounds. The pulsations in the popliteal arteries and in the dorsalis pedis were decreased bilaterally. Radiographically there was evidence of calcifications both in the aortic arch and the vessels of both lower extremities. The oscillometric readings and skin temperature over calves were decreased. His serum has been known for years to be milky and to have a total lipid level of more than 3000 mg. per 100 ml. with a cholesterol level of an average of 600 mg. per 100 ml. This level did not respond to estrogens or thyroid medication but decreased somewhat during a period of a diet entirely void of animal fats. Even then, however, the lowest level obtained was still 470 mg. per 100 ml. with the majority of the levels between 520 and 600, and no significant effect on the turbidity of the serum. During the past 5 months, the patient had been on a low fat diet without any medication and his serum cholesterol level had increased to values between 600 and 700 mg. per 100 ml. He felt tired, depressed and was unable to walk more than 2 blocks without pain in his calves. In addition, he had experienced a new, rather uncommon symptom of hyperalgesia in both hands. Immediately before starting the medication with the tablets DC 710, his serum cholesterol level was found to be as high as 1000 mg. and his total serum lipids were 12,400 mg. per ml. He was given 3 × 2 tablets daily for the first 2 weeks, at the end of which the serum cholesterol level had decreased to 520 mg. per 100 ml., with continued medication of 3 × 3 tablets daily for 3 weeks a level of 344 mg. per 100 ml. was obtained. His serum had lost most of its turbidity and its total lipid content was 1200 mg. per 100 ml. Moreover, within a few days on DC 710, the patient had lost the painful sensation in his hands and did not experience leg cramps when walking 5 or 6 blocks. He felt less tired and more ambitious. After another week a level of 256 mg. per 100 ml. serum cholesterol was obtained. This level has been maintained in spite of the fact that the fat intake was increased from 30 gm. to 60 gm. daily and the medication decreased after the 10th week to 4 tablets daily. The side effects of the medication (see below) which he noted were not only the rather common and unpleasant flushing, but also the occurrence of more voluminous soft and foul-smelling feces. It might be mentioned, however, that the glycosuria and hyperglycemia increased slightly but was controlled with 1 gm. Tolbutamide daily.

Similar gratifying results were obtained in each patient who has continued the medication for more than one week.

B. INTERRUPTED MEDICATION WITH LARGE DOSES. As soon as the drug was withheld, the initial hypercholesterolemia returned. Within 2 or 3 days, a marked rise of the serum cholesterol occurred but a new fall could be obtained as early as 48 hours after resumption of the effective dose. This rapid rise was as striking as the sustained decrease of the levels as long as medication was adequate. Illustrative curves are shown in Fig. 2.

Case Reports. Case 25. A 57-year-old man with arteriosclerotic Parkinsonism refused the medication after one week because of severe flushing, irritability and inability to sleep. His initial serum cholesterol level was 293 mg. per 100 ml.; at the last day of medication with 3 × 3 tablets it had decreased to 234 mg. per 100 ml. Another estimation done 48 hours later, was 317 mg. per 100 ml. Two weeks later the patient was willing to resume the medication. This time the initial level was 302 and after 2 days of medication of 3 × 3 tablets it had then decreased to 229 mg. per 100 ml. and 2 weeks later to 200 mg. per 100 ml.

Case 49. A 32-year-old woman with hypercholesterolemia had had an excellent response to 3 × 3 tablets daily; her serum

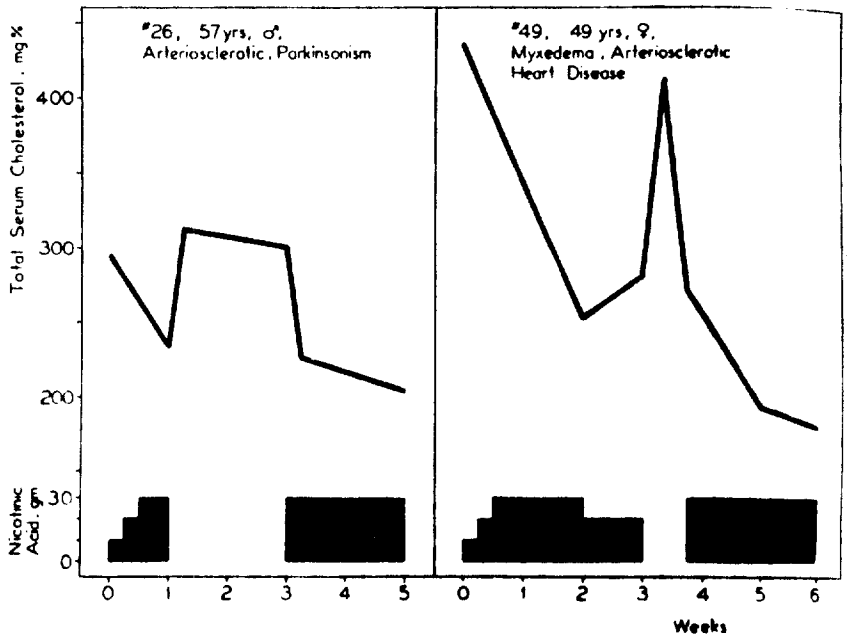

Fig. 2.—The effect of interrupted treatment with large doses of niacin supplemented with pyridoxine on the serum cholesterol level in 2 patients with hypercholesterolemia.

TABLE 3.—LIVER FUNCTION TESTS AND FASTING SUGAR LEVELS BEFORE AND DURING TREATMENT WITH NIACIN IN 12 PATIENTS. THE CASE NUMBERS REFER TO THE CASES IN TABLE 1

Case No.	*A/G Ratio*		*Ceph. Floc.*		*Thymol Turbidity*		*FBS*	
	Before	*During*	*Before*	*During*	*Before*	*During*	*Before*	*During*
1*	3.44/4.25	3.2/4.2	1+	2+	—	-	98	86
3†	3.76/3.30	3.50/3.90	-	-	2.8	1.59	102	95
5*	4.07/3.49	3.65/3.45		1+		—	89	93
7**	3.84/3.61	4.39/4.10		-	—	-	84	104
8**	4.39/3.00	4.50/3.10					77	82
9**	5.0/2.24	4.60/2.50			—	—	93	68
10‡	3.76/3.88	3.5/3.5	3+	2+	2.3	3.3	112	95
11‡	3.64/3.86	3.9/3.0		-	-	2.3	79	70
15**	4.64/2.10	4.0/3.3	2+	—	0.9	0.23	106	83
16**	3.65/3.84	3.70/3.81	2+	2+	3.4	3.9	110	89
18*	3.40/3.80	3.77/3.6	1+		2.8	3.4	104	90
20**	4.30/3.03	4.05/2.81			2.3	1.76	88	68

*Indicates 2nd test after 4 weeks of treatment.

†Indicates 2nd test after 6 weeks of treatment.

‡Indicates 2nd test after 8 weeks of treatment.

**Indicates 2nd test after 10 weeks of treatment.

The fasting blood sugar levels represent averages of 3 to 5 determinations done at interva

cholesterol level had fallen within 2 weeks from 435 to 251 mg. per 100 ml. The dose was then decreased to 3 × 2 tablets, whereupon only a small increase occurred to 280 mg. per 100 ml. At this time medication was withheld for 2 days with a subsequent rise to 410 mg. per 100 ml. Resumption of medication with the initial dose brought within 48 hours a fall to 270 mg. per 100 ml. which dropped further to 192 and 180 mg. per 100 ml. at subsequent weekly intervals.

C. STUDIES WITH SMALLER DOSES OF NIACIN. In view of Altschul's observation of some hypocholesterolemic effects with 1 gm. per day of niacin and our own finding that a few patients could be treated effectively with approximately 2 gm. per day, we attempted an evaluation of still lower doses. Patients received as little as 25 mg. niacin q.i.d. No response of the serum cholesterol could be noted. With increase of the dosage to 50 mg. q.i.d., however, small and sustained drops were obtained. Representative data for this group is shown in Table 3. The flotation fractions SF 12–20 and 20–100 of the beta-lipoproteins were estimated in these patients and are listed in the table for the 3 patients in whom a hypocholesterolemic effect appeared. Their changes are not significant.

SIDE EFFECTS. The most disturbing and most common side effect was flushing and the feeling of warmth and lightheadedness. Most every patient experienced it, at least during the first week of medication. It occurred with the small doses as well as with the largest. The addition of reserpine and pentylenetetrazole to the medication seems to have brought some improvement, but a large group of the patients continued to be bothered by these symptoms to some degree. The flushing usually persists for a half to one hour. Most patients got used to this annoying feeling or noted acquiescence with continued medication. Fifteen patients, however, refused continuation of the medication after one week.

Next common was the feeling of nausea and occasional vomiting. This occurred particularly when the medication was taken on an empty stomach. It could be prevented by offering the tablets together with the meals.

A number of patients observed that their feces had become more voluminous and foul smelling. Four patients complained in addition of persistent diarrhea. The feces had the shiny appearance of fatty stools. The diarrhea could easily be controlled with kaopek.

Other patients, 3 in the whole group of 53, noted transitory episodes of palpitation and a few complained about difficulties in falling asleep.

Outside of the flushing episodes, there was no impairment of the general well being. Many patients indicated that their appetite had increased and they showed in general more interest and activity. With regard to the cardiovascular system, there were no noticeable persistent changes in the peripheral circulation, no changes in the blood pressure or the electrocardiogram. It is noteworthy that none of the patients with previous coronary disease experienced any recurrence of precordial pain or angina.

Serial liver function tests were done in 12 of the 20 patients treated for 10 weeks or longer. The tests were done before treatment and either after 4, 6, 8, or 10 weeks of medication. No significant changes were noted in any of the tested parameters, at any interval. The data for the individual patients are given in Table 3. This table demonstrates also that the fasting blood sugar level was not significantly influenced by the medication. The values during treatment represent the averages of 3 or more determinations done at various intervals during the treatment period. In a few patients

the diurnal blood sugar level was followed and no significant alterations were observed.

As is to be expected in a prolonged study of elderly and seriously disabled patients, there occurred a few fatalities which most likely are not related to the drug. A 96-year-old woman with advanced atherosclerosis died of bronchopneumonia; a 60-year-old woman with atherosclerosis developed mesenteric thrombosis and died with peritonitis.

Discussion. Our observations leave no doubt that niacin in appropriate dosage, particularly in combination with pyridoxine, is a potent serum-cholesterol lowering agent. Our results during a 10 weeks' period with a dosage containing 3 gm. of niacin and 3 mg. pyridoxine per day were somewhat superior to those obtained by Parsons *et al.*[5,6]. The mean starting serum cholesterol level in our series of 20 patients decreased from 338 mg. to 203 mg. per 100 ml., and none of the patients failed to show a response. The two series are, of course, not identical in every respect, and, therefore, not easily comparable. Thirteen of our patients observed for a 10 weeks' period had an initial serum cholesterol level of 270 mg. per 100 ml., or higher, as the 23 patients observed by Parsons and Flinn[6] over 12 weeks. While in this latter series 9 showed no significant response, 6 had a reduction from the initial level of 40 mg. per 100 ml. or more, and 8 had a final level below 250 mg., all 13 of our series showed a significant response with a fall to or below 250 mg. per 100 ml. in 10 of them. The Chi-square test shows that this difference is statistically significant ($P = .02$).

The efficacy of this medication seems to be greater than that of any other known means of lowering the serum cholesterol level. It exerts its effect in the various types of hypercholesterolemia, the familial or idiopathic as well as the acquired, the hypercholesterolemia of uncontrolled diabetes and of myxedema.

The magnitude of the effect is so great that it cannot be explained by interference with the dietary or exogenous cholesterol alone. Relaxation of dietary restriction, as in Case 51, did not lead to a resurgence of the serum cholesterol. It is likely that the endogenous cholesterol metabolism is affected as well.

The absence of serious side effects makes niacin even in high doses an acceptable clinical drug though the unpleasant signs and symptoms of vasodilatation as flushing and feeling of heat may be a deterrent to many patients. Our attempts to combine it with an ataractic and tranquilizer have brought some, but not sufficient improvement with regard to the flushing effect. The addition of pyridoxine seems to have lowered the effective dose. It will be worth while to investigate whether other combinations or modifications can potentiate the serum cholesterol lowering effect of niacin and permit elimination of the hyperemia.

The possibility of liver impairment by prolonged medication of large doses of niacin has to be considered. Niacin, at least to a large extent, is transformed to nicotinamide and then methylated in the liver. The prime methyl-donor is methionine. Thus, this lipotropic factor may be deviated from its physiological function. Fatty livers have indeed been demonstrated in the animal experiment after administration of unusually large doses of nicotinamide (Krehl[4]). No such observations have been reported in man. No liver damage was demonstrated by the liver function tests in our patients. It appears, therefore, that a normal protein intake of 100 gm. per day, as

provided in our series, will give adequate protection against this danger.

Niacin has been reported recently to inhibit enzymatic insulin degradation and thus to induce hypoglycemia (Mirsky, Perisutti and Jinks[4]). Although we did not note in our patients any effect on the fasting blood sugar level, further studies are under way employing both the glucose tolerance and the insulin sensitivity tests. These will be reported later.

The mechanism of the action on cholesterol metabolism is as yet entirely unknown. The hypocholesterolemic effects can hardly be considered as the result of substitution therapy since the effective doses are far in excess of those required to correct nicotinic acid deficiency, for example, in pellagra. One must think of a pharmacological effect. This is reminiscent of Jolliffe's[2] earlier observation of the beneficial role of large doses of nicotinic acid in the treatment of alcoholic encephalopathies. Altschul[1] has suggested that niacin as part of the respiratory enzyme system might increase tissue oxidation and thus lead to faster oxidation of cholesterol, and that no reabsorbable oxycholesterols may be formed and excreted. An interference with the reabsorption or absorption of cholesterol from the gut would indeed explain the rapid development and the equally fast disappearance of the effect. One would have to assume that the endogenous cholesterol reaching the gut through the biliary tract as well as the dietary cholesterol are prevented from entering the intestinal mucosa. Our observation of the occurrence of voluminous foul-smelling and shiny stool seems to support this concept. These stools, however, occurred in only a few patients. The changes in the beta-lipoprotein fractions under niacin medication as reported from the Mayo Clinic, and our clinical observation in the case of familial idiopathic hyperlipemia and hypercholesterolemia (Case 51) of the clearing of the hyperlipemic serum may suggest that niacin has some effect on the clearing factor of the blood and on lipid transport. These problems deserve further investigation. Preliminary data on patients with biliary drainage seem to indicate that the niacin hypocholesterolemia is accompanied by an increased biliary bile flow[7].

Equally important will be clinical and pathological studies on the effect of niacin medication on the development and the course of degenerative vascular diseases. Evident as the relationship between hypercholesterolemia and atherosclerosis is, it is far from clear what role the hypercholesterolemia plays in the chain of causative factors.

Summary. In a group of 53 patients with hypercholesterolemia a marked and sustained lowering of the serum cholesterol level was obtained by medication of a combination of niacin and pyridoxine. The effective daily dose ranged between 2 and 3 gm. niacin and 2 to 3 gm. pyridoxine. The addition to the medication of Vitamin C, Rauwolfia and metrazole appears to have decreased the incidence and severity of the side effects of niacin, particularly the skin hyperemia and the feeling of heat and flushing. In 20 patients who were treated with this formula for more than 10 weeks, the serum cholesterol level fell from an initial average of 338 mg. per 100 ml. to an average of 203 mg. per 100 ml. after 10 weeks. Hypercholesterolemias of various types showed significant responses. Discontinuation of the drug was followed immediately by restoration of the pre-existent hypercholesterolemia.

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Developments leading to the metabolic role of vitamin B_6[1]

Paul György,[2] *M.D.*

In the late 1920's my interest was focused on dermatological conditions in infants, with special reference to seborrhoid dermatitis and acrodynia. We tried to approach the problem by feeding various experimental rations to rats. One study was devoted to investigations of "egg white injury," which is produced in rats on a ration containing dry, uncooked egg white as the sole source of protein (1, 2). This work, covering the extended period from 1929 to 1940, led to the identification of the known yeast-growth promoting factor, biotin, with the egg white injury factor. In the later years of this study, the cooperation of Dr. Vincent du Vigneaud, Professor of Biochemistry at the Cornell School of Medicine was truly essential for success. Biotin has since been proven a very important vitamin for all living organisms, not only, as was originally assumed, for yeast alone (3).

The second, more intensive and more progressive, study dealt with the isolation of vitamin B_2. In 1927, the British Committee on Accessory Food Factors had distinguished two separate components of the vitamin B complex: *1)* vitamin B_1, the antineuritic factor; and *2)* vitamin B_2, the antipellagra factor. The same committee defined vitamin B_2 as "the more heat stable, water soluble dietary factor recently described and named P-P (pellagra preventive) factor by Goldberger, Wheeler, Lillie and Rogers (4) and found necessary for maintenance of growth and health and prevention of characteristic skin lesions in rats, and considered by the latter workers to be concerned in the prevention of human pellagra."

In 1929, we proceeded with the chemical isolation of the so-called vitamin B_2 in collaboration with Professor Richard Kuhn and Dr. Theodor Wagner-Jauregg. For assay purposes, we used the growth response of rats fed a purified supplemented diet, containing cod liver oil as the source of vitamin A and D and an alcoholic extract of wheat (5) as the source of vitamin B_1. Modern research workers, accustomed to microbiological tests that give an answer within 20 to 48 hr and enable the simultaneous assays of scores of test substances, should be impressed by the fact that each assay for vitamin B_2 in rats required a testing period of 3 to 4 weeks, with a corresponding number of prepared experimental animals.

During the course of the isolation of "vitamin B_2," it was our collaborator Dr. Wagner-Jauregg who first noted that all concentrates prepared from cow's milk that proved to be active when used as a supplement to a vitamin B_1 concentrate such as an alcoholic extract from wheat (5), were colored and showed an intensive green-yellow fluorescence in direct proportion to their biological effect. Exposure to visible light destroyed the growth promoting activity of these concentrates (6). But the obvious working hypothesis, which identified vitamin B_2 with a yellow-green fluorescence, soon met serious difficulties. To the great disappointment and

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despair of the chemist, the concentrates became biologically inactive in the rat growth test as they were further purified, and became more highly colored. Here the biologist and animal experimentalist came to the rescue of the chemist (7). It was shown that, by supplementing the diet with a yeast concentrate (8) from which all colored material had been removed by charcoal absorption (the so-called Peters eluate), the biologic activity of the colored preparation was restored. Thus, what was at first sight disappointing, opened the gate to important new assaults on the problem of the vitamin B_2 complex. The pure crystalline, yellow compound first isolated in cooperation with Professor Richard Kuhn and Dr. Wagner-Jauregg from milk was originally named by our group "lactoflavin" (9–12). It has been identified chemically as a derivative of isoalloxazine with a ribose radical attached. Hence the present term, riboflavin.

Riboflavin was the first vitamin recognized as part of an enzyme system. Therefore, it was not only a vitamin but also a dietary pro-enzyme as a prosthetic group of the flavo enzymes. The yellow pigment isolated previously by Warburg and Christian (13) from their yellow enzyme was a photo-derivative of riboflavin and was inactive as a vitamin. Riboflavin bridged the gap between an essential nutrient and cell enzymes and cellular metabolism. In biochemical research it represented a special milestone. Today, with the general acceptance of this idea, it is not surprising that water-soluble vitamins have been found to be essential parts of enzyme systems.

Crystalline vitamin B_1 (thiamin) became available in 1933. The isolation of riboflavin in pure form gave us the key for a real breakthrough in the investigation of the vitamin B_6 problem. It was shown (7, 14) that after a few weeks on a diet free from the whole vitamin B complex and supplemented with required amounts of thiamin and riboflavin, young rats showed a reduced growth rate and developed a scaly symmetric dermatitis that was most pronounced on the peripheral parts of the body (paws, ears, snout) Inasmuch as the distribution of these cutaneous lesions somewhat resembled the skin lesions in human pellagra, the dermatitis seen in rats receiving a vitamin B-free diet supplmented only with pure thiamin and riboflavin was first called pellagra-like, "without prejudice as to their identity or non-identity with human pellagra" (7). This observation furnished exact proof for the conclusion that vitamin B_2 was not a single vitamin but a complex in itself, with riboflavin its first definitely established member. In order to avoid confusion with previously claimed and discussed in the literature, but not properly identified factors like vitamins B_3, B_4, B_5 (and Y), our "rat pellagra factor" was named vitamin B_6 (14).

Further investigations (15–17) led to the differentiation of riboflavin and vitamin B_6 from the specific pellagra preventive (P-P) factor of Goldberger and his associates, and definitely established the separate existence of these three members of the vitamin B complex. The designation "pellagra-like dermatitis" of vitamin B_6 deficiency has been changed to "rat acrodynia," again on the basis of the distribution of the cutaneous changes, without any reference to human acrodynia (15).

The isolation of pure crystalline vitamin B_6 was first reported by Lepkovsky (18) barely 4 years after recognition of this specific member of the vitamin B complex. Independently, but slightly later, several other groups (19–22) also reported the isolation of vitamin B_6. It is appropriate to recall the fine (in general, quite unusual) friendly gesture of Dr. Lepkovsky, who knowing my active interest in the isolation of vitamin B_6, advised me early in 1938 that he and Keresztesy were ready to submit independently their papers on the successful isolation of vitamin B_6 for publication. This enabled me, with some accelerated urgency, to make ready and submit my own publication in time.

Within a year, the exact chemical structure of vitamin B_6 as a pyridoxine derivative had been elucidated (23, 24). The term pyridoxine, proposed by us (25) for this compound, has received general acceptance.

At this stage of the historical development, microbiological research entered the scene. Credit is due to Snell and his associates (26) for first recognizing the existence of other

after delivery is significantly higher than that in the maternal blood (39).

The irregular metabolites of tryptophan in a positive load test may have other clinical and metabolic consequences, such as migraine and depression of various grades in women receiving contraceptive pills (40) (requiring at least 50 mg pyridoxine daily compared with the usual daily recommended allowance of 2 mg and even in pregnancy only 2.5 mg). In animal experiments, tryptophan metabolic intermediates have been reported to be carcinogenic (41). Xanthurenic acid (XA) proved to be diabetogenic. High fat content in the diet increases the diabetogenic effect of XA, which first reduces the glutathione content of the blood, binds insulin, and may lead to chronic diabetes through exhaustion of the pancreatic β-cells (42, 43). Administration of pyridoxine will prevent all manifestations of the diabetogenic effect of XA, which in itself is analogous to that of alloxan in animals. Of course, complete destruction of the β-cells cannot be reversed by pyridoxine. The increased capacity of the liver and adipose tissue to synthesize fat (44), just as high dietary fat intake (45) in (absolute or relative) B_6 deficiency, may act as supporting factors in the development of diabetes. It is of special interest, not yet clarified, that XA in free form was invariably present in the urines of diabetic patients (45). These tryptophan intermediates can interfere also with gluconeogenesis (46) and energy metabolism (47).

The very common premenstrual edema and late massive edema of pregnancy is preventable or therapeutically influenced with high doses of pyridoxine (48) without the use of pharmaceutical antidiuretics.

Of even greater theoretical and practical importance than the disarranged B_6 enzymes in pregnancy or in women using oral contraceptives is a similar physiological disturbance of B_6-enzyme activity with increasing age in geriatrics. The levels of PLP in the blood plasma show gradual reduction in the aged, accompanied by a positive tryptophan load test. The serum-glutamic-oxalic acid transaminase (SGOT) was found also lower in old individuals than those of the young. Large doses of pyridoxine restored all metabolic changes mentioned to normal (49, 50). It has also been claimed that acute rheumatism, as well as osteoarthritis (the disease mainly of the aged), in its early stages may be beneficially influenced by 50 to 100 mg of pyridoxine daily (51).

In all the above clinical conditions, the amino acid load test was carried out with tryptophan. More recently, attention was directed toward methionine with expected changes in the transulfuration pathway observed in subjects kept on a low vitamin B_6 diet for an extended period of time. As a result of reduced activity of B_6-dependent cystathionase and cystathionine synthetase (Fig. 2), high excretion of cystathionine or homocysteine (homocystine), or both, was observed (52). It is of special pediatric interest that the enzyme cystathionase is absent in human fetal liver or in the liver of premature infants. In consequence, the fetus and the premature infant (at least at birth) are not capable of utilizing methionine and must depend on cystine as the essential sulfur-containing amino acid (53). In this connection, it must be called remarkable that human milk is the only source of animal protein with the lowest ratio methionine:cystine. Or, in other words, human milk is relatively rich in cystine, whereas cow's milk, meat, or any other animal protein have a great excess of methionine (two to five times more than cystine), which represents a special stress for the pregnant mother and thus, for the fetus and the premature infant, another glaring example of teleology.

Typical arteriosclerotic lesions were described more than 20 years ago (54) in B_6-deficient monkeys. More recently, accelerated arteriosclerosis has also been found in children in association with the inborn metabolic error of homocystinuria (55, 56). In cell cultures obtained from the skin of such individuals, an abnormal proteoglycan substance was found that is granular, aggregated, and flocculent (57). In the arterial wall, the normal fibrillar structure was altered in children with homocystinuria, showing the abnormal proteoglycan substance. The same changes were observed in rabbits after repeated injections of homocysteine. "Moreover, addition of cholesterol to the diet of

forms of pyridoxine, i.e., pyridoxal and pyridoxamine, both (especially pyridoxal in its phosphorylated form) acting as coenzymes. It became customary (27) to speak of vitamin B_6 as a subgroup of the vitamin B_2 complex, with pyridoxine, pyridoxal, and pyridoxamine as its particular chemical representatives.

With all the rich history of vitamin B_6, approximately 20 years passed before its requirement by the human organism had been definitely established and recognized. At present we are facing an almost explosive interest in the metabolic role of vitamin B_6 in man. There are large numbers of findings of various types accumulating, but a clear, overall picture of all connections and of their role is still missing.

The role of vitamin B_6 in maintaining and regulating a normal physiological and neurological state has been discussed; apart from convulsions, the oldest metabolic observations on the role of vitamin B concern pregnancy (28). They originated from the demonstration of increased amounts of xanthurenic acid in the urine of vitamin B_6-deficient rats after oral administration of tryptophan (29). Xanthurenic acid, together with kynurenine, anthranilic acid, et cetera are the metabolites of tryptophan on the way to the formation of nicotinic acid (Fig. 1). Abnormally high excretion of xanthurenic acid and related metabolites of tryptophan was also found in pregnancy, beginning from the 12th to 14th week (30–33). This disturbance in the metabolism of tryptophan must be due to the deficiency or malfunction of not one but more B_6 enzymes involved in the enzymatic pathway of tryptophan to nicotinic acid.

This first metabolic study coincided with the work of enzymologists. The above findings were the not unexpected consequence of the statement of Braunstein, the famous Soviet biochemist: "Pyridoxal phosphate holds an exceptional place among the coenzymes with regard both to the unparalleled diversity of its catalytic function and to their paramount significance in biochemical transformations of amino acids and in integral pattern of nitrogen metabolism" (33).

The positive tryptophan load test in pregnancy was ascribed to a relative vitamin B_6 deficiency (28). Considering the more recent observations on similar derangement observed in women receiving contraceptive pills (34–37) and also the analytical findings on reduced pyridoxal phosphate (PLP) in the plasma of pregnant women and women on contraceptive pills, it appears to be more logical to consider an antagonism between hormones in pregnancy and in contraceptive pills to the coenzymes involved in the degradation of tryptophan, and (as discussed later) in other examples of metabolism of amino acids. With this assumption, the requirement of high doses of pyridoxine for the restoration of disturbed tryptophan metabolism (30 mg (!) of pyridoxine daily) (38) is in good accord.

The pyridoxal levels in the cord blood

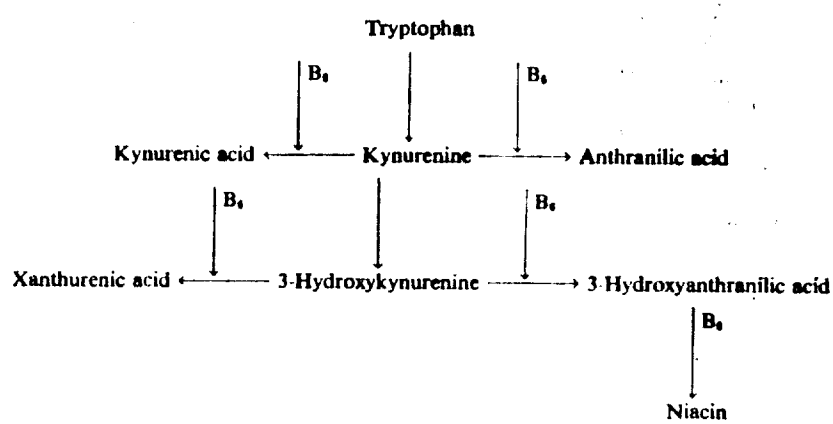

FIG. 1. Tryptophan-niacin pathway.

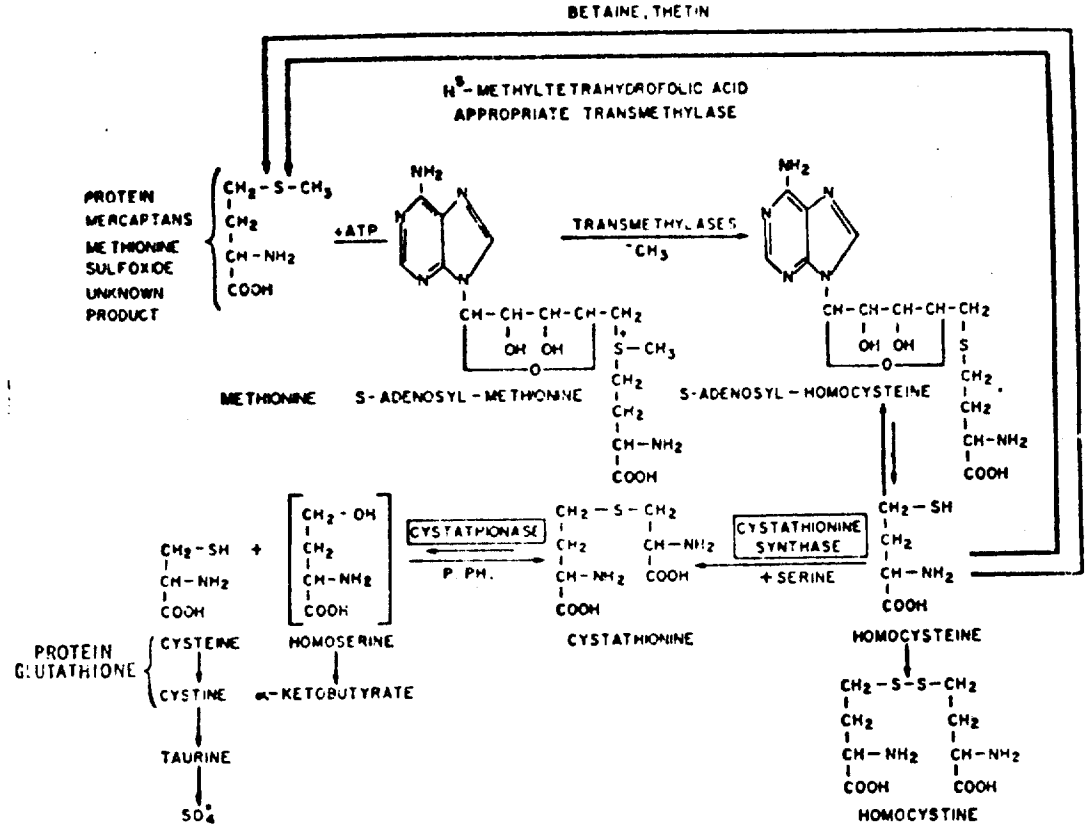

FIG. 2. Transulfuration pathway (53).

homocysteinemic rabbits resulted in deposition of lipids in the aortic lesions, suggesting that lipid accumulation is a complication of the primary vascular alteration" (58). In other words, hypercholesteremia is not the leading pathogenetic factor.

All this opens the way to further studies, bringing the high requirement for B_6 with increasing age and arteriosclerosis (plus other geriatric diseases) into proper focus.

In the past, a positive tryptophan load test (and this applies also to the most recently introduced methionine load test) was considered an interesting biochemical curiosity, without special medical value. With the recently acquired knowledge of the possible harmful effect of some abnormal metabolites formed in the course of these load tests, especially when of extended duration (such as in geriatrics or in women on contraceptive pills for years), important and urgent practical conclusions are in order. The present daily dietary allowance for B_6 should be changed from 2 to 2.5 mg to 25 mg/day as a precaution to prevent possible serious pathological conditions.

In summary, the history of vitamin B_6 is a further proof that success usually is preceded by trials, tribulations, and recurrent disappointment. The most helpful factor, apart from perseverance and timeliness of the line of research, is the deliberate recognition of a principle that is paramount in scientific research; it is often almost beyond our control, and touches closely on intuition. It is Walter B. Cannon's "serendipity."

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Studies on the Induction of Tyrosine Transaminase by Large Dose Administration of Vitamin B_6[1]

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Intraperitoneal as well as oral administration of pyridoxine 50 mg per 100 g body weight to rats resulted in a 2 to 3-fold rise of hepatic tyrosine transaminase (L-tyrosine 2-oxoglutarate aminotransferase EC 2.6.1.5.), the maximal activity being attained 4 hours after administration. This rise was also observed by intraperitoneal injection of some other vitamins.

Bilateral adrenalectomy curtailed the rise by pyridoxine or pyridoxal administration but still a 150 % increase of the activity was observed 2 to 4 days after the operation, while injection of pyridoxamine and other vitamins had no effect on the adrenalectomized rats. The induction of tyrosine transaminase by vitamin B_6 derivatives, which was confirmed by Kenney *et al.* due to an increased rate of its *de novo* synthesis, was no more seen 7 days after adrenalectomy. The inducibility was restored by the pretreatment of hydrocortisone 5 mg per 100 g body weight of rats 15 hours before the injection of pyridoxine.

This phenomenon together with the fact that the day course of the induction by pyridoxine after adrenalectomy seems to reflect the serum content of corticosterone of adrenalectomized rats as reported by Fazekas, supports the conclusion that the induction of tyrosine transaminase by pyridoxine is a secondary change mediated by glucocorticoid.

Tyrosine transaminase (TTA) is a pyridoxal enzyme and it has been established that its hepatic level is controlled by adrenal glucocorticoids and severeal other factors (1-3). In 1963, Greengard and his coworkers reported that parenteral administration of a large dose of pyridoxine to adrenalectomized rats caused a 3-fold rise of the hepatic enzyme and proposed to call the "cofactor or coenzyme induction" different from the hormonal control (4, 5). Afterwards Kenney (6) concluded that the elevation of activity of TTA caused by a large dose administration of vitamin B_6 derivatives was to be attributed to the increased production of apo-enzyme (enzyme synthesis) on a fact that the administration of B_6 to adrenalectomized rats facilitated the uptake of ^{14}C-labeled amino acid into the enzyme protein in the liver and that the induction was inhibited by actinomycin D. As there

[1] This paper was partly presented at the 39th Annual Meeting of Japanese Biochemical Society in Tokyo, October 28, 1968. Following abbreviations are used: TTA, tyrosine transaminase; EDTA, ethylenediaminetetraacetate; PAL-P, pyridoxal phosphate; *a*-KGA, *a*-ketoglutaric acid; *p*-HPP, *p*-hydroxyphenylpyruvate.

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has been limited reports about enzyme induction accompanied by the increase of coenzyme, it excited our interest to make a further investigation *in vivo* of the mechanism using adrenalectomized rats. We have obtained some informations suggesting that the increased activity of hepatic TTA in rats administered by a large dose of the vitamin might be due to an effect mediated by adrenal glucocorticoid.

Materials and Methods

Chemicals —— Actinomycin D and hydrocortisone sodium succinate were purchased from Sigma Chemical Company. Pyridoxine hydrochloride and other reagents were all of analytical reagent grade.

Treatment of Animals —— Male, Wistar strain rats weighing 150 to 200 g were used in this study. Bilateral adrenalectomy was carried out under anesthesia with pentobarbital. The animals were maintained on 0.9% NaCl and were used for experiments on the indicated days after the operation. Administrations of the B_6 derivatives and other vitamins were by intraperitoneal or oral route after dissolving in 0.9% NaCl solution. Hydrocortisone was suspended in the saline and injected intraperitoneally.

Preparation of Enzyme —— The animals were decapitated 4 hours after the administration and their hepatic perfusion was rapidly performed with saline through portal vein. The liver was removed immediately and homogenized with glass homogenizer in 4 volumes of ice cold 0.15 M KCl-10^{-3}M EDTA. The homogenate was centrifuged at 20,000×g for 20 minutes and the supernatant (diluted to 6-fold) was subjected to enzyme assay of TTA.

Assays —— TTA activity was assayed essentially according to Kerney (7) as follows: A 4.5 ml of reaction mixture consists of enzyme solution containing 2-3 mg protein, phosphate buffer (pH 7.6) 300 μmoles, PAL-P 24.8 μmoles, tyrosine 12 μmoles and diethyldithiodicarbamate 10 μmoles. Diethyldithiodicarbamate was added for preventing the oxidation of *p*-hydroxyphenylpyruvate produced during the reaction. After the reaction mixture was preincubated at 37° for 5 minutes, the reaction was initiated by addition of *α*-ketoglutarate 60 μmoles. After ten minutes, the reaction was terminated with 0.5 ml of 30% trichloroacetic acid, and centrifuged at 3,000 rpm for 5 minutes. To 3 ml of the supernatant were added 2 ml of water and 2 ml of the "mixed reagent". The "mixed reagent" was freshly prepared on an equivolume basis from stock solutions of 3% ammonium molybdate and 1% aqeous KH_2PO_4. The optical density of the resulting colored solution was read after one hour on a Hitachi spectrophotometer at 850 mμ. One unit of enzyme is defined as the amount that will catalyze the formation of 1 μg of *p*-HPP under these conditions. Protein was estimated by the biuret procedure (8).

Results

Investigation of Enzyme Assay

In order to obtain an optimal condition for enzyme assay, amounts of substrate and coenzyme, and reaction time of the Kenney's method were scrutinized: The activity of TTA was examined with the addition of various amounts of *α*-KGA to 10 μmoles of tyrosine in the reaction mixture. With the addition of more than 20 μmoles of *α*-KGA and 2.48 μmoles of PAL-P, the activity reaches the plateau as illustrated in Fig. 1 and Table 1. The relation between the enzyme activity and reaction time or enzyme amount was also examined. Under these conditions, the reaction proceeds almost linearly up to 15 minutes and the enzyme activities were proportional to enzyme concentrations as shown in Fig. 2a, b. As most of the enzyme activity

Table 1
TTA activity and PAL-P concentration in the reaction mixture

PAL-P added	Optical density	P-HPP formed
μmoles		*μg*
0.248	0.51	147
0.62	0.55	165
2.48	0.64	195
6.2	0.64	195

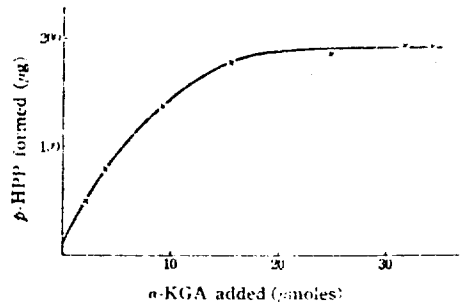

Fig. 1 *TTA activity and α-KGA concentration*

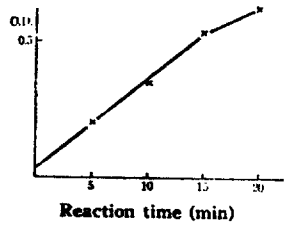

FIG. 2a *TTA activity and reaction time*

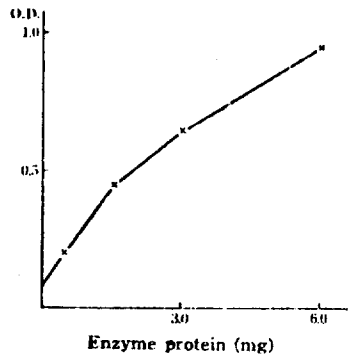

FIG. 2b *TTA activity and enzyme concentration*

resided in the 20,000×g supernatant of liver homogenate, the enzyme solution was used after appropriate dilution of the supernatant.

Experiments with Unoperated Rats

1. Change of Hepatic TTA Activity after Intraperitoneal Injection of Pyridoxine

Rats were given pyridoxine 50 mg per 100 g body weight intraperitonealy and time course of hepatic TTA activity was followed. As shown in Fig. 3 the activity reached maximum 4 hours after the injection and gradually declined thereafter. Although Greengard or Kenney (4, 5) administered pyridoxine 100 mg per 100 g body weight intraperitoneally twice at zero time and 2 hours after the injection and observed a 2-3 fold rise of TTA activity 4 hours after the first injection, a similar incrase of activity could be obtained by a single injection in this experiment. The administration of pyridoxine and its derivatives was made according to this method in all the subsequent experiments.

2. Dose Response of Hepatic TTA Activity to the Administration of Pyridoxine

The response of hepatic TTA activity

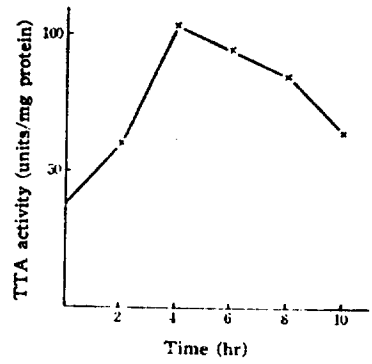

FIG. 3 *Change of hepatic TTA activity in unoperated rats after intraperitoneal injection of pyridoxine*

Each value in this figure and the subsequences is a mean of results obtained with 3 rats.

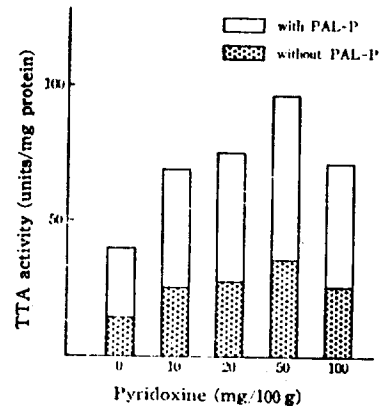

FIG. 4 *Change of TTA activity by varying amounts of pyridoxine in unoperated rats*

to various dose of pyridoxine injected intraperitoneally was examined and the activities with and without addition of PAL-P to the reaction mixture were compared. As shown in Fig. 4 the administration of 50 mg per 100 g body weight elicited the maximal response of total enzyme activity which was obtained with the addition of excess of PAL-P. Apoenzyme activity was defined as the value substracted holoenzyme activity which is obtained without adding PAL-P (activity produced by endogenous PAL-P) from total enzyme activity.

There was not much rise in holoenzyme activity 4 hours after the injection of pyridoxine and little increase of endogenous

PAL-P was seen. This change mimicked the pattern of enzyme induction by the administration of hydrocortisone by Greengard (5).

3. Change of TTA Activity by the Administration of Some Other Vitamins

To investigate whether the increase of TTA activity with pyridoxine in unoperated rats is specific for this vitamin, thiamine 5 mg, riboflavin 10 mg, nicotinic acid 40 mg per 100 g body weight respectively, dissolved in saline, were injected intraperitoneally and the hepatic TTA activities 4 hours after injections were determined. All the injection doses of each vitamins are corresponding to one fourth of LD_{50} to rats following the dose of pyridoxine. In all cases marked elevation of total enzyme activity was observed and there was no difference in the extent of the increase of holoenzyme activity among these vitamins (Fig. 5). These materials through intraperitoneal route would contribute to the induction of the enzyme as nonspecific metabolic stress as already reported by Kenney and Flora (10).

4. Change of Hepatic TTA Activity by Oral Administration of Pyridoxine

Since the augment of the enzyme activity by pyridoxine administration through intraperitoneal route was assumed to be a result of nonspecific peritoneal irritation, the change of hepatic TTA activity by oral administration was studied.

The time course of TTA activity after the oral administration of pyridoxine was nearly the same as that of intraperitoneal route and 50 mg dose elicited the maximal response (Fig. 6). The extent of the elevation of the enzyme activity by oral administration of several doses is similar to that of intraperitoneal injection. However, thiamine and other vitamins by oral administration did not produce any increase of hepatic TTA activity.

Experiments with Adrenalectomized Rats

1. Day-course of the Hepatic TTA Activity by Pyridoxine Administration after Adrenalectomy

Greengard (4) and Kenney (6) reported the induction of hepatic TTA of the rats 4 days after adrenalectomy on a assumption that its induced production was due to different mechanism from adrenocortical hormone.

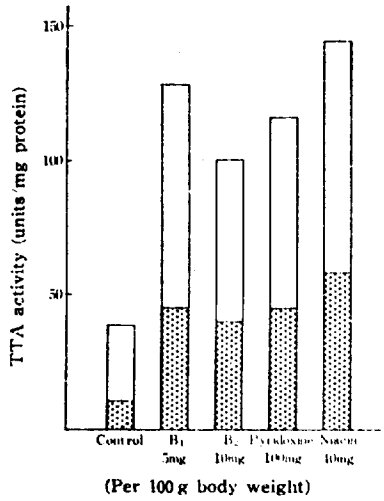

FIG. 5 *Change of TTA activity in unoperated rats 4 hours after intraperitoneal injection of some vitamins*

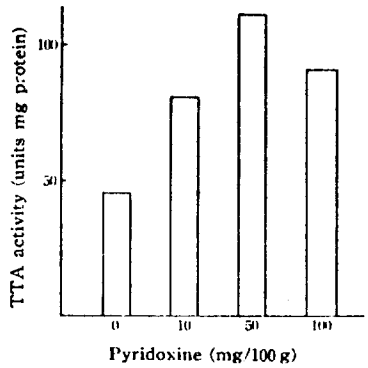

FIG. 6 *Change of TTA activity 4 hours after oral administration of pyridoxine*

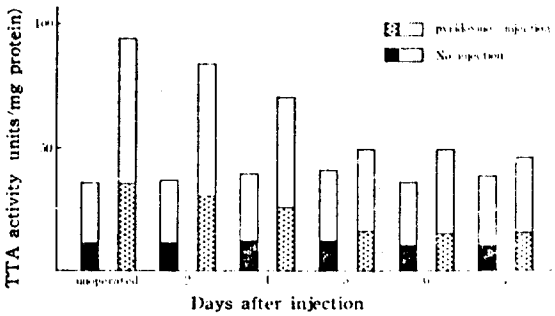

FIG. 7 *Change of TTA activity with pyridoxine injection after adrenalectomy*

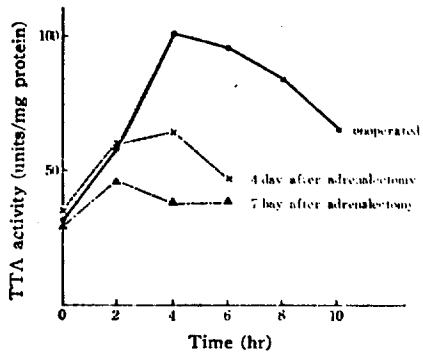

FIG. 8 *TTA activities after pyridoxine injection in adrenalectomized and unoperated rats*

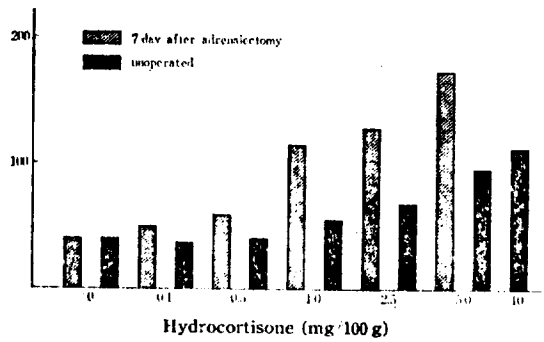

FIG. 9 *Change of TTA activity by varying amounts of hydrocortisone in 7 day after operation and unoperated rats*

The relationship between the days after adrenalectomy and change of TTA activity by pyridoxine administration was studied. As shown in Fig. 7, at 2 to 7 day intervals after adrenalectomy as indicated, 50 mg of pyridoxine per 100 g body weight was administered intraperitoneally and its hepatic TTA activity 4 hours after injection was estimated. In the rats of 4 days after adrenalectomy, the elevation of hepatic TTA by pyridoxine was observed as reported by Greengard and Kenney which was about 60 % of that of the unoperated. However, little increase was seen in the rats 7 days after adrenalectomy of TTA activity when compared with unoperated controls (Fig. 8). The inducibility of hepatic TTA by pyridoxine administration gradually diminished with the days after adrenalectomy and 7 days after the operation it almost disappeared, which seemed to imply the correlation of response by pyridoxine administration with remaining adrenocortical hormone in rats.

2. Effect of Hydrocortisone and Pyridoxine on the Hepatic TTA Activity of the Rats 7 Days after Adrenalectomy

The response of hepatic TTA of the adrenalectomized rats to the administration of pyridoxine gradually declined and it was scarcely seen on the 7th day after the operation, which mimicks the phenomena reported by Fazekas (10) that the content of serum corticosterone of adrenalectomized rats gradually diminished and was barely detected on the 7th day. However, the decrease of amino acid pool in the body tissue, due to the deficiency of dietary protein of rats, should be also considered; the TTA activity of 4 hours after intraperitoneal injection of hydrocortisone to the rats 7 days after adrenalectomy and unoperated rats was compared.

As shown in Fig. 9, the administration of hydrocortisone 5 mg per 100 g body weight elicited a 3-fold increase of total enzyme activity and the unoperated group properly showed a similar response. In either group the maximal response was attained after 4 hours and declined thereafter to reach the basal level after 10 hours. Fifty mg dose of pyridoxine was injected intraperitoneally with and without administration of hydrocortisone 5 mg per 100 g body weight 15 hours in advance. As shown in Fig. 10 the rats which had not received hydrocortisone beforehand

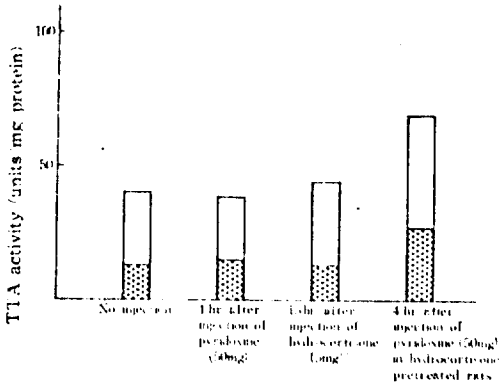

FIG. 10 *Effect of hydrocortisone and pyridoxine on TTA activity in 7 day after adrenalectomy rats*

showed no respose to pyridoxine, but those with pretreatment of hydrocortisone betrayed induced synthesis of TTA 4 hours after the injection of pyridoxine and the extent of the induction was nearly the same as that observed in the rats 4 days after adrenalectomy, which is to say that pyridoxine is effective when adrenocortical hormone is retained in the body tissue.

3. Effect of Administration of Pyridoxine and Some Other Vitamins on TTA Activity of Rats 4 Days after Adrenalectomy

Intraperitoneal injection of 50 mg dose pyridoxine to rats 4 days after adrenalectomy elicited a considerable increase of TTA activity. Thiamine and nicotinic acid which betrayed the same extent of effect as pyridoxine on the unoperated rats were intraperitoneally administered to rats 4 days after adrenalectomy and the effect on the hepatic TTA activity was compared 4 hours after the injection. As shown in Fig. 11, thiamine 5 mg or nicotinic acid 40 mg per 100 g body weight had no effect on the enzyme activity but 160 % enhancement over control was observed by even a 10 mg dose of pyridoxine. This shows that the effect of pyridoxine on hepatic TTA activity is considerably specific after adrenalectomy.

4. Effect of Vitamin B_6 Derivatives on TTA Activity of Rats 4 Days after Adrenalectomy

Pyridoxine, pyridoxal and pyridoxamine which are equally active biologically on rats, 50,10 and 50 mg per 100 g body weight respectively were injected intraperitoneally to rats 4 days after adrenalectomy and unoperated ones, and four hours after the injection the hepatic TTA activities were estimated. The injection doses of the three compounds were all one fourth of LD_{50} to rats. As shown in Fig. 12, although these compounds gave similar stimulative effects on the TTA activity in the unoperative rats, in the case of the operated rats, pyridoxine and pyridoxal had similar effects while pyridoxamine failed even at a dose 5 times as much as pyridoxal. It was of interest that the activity of a 10 mg pyridoxal dose coresponded to that of a 50 mg pyridoxine dose.

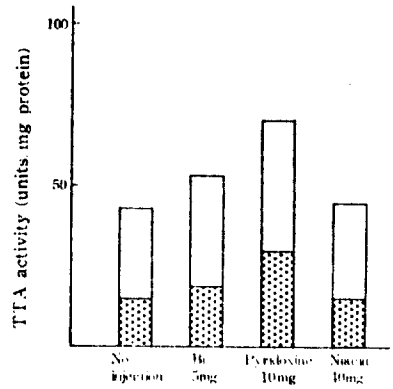

FIG. 11 *Change of TTA activity 4 hours after injection of B_1, pyridoxine and niacin in 4 day after adrenalectomy rats*

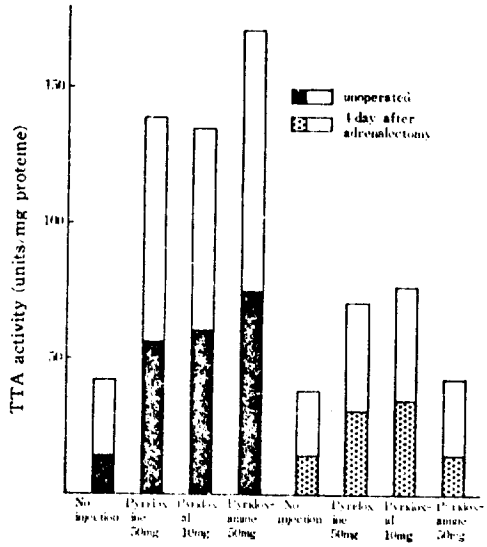

FIG. 12 *Change of TTA activity 4 hours after injection of B_6-derivatives in unoperated and 4 day after adrenalectomy rats*

5. Influence of Actinomycin D on the Induction of Hepatic TTA by Pyridoxine

Actinomycin D, 100 μg per 100 g body weight, which had an inhibitory effect on the mRNA snythesis concerned in the protein synthesis was injected intraperitoneally to the rats 4 days after adrenalectomy and unoperated ones. The TTA activity of 4 hours after intraperitoneal injection of the drug gave a nonspecific action on hepatic TTA resulting in elevation of total enzyme activity compared

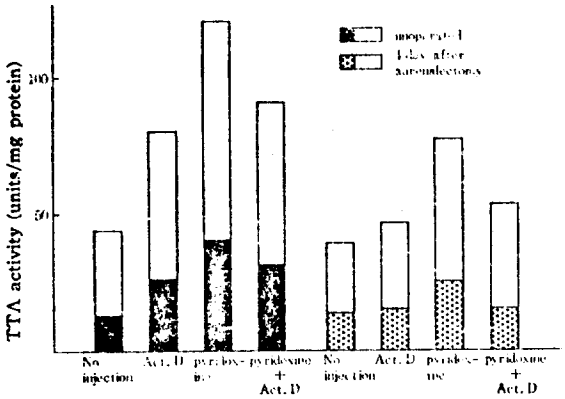

FIG. 13 *Inhibitory effect of actinomycin D on the induction of TTA by pyridoxine injection*

to the controls. But its simultaneous administration with pyridoxine depressed the induction by pyridoxine (Fig. 13).

6. Induction of Hepatic TTA of Adrenalectomized Rats by Tryptophan

There are many reports that either oral or intraperitoneal administration of tryptophan produces an increase of hepatic TTA activity of adrenalectomized rats (5, 9, 11). But the extent of the induction is not constant among those reports and its correlation with adrenocortical hormone has not been clarified. Therefore, we administered L-tryptophan 50 mg per 100 g body weight through oral route to rats 7 days after adrenalectomy and estimated the hepatic TTA activity 4 hours after the administration. As shown in Fig. 14, although pyridoxine and tyrosine gave no detectable

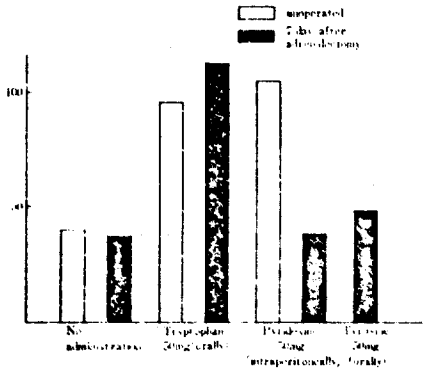

FIG. 14 *TTA activity 4 hours after administration of tryptophan, pyridoxine and tyrosine in unoperated and 7 day after adrenalectomy rats*

effect, remarkable increase of TTA activity was brought about by tryptophan uptake, from which it was assumed that the effect of tryptophan on TTA induction was due to a different mechanism from that of pyridoxine.

DISCUSSION

As TTA is a pyridoxal enzyme, the mechanism of its induced synthesis by a large dose of vitamin B_6 is a matter of great interest. In this respect Greengard (5) and Kenney (6) presented different views in the studies with adrenalectomized rats. Greegard seeked the action point of the vitamin on the TTA induction on "translation" step by a reason that the increase of the activity was blocked by puromycin but not by actinomycin D, while Kenney on "transcription" step as it was also inhibited by actinomycin D. Our experiment reached the conclusion that the induction by the vitamin was mediated by adrenal cortex hormone. The induction was actinomycin D-sensitive in our experiment, in which respect our result is consistent with the report by Kenney. We assume that this contradiction of the findings about the inhibitory effect of actinomycin D may be due to the difference in the dose administered, that is, Greengard administered 70 μg per 100 g body weight of rats and Kenney 120 μg. We used 100 μg per 100 g body weight according to a Labrie's report (12). Mechanism of TTA induction by pyridoxine is similar to that by adrenal glucocorticoid. Although the similar increase of TTA activity is observed by the intraperitoneal injection of some other vitamins in unoperated rats, it is no more seen after adrenalectomy when the effect of pyridoxine is still persisting. In this respect this effect is to be considered specific for pyridoxine.

In fact, the effect of pyridoxine on the rats 2 or 4 days after adrenalectomy is consistent with the reports by Greengard and Kenney that hepatic TTA of adrenalectomized rats responded well to pyridoxine administration. However, 7 days after adrenalectomy this response to pyridoxine was no more observed. The change of inducibility by pyridoxine after

adrenalectomy is coincident with a vicissitude of serum corticosteroid seen in a Fazekas' report (10), hence we assumed that pyridoxine might effect on the adrenocortical hormone remaining in the blood or body tissue. Ninety per cent of the corticosteroid is thought to be bound to serum protein (13) which is intesified after adrenalectomy (14). According to Fazekas, corticosteroid is also deposited in various body tissue bound to protein. When pyridoxine 100 mg per 100 g body weight is intraperitoneally given to a rat, about 200 fold increase of serum vitamin B_6 content and about 10 fold of hepatic content are observed. This large amount of the vitamin B_6 may concern setting free the protein bound corticosteroid. This concept can be somewhat supported by the finding that on the 7th day after adrenalectomy, corticosteroid in the body tissue might have disappeared displaying no more effect on the induction of TTA by a large dose administration of pyridoxine and the induction of TTA by pyridoxine is resumed by the injection of hydrocortisone 14 hours in advance. Among the three derivatives of B_6 which have biologically the same effect on rats, differences were observed in the extent of induction of TTA. It seems to be in parallel with the strength of affinity of the derivatives with protein. Acid antiinflammatory agents such as phenylbutazone which are capable to bind with serum protein, elevate the TTA activity of the rats 4 days after adrenalectomy, just as same as seen by the pyridoxine injection. This effect of the agents is no more observed in the rats 7 days after adrenalectomy (17).

According to Nakao (15) about 20 % of rats bear accessory adrenal gland and it compensatively enlarges after adrenalectomy. In our experiment 20-30 % cases of adrenalectomized rats showed erratic value in TTA activity which we assume to be due to accessory adrenal gland.

There are several reports about induction synthesis of TTA by amino acid mixture or tryptophan (11). The daily rhythmic change of TTA activity is also recently reported relating to the diet (16). Remarkable induced synthesis of hepatic TTA was observed dy oral administration of tryptophan to the rats 7 days after adrenalectomy when the induction by pyridoxine was no more detected. This fact lends support to a conclusion that the induction by tryptophan is not mediated by adrenal cortex hormone, but by a different mechanism from that of vitamin B_6.

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Metabolism of [$^3H_8$]Pyridoxine in Mice*

Stig Johansson, Sven Lindstedt,†,‡ and Hans-Göran Tiselius†

ABSTRACT: [$^3H_3$]Pyridoxine has been administered by the intravenous route to mice, and perchloric acid extracts of liver and carcass have been fractionated by an ion-exchange chromatographic procedure. The distribution of isotope between pyridoxine 5′-phosphate, pyridoxal, pyridoxal 5′-phosphate, pyridoxamine, and pyridoxamine 5′-phosphate has been calculated at different times after the administration of pyridoxine. Labeled pyridoxine disappeared from the liver within 60 min after the injection. During this period the labeling of pyridoxine 5′-phosphate first increased and reached a maximum after 10–15 min when 40% of the recovered isotope appeared as pyridoxine 5′-phosphate. Concomitantly with the following decrease in labeling of the pyridoxine 5′-phosphate, the labeling of pyridoxal 5′-phosphate increased. The incorporation of isotope into pyridoxamine 5′-phosphate occurred at a slower rate; between days 1 and 7 the ratio between pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate remained constant. In carcass the same processes occurred as in liver but at a slower rate. The specific radioactivity was determined in pyridoxal 5′-phosphate, pyridoxal, pyridoxamine 5′-phosphate, and pyridoxamine between days 1 and 7 after the administration of [$^3H_3$]pyridoxine. It remained approximately constant between days 4 and 7 indicating a slow turnover of the body pool of vitamin B_6.

In animal tissues vitamin B_6 occurs mainly in the form of pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate (Lyon *et al.*, 1962). The requirement for vitamin B_6 is covered to a large extent by vitamin B_6 in cereals, in which pyridoxine is a large fraction of the total vitamin B_6 content (Toepfler and Polansky, 1964). Little is known about the interconversion of the different forms of the vitamin *in vivo* in animals and humans (*cf.* Snell, 1964). It has been suggested that a functional vitamin B_6 deficiency may arise through impaired conversion of injected pyridoxine into a metabolically active form and that such a mechanism may, at least partially, be responsible for the antivitamin effect of certain carbonyl reagents, *e.g.*, isoniazid (Snell, 1964). Possibly, such mechanisms may also be involved in aberrations of vitamin B_6 metabolism such as the dependency syndrome (Harris *et al.*, 1956; Harris and Horrigan, 1964) or vitamin B_6 responsive anemia (Hunt *et al.*, 1954; Coursin, 1964).

The purpose of the present study was to obtain information on the conversion of pyridoxine into other forms of the vitamin *in vivo* in a normal animal.

Material and Methods

Compounds. [$^3H_3$]Pyridoxine was obtained from the Radiochemical Centre (Amersham, Bucks, England) with a specific radioactivity of about 1 μCi/μg. According to the manufacturer this preparation was more than 97% radiochemically pure by the following criteria: paper chromatography in (i) 1-butanol–dioxane–ammonia, (ii) 1-butanol–acetic acid–water, and (iii) ethanol–ammonia–water; thin-layer chromatography in acetone–dioxane–ammonia. This was also verified by ion-exchange chromatography on a column of Dowex 50 (Figure 1) and by high-voltage electrophoresis (Clotten and Clotten, 1962). Pyridoxine-HCl, pyridoxamine-HCl, pyridoxal-HCl, pyridoxamine 5′-phosphate, and pyridoxal 5′-phosphate were gifts from Merck Sharp and Dohme, Rahway, N. J. Pyridoxine 5′-phosphate was purchased from Calbiochem, Luzerne, Switzerland.

Animals and Preparation of Tissues. Male mice of the N. M. R. I. strain, weighing about 25 g, which had been kept on a commercial mouse diet (Harld Forss Co., Stockholm, Sweden), were injected in a tail vein with 0.1 ml of a solution of [$^3H_3$]pyridoxine which contained 200 μg/ml. The mice were killed by a blow on the head at different times after the injection.

Tissues were homogenized in an equal weight of water in a VirTis homogenizer (The VirTis Co., Inc., Gardiner, New York, N. Y.). The proteins were precipitated by the addition of an equal volume of 2 N perchloric acid, the mixture was centrifuged, and the proteins were washed twice with 1 N perchloric acid. The combined solutions were titrated to pH 4.2 with 3 M potassium hydroxide and left overnight at 0°. Potassium perchlorate was filtered in the cold and the filtrate was evaporated to dryness *in vacuo* at a bath temperature of 40°.

Chromatographic Procedure. A suitable aliquot of the perchloric acid extract was dissolved in 1–2 ml of 0.05 M ammonium formate buffer at pH 4.25 and put onto a column (diameter 9 mm, height 400 mm) of Dowex 50-X8. The column was eluted first with 100 ml of 0.05 M ammonium formate at pH 4.25. A gradient was then

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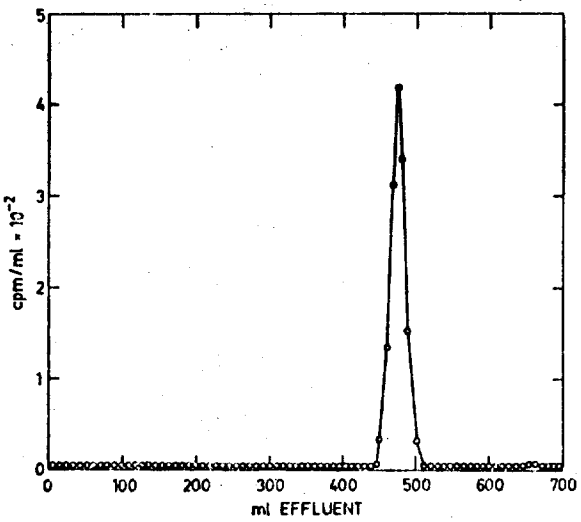

FIGURE 1: Chromatography of [$^3H_5$]pyridoxine on a column (height 40 cm; diameter 0.9 cm) of Dowex 50-X8 (200–400 mesh). The column was equilibrated with 0.05 M ammonium formate at pH 4.25 and eluted with 100 ml of the same buffer, followed by 0.5 M ammonium formate with an increasing pH gradient (4.25–7.5).

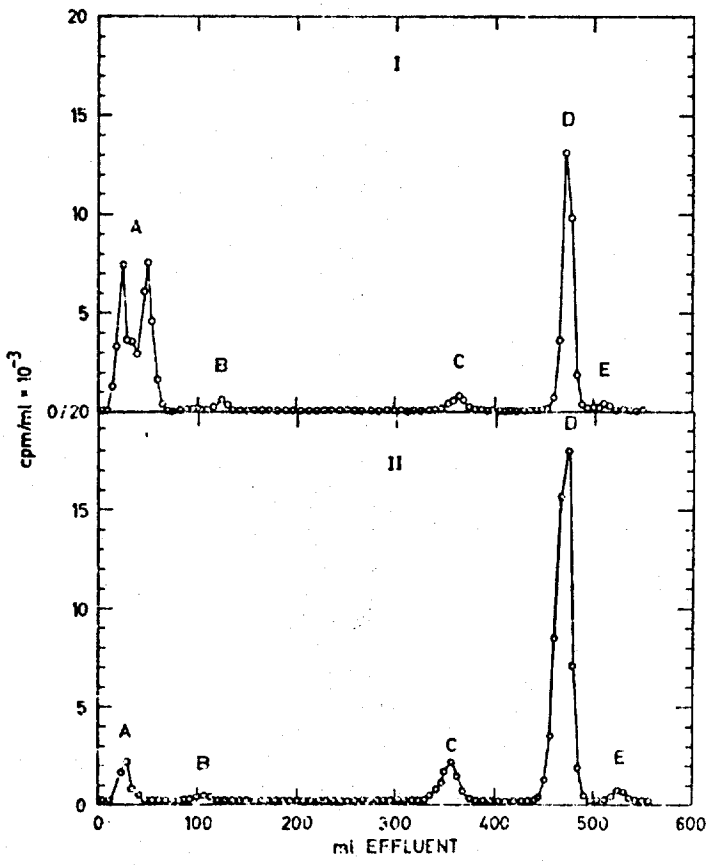

FIGURE 2: Chromatography on 2 columns (height 40 cm; diameter 0.9 cm) of Dowex 50-X8 of a perchloric acid extract of mouse liver 12 min after the injection of [$^3H_5$]pyridoxine. I is the unhydrolyzed extract and II the same extract after hydrolysis. The peaks are: (A) pyridoxine 5′-phosphate and pyridoxal 5′-phosphate, (B) pyridoxamine 5′-phosphate, (C) pyridoxal, (D) pyridoxine, and (E) pyridoxamine.

started with 100 ml of 0.5 M ammonium formate at pH 4.25 in a closed chamber to which was added 0.5 M ammonium formate at pH 7.5. The eluate was collected in fractions of 5-ml volume at a flow rate of about 0.5 ml/min.

Isotope Determination. The isotope content was determined on each chromatographic fraction in a liquid scintillation spectrometer (Tri-Carb, Packard Co., La Grange, Ill.). To 0.4 ml of each fraction was added 16 ml of a mixture of the following composition: 2,5-diphenyloxazole (10 g), 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (0.3 g), toluene (1000 ml), and methyl Cellosolve (600 ml). The efficiency for tritium in this system was about 15%.

Assay of Vitamin B_6 Activity. Vitamin B_6 activity was determined with a microbiological technique with *Saccharomyces carlsbergiensis* ATCC 9080, strain 4288. An aliquot of each chromatographic fraction (1 ml) was added to 1 ml of 0.4 M sulfuric acid and the solution was kept in an autoclave at 120° for 1 hr. The solution was then adjusted to pH 5.0–5.2 with a potassium citrate buffer at pH 12.3. Part of this solution (1 ml) was then used in the microbiological assay, which was carried out essentially as described by Atkin *et al.* (1943).

Results

Short-Time Experiments. A series of mice were injected intravenously with 20 μg (20 μCi) of [$^3H_5$]pyridoxine and killed at different times within the first hour. Figure 2 shows ion-exchange chromatograms of the perchloric acid extract of liver from the experiment in which homogenization of the liver was started 12 min after the injection.

The main radioactive compound seen in Figure 2 is pyridoxine (peak D) but radioactive material also appears at the position of pyridoxal 5′-phosphate and pyridoxine 5′-phosphate (peak A) and in small amounts at the position of pyridoxamine 5′-phosphate (peak B), pyridoxal (peak C), and pyridoxamine (peak E). Figure 3 illustrates the changes in the percentage distribution of isotope between the different forms of vitamin B_6 in mouse liver during the first hour after injection. Labeled pyridoxine disappeared rapidly and accounted for less than 1% of the total isotope after 60 min. The labeling of pyridoxine 5′-phosphate reached its maximum about 10 min after injection of the [$^3H_5$]pyridoxine and then declined. In contrast, isotope in pyridoxal 5′-phosphate increased and after 60 min this compound accounted for about 70% of the recovered isotope. The amount of isotope in the other forms of the vitamin was small.

Figure 4 shows an ion-exchange chromatogram of a perchloric acid extract of the carcass from a mouse which had been killed 35 min after injection of labeled pyridoxine. The top curve shows the distribution of isotope in the chromatogram and the bottom curve the result of microbiological assay on the individual fractions. The main labeled compound at this time is pyridoxine (peak D), followed by pyridoxine 5′-phosphate and pyridoxal 5′-phosphate (peak A).

The distribution of isotope between the different forms of the vitamin in carcass is illustrated in Figure 5. The labeling of pyridoxine declined immediately after the injection, but after 60 min 60% of the total isotope was still present as pyridoxine. The labeling of pyridoxine 5′-phosphate increased, and this form of the vitamin accounted for 25% of the recovered isotope after 60 min. At this time only small amounts of isotope had been incorporated into other forms of the vitamin.

Long-Time Experiments. Figure 6 shows the distribution of recovered isotope between the different forms of the vitamin in the liver from days 1 to 7 after the intravenous administration of [$^3H_3$]pyridoxine. The initial values in this figure are the 60-min values from Figure 2. Twenty-four hours after the administration of labeled pyridoxine the isotope appeared mainly in pyridoxal 5′-phosphate and in pyridoxamine 5′-phosphate; less then 10% appeared as free pyridoxal or pyridoxamine. From days 1 to 7 there was no significant change in the distribution of isotope between the different forms of the vitamin.

In carcass (Figure 7) about 7% of the total isotope remained as pyridoxine 1 day after injection of [$^3H_3$]pyridoxine. At this time about equal amount of isotope (~25%) appeared in pyridoxal 5′-phosphate and in pyridoxamine 5′-phosphate and about 20% of the isotope appeared in each of pyridoxal and pyridoxamine. The distribution of isotope between the different forms of vitamin B_6 was essentially unchanged from days 1 to 7. Figure 8 shows a plot of the specific radioactivity of pyridoxamine 5′-phosphate, pyridoxamine, pyridoxal 5′-phosphate, and pyridoxal in the liver from days 1 to 7 after the injection of [$^3H_3$]pyridoxine. Figure 9 shows the specific radioactivity of the same compounds in carcass. It is seen from these figures that the specific radioactivity was approximately the same in the different forms of the vitamin, and also that there was no marked change in the specific radioactivity from days 4 to 7 after the administration of the labeled pyridoxine.

Discussion

Tritium-labeled pyridoxine has been used to study the excretion of vitamin B_6 in the rat (Cox *et al.*, 1962), the absorption of pyridoxine from the gut in rats (Booth and Brain, 1962) and humans (Brain and Booth, 1964), and the turnover and body pool of the vitamin (Johansson *et al.*, 1966a,b). It has been pointed out by Argoudelis and Kummerow (1966) that commercially available pyridoxine labeled by the Wilzbach (1957) technique may contain appreciable amounts of labeled impurities and that results obtained with such preparations may be invalid. Our preparation was obtained from another source than that referred to by Argoudelis and Kummerow and was radiochemically pure as evidenced by paper and thin-layer chromatography, ion-exchange chromatography, and high-voltage electrophoresis. Only after prolonged storage and repeated thawings of frozen solutions have we occasionally observed labeled degradation products of pyridoxine. These are however easily removed by ion-exchange chromatography. In a study of pyridoxine metabolism in animals it would be desirable to administer pyridoxine in "trace" amounts in order to conserve a physiological steady state. It has for instance been demonstrated that a proportionally larger amount of isotope is excreted in the urine when the

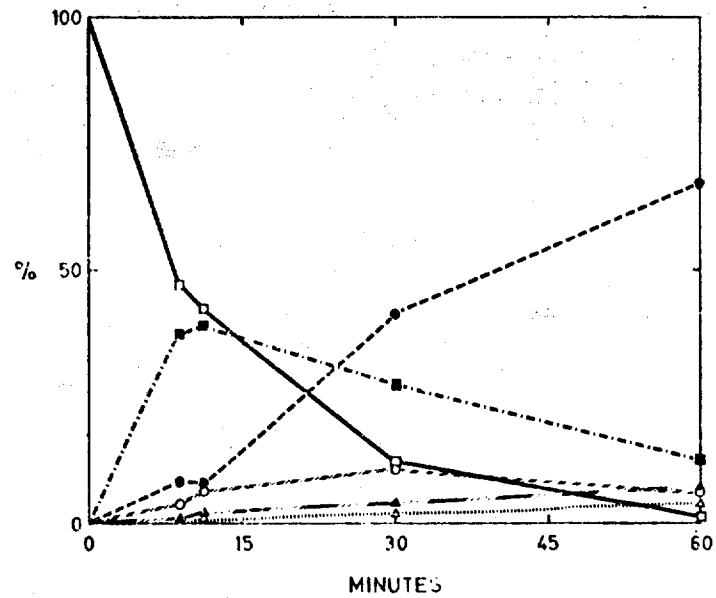

FIGURE 3: Distribution of radioactivity in mouse liver between different forms of vitamin B_6 during the first hour after intravenous administration of [$^3H_3$]pyridoxine. Pyridoxine (□), pyridoxine 5′-phosphate (■), pyridoxal (○), pyridoxal 5′-phosphate (●), pyridoxamine (△), and pyridoxamine 5′-phosphate (▲).

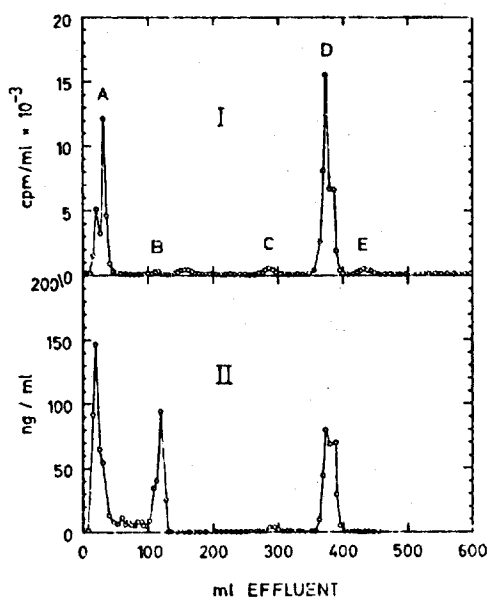

FIGURE 4: Chromatography on a column (height 40 cm; diameter 0.9 cm) of Dowex 50-X8 (200–400 mesh) of a perchloric acid extract of mouse carcass 35 min after the injection of [$^3H_3$]pyridoxine. The column had been equilibrated with 0.05 M ammonium formate at pH 4.25. It was eluted first with 100 ml of the same buffer, followed by 0.5 M ammonium formate with an increasing pH gradient (4.25–7.5). The *top curve* shows isotope determination and the *bottom curve* the microbiological assay with *S. carlsbergiensis*. The peaks are (A) pyridoxine 5′-phosphate and pyridoxal 5′-phosphate, (B) pyridoxamine 5′-phosphate, (C) pyridoxal, (D) pyridoxine, and (E) pyridoxamine.

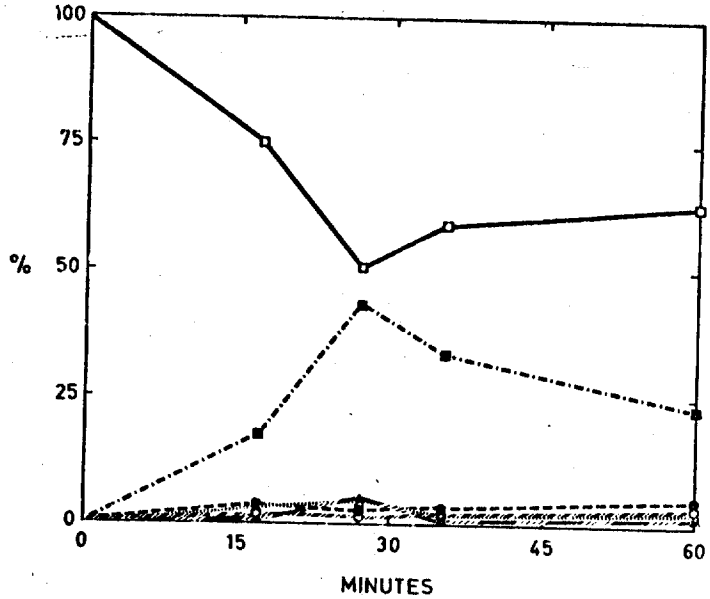

FIGURE 5: Distribution of isotope between the different forms of vitamin B_6 in mouse carcass during the first hour after the intravenous administration of [$^3H_6$]pyridoxine. Pyridoxine (□), pyridoxine 5′-phosphate (■), pyridoxal (○), pyridoxal 5′-phosphate (●), pyridoxamine (△), and pyridoxamine 5′-phosphate (▲).

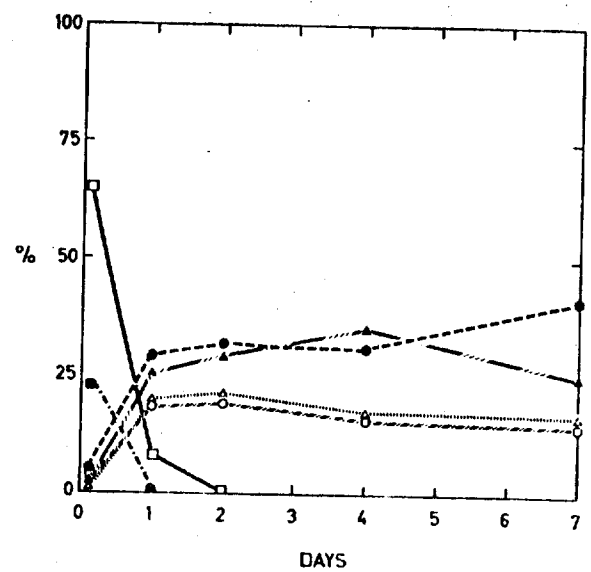

FIGURE 7: Distribution of isotope between different forms of vitamin B_6 in mouse carcass from days 1 to 7 after the intravenous administration of [$^3H_6$]pyridoxine. Pyridoxine (□), pyridoxine 5′-phosphate (■), pyridoxal (○), pyridoxal 5′-phosphate (●), pyridoxamine (△), and pyridoxamine 5′-phosphate (▲).

amount of administered pyridoxine is increased (Johansson *et al.*, 1966a). In this study 20 μg of pyridoxine was injected as a compromise between the desire to keep the injected amount as low as possible and the need for sufficient amount of isotope in the chromatographic separations.

Since a high specific radioactivity of the pyridoxine was required, tritium-labeled pyridoxine had to be used. We have recently found that about 20% of the tritium atoms is located at the carbon in the 4-hydroxymethyl group (S. Lindstedt, S. Johansson, and H.-G. Tiselius, unpublished data). This tritium is metabolically labile and is, *e.g.*, lost in the conversion into 4-pyridoxic acid; the remaining tritium is apparently metabolically stable in animals in the reactions discussed in this paper.

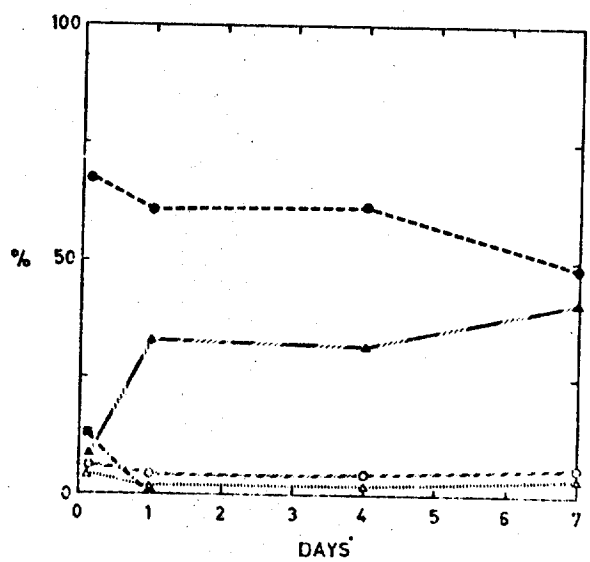

FIGURE 6: Distribution of isotope between different forms of vitamin B_6 in the liver from days 1 to 7 after the intravenous administration of [$^3H_6$]pyridoxine. Pyridoxine (□), pyridoxine 5-phosphate, (■) pyridoxal (○), pyridoxal 5-phosphate, (●) pyridoxamine (△), and pyridoxamine 5-phosphate (▲).

Problems involved in the extraction of vitamin B_6 from biological material have been discussed by Storvick and Peters (1964). The method used in this study is essentially that which has been used by Bain and Williams in their chromatographic studies of the concentration of different forms of vitamin B_6 in animal tissues (Bain and Williams, 1960; Lyon *et al.*, 1962). In our experience at least 85% of the total amount of labeled compounds is extracted by this procedure.

A chromatographic system for the fractionation of the different forms of vitamin B_6, described by Bain and Williams (1960), utilizes a two-layered column of the anion exchanger Dowex 1 and the cation exchanger Dowex 50 and a series of different buffers for elution. In our hands, with large volumes of tissue extract, this system did not give consistently reproducible results. A simpler chromatographic system with the cation exchanger Dowex 50 and volatile buffers was developed, but this system did not completely separate pyridoxine 5′-phosphate and pyridoxal 5′-phosphate. When both these compounds were present their relative amounts could be calculated either from a comparison of chromatograms of hydrolyzed and unhydrolyzed tissue extracts (*cf.* Figure 2) or by rechromatography of pyridoxine and pyridoxal obtained by hydrolysis of the unresolved mixture.

Present-day concept of the interconversion of the different forms of vitamin B_6 is mainly based on *in vitro* experiments and on the isolation of different enzymes responsible for the interconversions (Snell, 1964). However, Rabinowitz and Snell (1949) administered pyri-

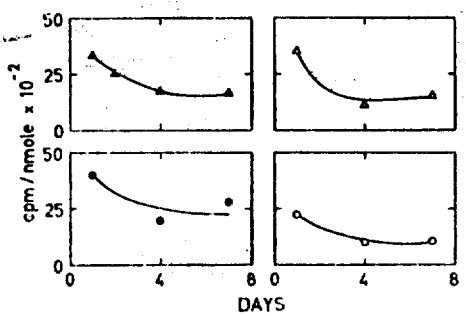

FIGURE 8: Specific radioactivity of pyridoxal (○), pyridoxal 5-phosphate (●), pyridoxamine (△), and pyridoxamine 5-phosphate (▲) in mouse liver after the intravenous administration of [^{3}H$_3$]pyridoxine.

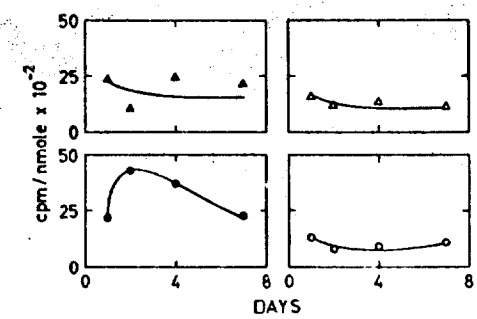

FIGURE 9: Specific radioactivity of pyridoxal (○), pyridoxal 5-phosphate (●), pyridoxamine (△), and pyridoxamine 5-phosphate (▲) in mouse carcass after the intravenous administration of [^{3}H$_3$]pyridoxine.

doxine, pyridoxamine, and pyridoxal to humans and determined these compounds in urine with a differential microbiological assay. The excretion of 4-pyridoxic acid was increased when either of these compounds was administered. Injection of pyridoxine resulted in a greatly increased excretion of pyridoxal and pyridoxamine, but pyridoxine excretion was not increased after the feeding of pyridoxal or pyridoxamine. Since the microbiological assay was carried out on hydrolyzed samples it could not be decided if these compounds were excreted in free or phosphorylated forms.

The present study has demonstrated a rapid phosphorylation of pyridoxine in the liver; practically no free pyridoxine could be detected 60 min after the intravenous injection of [^{3}H$_3$]pyridoxine. Ten minutes after the injection of [^{3}H$_3$]pyridoxine about 40% of the recovered isotope appeared as pyridoxine 5′-phosphate and the relation between pyridoxine and pyridoxine 5′-phosphate resembled a classic precursor–product relationship. ATP:pyridoxal 5′-phosphotransferase (EC 2.7.1.35) has been partially purified from rat liver and brain, from beef and human brain, and also from several microorganisms (McCormick and Snell, 1959, 1961; McCormick *et al.*, 1961). Pyridoxal was the preferred substrate, but pyridoxine and pyridoxamine were also phosphorylated; the K_m values for these substrates were reported as 0.15, 0.25, and 1.5×10^{-4} M for the rat liver enzyme. The same authors also studied the distribution of the enzyme in rat tissue and found high activity in liver, kidney, spleen, and brain. The activity in skeletal muscle was low, which agrees with our finding that labeled pyridoxine disappeared considerably slower from carcass than from liver. It is plausible that some of the pyridoxine 5′-phosphate which is found in carcass may have originated in liver or other parenchymatous organs, but present data do not allow any estimate of such transfer.

The isotope content of the pyridoxal 5′-phosphate in liver started to rise immediately after the injection, but very little pyridoxal 5′-phosphate had appeared in carcass within the first 60 min after injection of labeled pyridoxine although a fairly high concentration of labeled pyridoxine 5′-phosphate had been attained at this time. An enzyme catalyzing the oxidation of pyridoxine 5′-phosphate and of pyridoxamine 5′-phosphate to pyridoxal 5′-phosphate was first demonstrated in rabbit liver (Pogell, 1958). This enzyme pyridoxamine 5′-phosphate:O_2 oxidoreductase (deaminating), (EC 1.4.3.5) has been purified about 65-fold (Wada and Snell, 1961). Its distribution in different tissues has not been studied, but its concentration in muscle is apparently low.

In carcass, and to a smaller extent in liver, isotope was recovered in pyridoxal and pyridoxamine. A direct conversion of pyridoxine into pyridoxal has been demonstrated (Braunstein and Bukin, 1956), but these compounds may also originate from the action of phosphatases on the respective phosphorylated forms.

In liver the appearance of isotope in pyridoxamine 5′-phosphate was slower than in pyridoxal 5′-phosphate, whereas in carcass these two forms of the vitamin increased almost in parallel in isotope content. The enzymatic mechanisms behind the formation of pyridoxamine and pyridoxamine 5′-phosphate are not as obvious as those involved in the interconversions discussed above. In the course of enzymatic transamination enzyme-bound pyridoxal 5′-phosphate would be transiently converted into pyridoxamine 5′-phosphate. An enzyme catalyzing the reversible transamination between pyridoxamine and oxaloacetate has been purified from rabbit liver (Wada and Snell, 1962). Since the results strongly support the idea that phosphorylation is the primary step in the metabolic transformation of pyridoxine, this reaction is probably of minor significance *in vivo*.

Rabinowitz and Snell (1949) in their study on the metabolism of vitamin B_6 in humans failed to account for more than about 45% of the administered amount in the urine. Later studies with labeled pyridoxine have demonstrated that a variable fraction of administered pyridoxine is retained in the body and excreted at a slow rate (Johansson *et al.*, 1966a,b). Based on the shape of the isotope retention curve a metabolic model for the *in vivo* metabolism of pyridoxine has been proposed, and it has been suggested that the total amount of vitamin B_6 in the organism may be estimated by the principle of isotope dilution from knowledge of the total administered isotope and the specific radioactivity of the urinary 4-pyridoxic acid. This procedure requires that the different forms of the vitamin reach the same specific radioactivity after an initial period of equilibration. To test this hypothesis the specific radioactivity of pyridoxal 5′-phosphate, pyridoxal, pyridoxamine 5′-phosphate,

and pyridoxamine was determined at different times after the administration of [$^3H_8$]pyridoxine. They were found to reach approximately the same specific radioactivity. However, the values are probably not very accurate since the microbiological assay has a fairly large methodological error. For this reason, no corrections were used for loss of tritium atoms in the conversion of pyridoxine into pyridoxal and pyridoxamine or for the slightly different growth response of *S. carlsbergiensis* to the three forms of the vitamin. The slow decline in specific radioactivity of the different forms of the vitamin between days 4 and 7 confirms previous results, demonstrating a slow turnover of the body pool of vitamin B_6 (Johansson *et al.*, 1966a,b).

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Studies on the Metabolism of Labeled Pyridoxine in Man

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THE CLINICAL symptoms of vitamin B_6 deficiency in human subjects include seborrhea-like dermatitis, cheilosis and glossitis, but other manifestations of a deficiency, or possibly disturbance in the normal metabolism of the vitamin, have been reported.[2] Harris and co-workers[3] in 1956 described the first case of a pyridoxine-responsive anemia; other cases have since been reported.[4] A number of cases of convulsive disorders in children have been reported which responded to treatment with vitamin B_6.[5-8] Abnormalities in the vitamin B_6 nutritive status of mongoloid children have been reported[9] and recently it was suggested that vitamin B_6 therapy may be of value in patients with urinary calculi,[10] since it has been shown that urinary calculi formed of calcium oxalate frequently develop in cats[11] and rats[12] consuming a diet deficient in vitamin B_6. Lepkovsky and co-workers[13] demonstrated in 1943 that rats deficient in vitamin B_6 excrete xanthurenic acid in the urine, a sign of abnormal tryptophan metabolism. The same observation has been made in other species including man,[14] and a "tryptophan tolerance test" has been used widely to assess the vitamin B_6 nutritive status of human subjects.[14,15] For example, with this test it has been shown that pregnant women have a relative deficiency of vitamin B_6.[16,17] Determinations of the blood level of glutamic oxaloacetic transaminase[18,19] or pyridoxal phosphate[19-21] have also been used to assess vitamin B_6 nutritive status. Still, the optimal daily requirement of vitamin B_6 cannot be regarded as well established and additional methods are necessary for the study of the metabolism of vitamin B_6 and the determination of vitamin B_6 nutritive status. It appeared possible that further information could be obtained by a study of the over-all metabolism of pyridoxine. Therefore we administered tritium-labeled pyridoxine to three subjects and estimated the excretion of isotope in the urine for up to twenty-five days. Furthermore, 4-pyridoxic acid was isolated from each 24 hour urine specimen and its specific radioactivity was determined. On the basis of the results obtained we propose a simple metabolic model for the over-all metabolism of pyridoxine from which an elimination rate of vitamin B_6 from the body reservoir may be calculated, and which may be used to estimate total vitamin B_6 in the body.

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MATERIALS AND METHODS

Three subjects were studied. S. S., a forty-seven year old man with recurrent kidney stones consisting of calcium oxalate, was hospitalized during the first week of the study. W. W. was a thirty seven year old man who had passed 100 calcium oxalate stones in the course of ten years. Blood chemistry and kidney functions were normal in both these subjects. R. E. was a fifty year old man with chronic schizophrenia of thirty years' duration. At the time of the study R. E. was in a mental hospital but he was in good physical condition. The subjects consumed their regular diet.

Pyridoxine, universally labeled with tritium was purchased from the Radiochemical Center (Amersham, Buckinghamshire, Great Britain). It had a specific radioactivity of about 1 μc. per μg. and gave one single isotope-containing peak when chromatographed on the Dowex 50 cation exchange resin column (described subsequently). Subjects S. S. and R. E. were given an intravenous injection of 400 μg. of labeled pyridoxine dissolved in physiologic saline solution, whereas subject W. W. was given 250 μg. orally in a glass of water. Urine was collected in bottles containing formic acid as a preservative in 4 to 8 hour samples during the first day after administration of the labeled pyridoxine and in 24 hour samples during the succeeding days.

Analytical Procedures

From each urine sample 10 ml. was adjusted to pH 4.0 to 4.2 with ammonium hydroxide and put onto a column (diameter 14 mm., height 130 mm.) of Dowex 2-×8, (200 to 400 mesh, formate form). The column was washed with 40 ml. of water and eluted with 0.6 M formic acid in fractions of 2 ml. volume. The fluorescence of each fraction was determined at 425 nm. (activating wavelength 325 nm.) in a spectrophotofluorometer (Aminco-Bowman, American Instrument Co., Inc., Silver Spring, Maryland). The fractions containing 4-pyridoxic acid were combined and kept frozen for further analysis. The effluent from the column corresponding to the sample and the wash water was put onto a column (diameter 14 mm., height 130 mm.) of Dowex 50-×8 (200 to 400 mesh, ammonium form). Forty milliliters of 0.05 M ammonium formate at pH 4.0 was passed through the column, which was then eluted in 2 ml. fractions with 100 ml. of 0.25 M ammonium formate at pH 7.0, followed by 60 ml. of 1 M ammonium formate at pH 7.0. The fluorescence of the fractions was determined at 400 nm. with an activating wavelength of 330 nm. Aliquots of 0.4 ml. of the fractions were used for the determination of isotope.

High-voltage electrophoresis was performed in a water cooled apparatus (AB Analysteknik, Vallentuna, Sweden) on Munktell 302 filter paper (60 volts per cm.) in a pyridine acetic acid buffer at pH 3.6 (pyridine 10 ml., acetic acid 100 ml., water 890 ml.).[22] Pyridoxine and its derivatives were made visible by dipping the dried papers in a 0.1 per cent solution of 2,6-dichloroquinone chloroimide[23] in benzene.

The quantitative determination of 4-pyridoxic acid was performed after conversion of the acid to the lactone by the principle of Huff and Perlzweig[24] and Sarett.[25] Aliquots of 1 to 8 ml. of the combined fractions containing 4-pyridoxic acid, isolated by chromatography on a Dowex 2 column, were placed in sample and blank tubes, and the volume was brought to 10 ml. with distilled water. To each tube was added 2 ml. of 6 M hydrochloric acid and the sample tubes were placed in a boiling water bath for 15 minutes. The sample tubes were cooled to room temperature and 4 ml. of 6 M ammonium hydroxide was added to sample and blank tubes. After mixing, water was added to a total volume of 25 ml. The fluorescence was then determined at 440 nm. with an activating wavelength of 355 nm., and the reading from the blank tube was subtracted from the reading from the sample tube. The standard used was 4-pyridoxic acid (Sigma Chemical Co., St. Louis, Missouri).

Radioactivity was determined in a liquid scintillation spectrometer (Tri-Carb[8], Packard Co., La Grange, Illinois). Of each urine sample or chromatographic fraction, 0.4 ml. was added to 16 ml. of a counting mixture of the following composition: 10 gm. PPO (2,5-diphenyloxazol), 0.3 gm. dimethyl-POPOP (1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene), 1,000 ml. toluene and 600 ml. methyl cellosolve. The counts were corrected for quenching by the addition of 0.1 ml. of a water solution of labeled pyridoxine as an internal standard. The efficiency of the counting procedure was about 15 per cent for tritium. A sufficient number of counts to give a statistical error of less than 5 per cent were recorded for each sample.

RESULTS

Rate of Excretion and Turnover of Vitamin B_6

The cumulative amount of isotope excreted in the urine was subtracted from that administered to obtain the amount of isotope remaining in the body at different times after tritium-labeled pyridoxine was given, since experiments with animals have shown that the excretion of isotope in the feces is negligible.[26] The percentage of administered isotope remaining in the body was plotted versus time on a semilogarithmic scale for the three subjects (Fig. 1 to 3). These curves may be resolved into two linear components with different slopes; thus, the equations for the curves are of the general form of equation 1:

$$R_t = R_0 (Me^{-\gamma 1^t} + Ne^{-\gamma 2^t}) \qquad (1)$$

in which R_t is the amount of isotope in the body

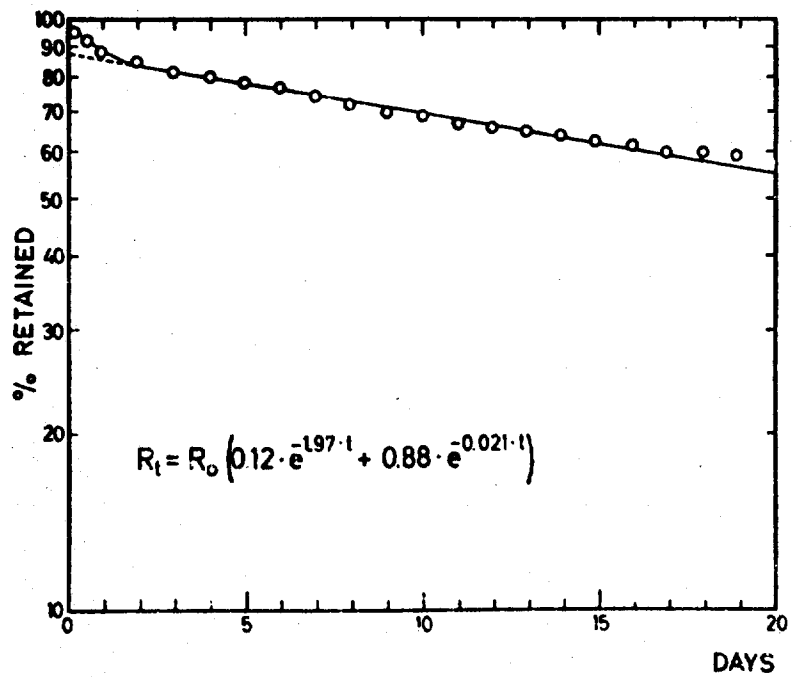

FIG. 1. Isotope retention curve obtained after the intravenous administration of 400 μg. of tritium-labeled pyridoxine to subject S. S. The per cent of administered isotope retained in the body at different times (R_t) has been calculated by subtraction of the cumulative urinary excretion of isotope from the amount administered (R_0 = 100).

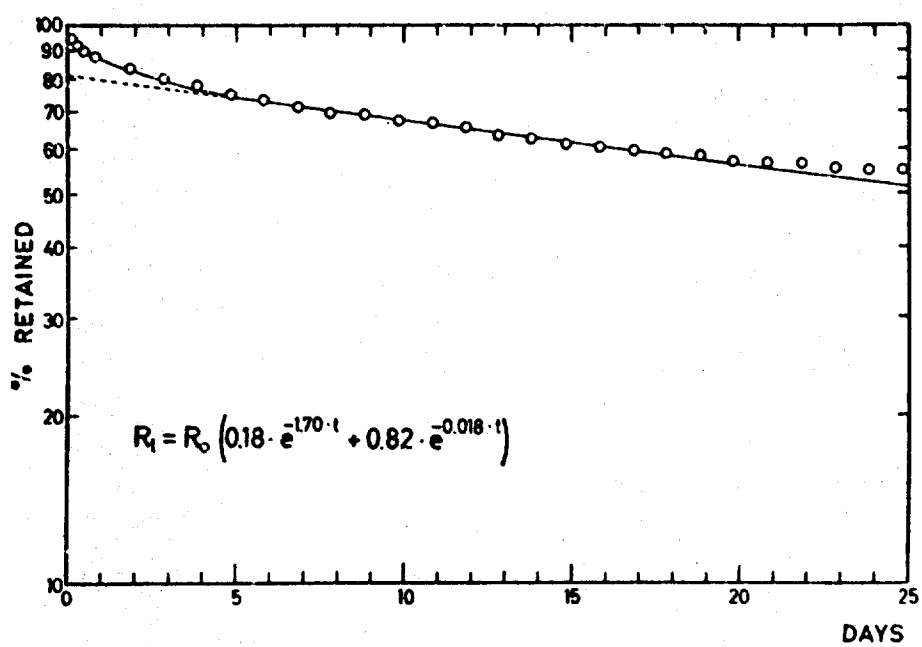

FIG. 2. Isotope retention curve obtained after the intravenous administration of 400 μg. of tritium-labeled pyridoxine to subject R. E. The per cent of administered isotope retained in the body at different times (R_t) has been calculated by subtraction of the cumulative urinary excretion of isotope from the amount administered (R_0 = 100).

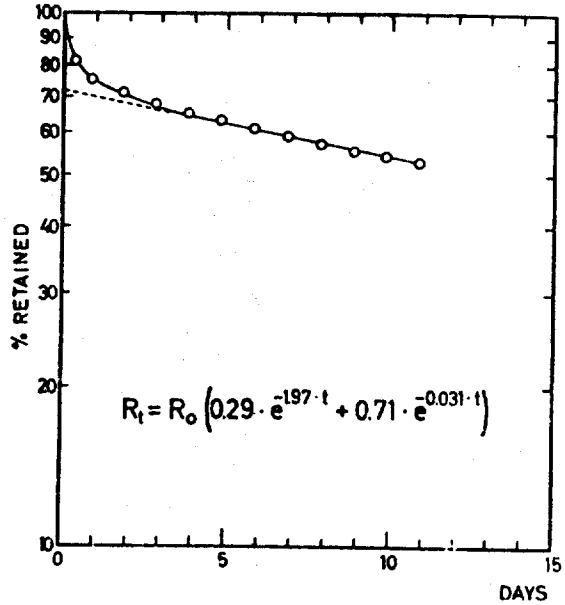

FIG. 3. Isotope retention curve obtained after the oral administration of 250 μg. of tritium-labeled pyridoxine to subject W. W. The per cent of administered isotope retained in the body at different times (R_t) has been calculated by subtraction of the cumulative urinary excretion of isotope from the amount administered (R_0 = 100).

at time t (per cent); R_0 is the amount of isotope in the body at zero time (= 100 per cent); M is the intercept of the first linear component with ordinate; N is the intercept of the second linear component with ordinate; γ_1 is the slope of the first linear component; γ_2 is the slope of the second linear component.

This type of relation between the amount of isotope in the body and the time elapsed after administration of the isotope would be obtained if the over-all metabolism of pyridoxine and its derivatives could be described by a two-compartment system of the form illustrated in Figure 4. The rate constants for the transfer

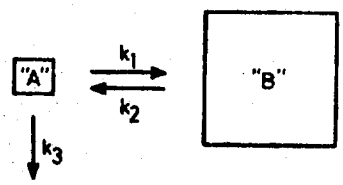

FIG. 4. Proposed model for the over-all metabolism of pyridoxine. "A" and "B" represent two compartments; k_1, k_2 and k_3 are the rate constants for the transfer of vitamin B_6 between the compartments and for the excretion of vitamin B_6 or its metabolites (in day^{-1}).

between compartments "A" and "B" are k_1 and k_2, and the rate constant for the excretion in the urine is k_3. The numeric value of these rate constants may be calculated from the parameters of the experimental curve by the following relations:

$$k_1 = \gamma_1 + \gamma_2 - k_2 - k_3; \quad k_2 = \gamma_1 \gamma_2 / k_3; \quad k_3 = M \gamma_1 + N \gamma_2$$

The ratio between the amounts of pyridoxine and its derivatives present in compartments B and A is k_1/k_2 and it may be shown that the fractional turnover rate of the whole body pool of pyridoxine and its derivatives is $k_2 \cdot k_3/(k_1 + k_2)$. The values of the parameters of the experimental curves and the calculated values for the rate constants are given in Table I.

The slope of the second component of the curve ranged from −0.031 to −0.018 day^{-1}, which corresponds to a half-life of twenty-two to thirty-eight days. The slope of the first component was at least fifty times larger (−1.70 to −1.97 day^{-1}). The rate constants for the transfer between compartments and the excretion in the urine were of the same magnitude in all three subjects, as was the ratio between compartment sizes, which showed that 90 per cent or more of total vitamin B_6 in the body was present in compartment B. The fractional turnover rate ranged from 2.2 to 4.4 per cent of the total body pool per day.

To obtain the absolute amounts present in and transferred between the compartments one would have to know the specific radioactivity of pyridoxine or its derivatives in the respective compartments at different times during the experiments. Since this information is not easy to obtain we made the assumption that, at least during the later phase of the experiment, the specific radioactivity of urinary 4-pyridoxic acid is the same as that of vitamin B_6 in pool A. Equation 2 describes the specific radioactivity in compartment A:

$$S_{At} = S_{A0}\left(\frac{\gamma_1 - k_2}{\gamma_1 - \gamma_2} e^{-\gamma_1 t} + \frac{\gamma_2 - k_2}{\gamma_2 - \gamma_1} e^{-\gamma_2 t}\right) \quad (2)$$

Plots of the specific radioactivity of 4-pyridoxic acid versus time in the three subjects are shown in Figures 5 to 7. A curve drawn ac-

TABLE I
Parameters of Experimental Isotope Retention Curves

Subject	M	N	γ_1 Day^{-1}	γ_2 Day^{-1}	k_1 Day^{-1}	k_2 Day^{-1}	k_3 Day^{-1}	k_1/k_2*	$k_2 k_3/(k_1 + k_2)$† Day^{-1}
S. S.	0.12	0.88	1.97	0.021	1.57	0.15	0.26	9.7	0.024
R. E.	0.18	0.82	1.70	0.018	1.30	0.10	0.32	13.7	0.023
W. W.	0.29	0.71	1.97	0.031	1.31	0.10	0.59	12.7	0.044

NOTE: Curves were obtained after administration of labeled pyridoxine. Rate constants were calculated in the proposed model to determine the over-all metabolism of vitamin B_6 in human subjects. For explanation of symbols see "Results."

* Ratio of amounts present in compartments A and B.

† Fractional turnover rate of total body pool.

cording to equation 2, with the values for the parameters as given in Table I, was fitted to the experimental values for each subject. Extrapolation of the second component of these curves to zero time gives $S_{A_0} \cdot (\gamma_2 - k_2)/(\gamma_2 - \gamma_1)$. The dilution of the administered amount of isotope in compartment A, obtained from the value of S_{A_0}, gives the size of compartment A; the size of compartment B is obtained from the relation $B = A \cdot k_1/k_2$. Compartment sizes, amounts transferred between the compartments and calculated values for the excretion in the urine are shown for each subject in the inserts in Figures 5 to 7. The total amount of vitamin B_6 ranged from 40 to 150 mg., the amount transferred between compartments from 9.8 to 22.0 mg. per day and the amount excreted in the urine from 1.7 to 3.6 mg. per day.

4-Pyridoxic acid was determined in the 24 hour urine samples using a fluorometric method; it varied between 647 and 986 μg. per day in the three subjects (Table II).

Effect of Increasing Daily Intake of Pyridoxine on Excretion of Isotope

Subject S. S. received a daily supplement of 80 mg. of pyridoxine beginning fifty-six days after administration of the labeled pyridoxine. A plot of the per cent of administered isotope excreted per day in this subject is shown in Figure 8. Although the excretion was not determined daily between days 20 and 56, it is obvious that the additional amount of pyridoxine in the diet caused an immediate increase in the amount of isotope excreted per day to values that were three to five times higher than the previous basic level.

Urinary Metabolites

A chromatogram of urine from subject S. S. collected between 4 and 8 hours after administration of the labeled pyridoxine is shown in

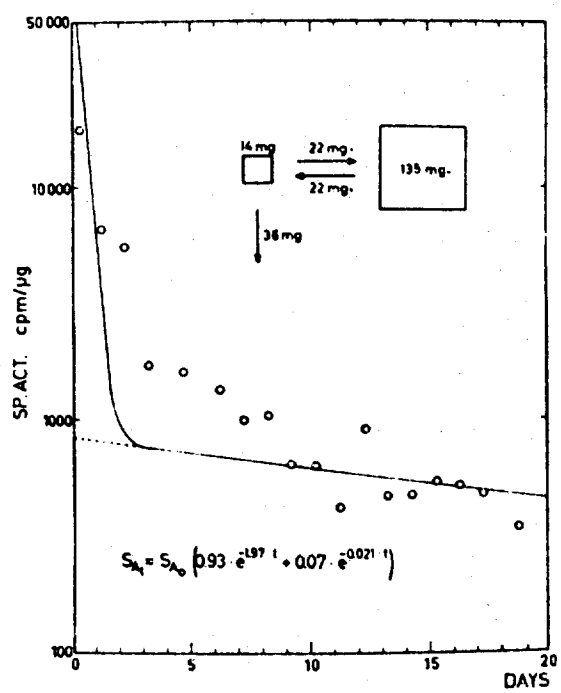

FIG. 5. Specific activity of urinary 4-pyridoxic acid in subject S. S. The equation for the solid line has been calculated according to equation 2 (see "Results"). The insert shows the proposed model for the metabolism of vitamin B_6, with the calculated values for compartment sizes, transfer between compartments and excretion.

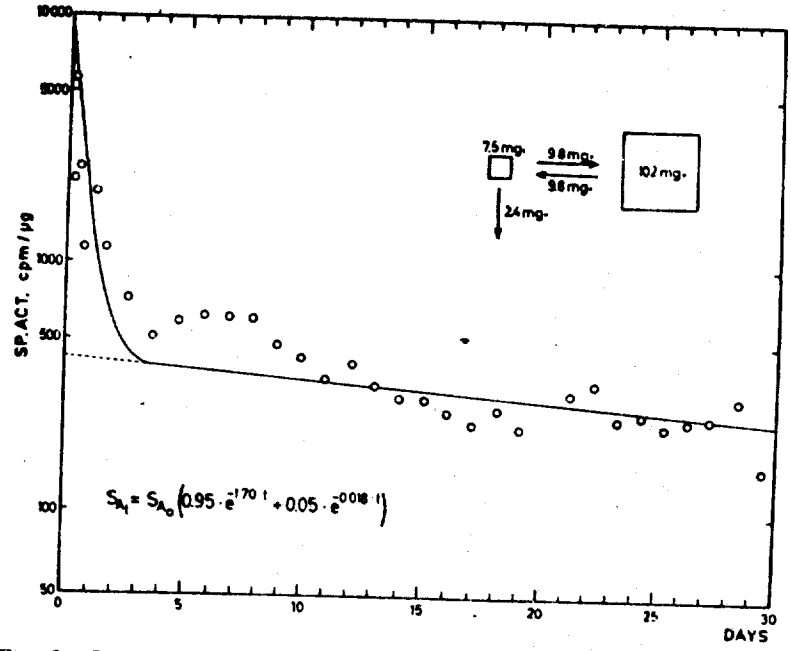

FIG. 6. Specific activity of urinary 4-pyridoxic acid in subject R. E. The equation for the solid line has been calculated according to equation 2 (see "Results"). The insert shows the proposed model for the metabolism of vitamin B_6, with the calculated values for compartment sizes, transfer between compartments and excretion.

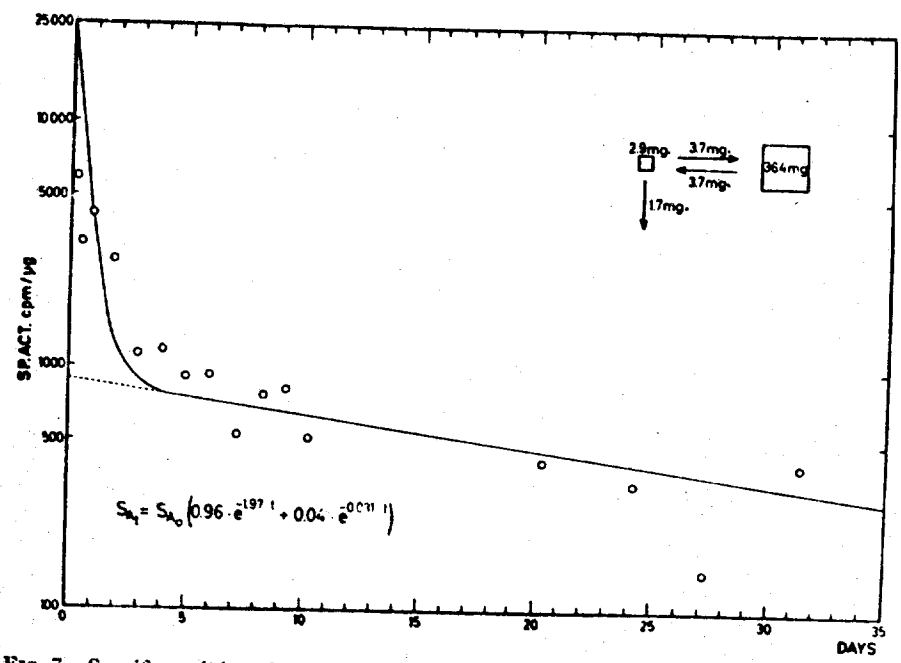

FIG. 7. Specific activity of urinary 4-pyridoxic acid in subject W. W. The equation for the solid line has been calculated according to equation 2 (see "Results"). The insert shows the proposed model for the metabolism of vitamin B_6, with the calculated values for compartment sizes, transfer between compartments and excretion.

TABLE II
Daily Urinary Excretion of 4-Pyridoxic Acid

Subject	4-Pyridoxic Acid*	
	μg./day	% Urinary Metabolites
S. S.	799 ± 88	28.4 ± 1.9
R. E.	986 ± 70	36.0 ± 3.5
W. W.	647 ± 20	40.7 ± 4.1

NOTE: Excretion was determined by a fluorometric method and as per cent of total excretion of isotope.
* Mean ± standard error of the mean.

Figure 9. Two main isotope-containing peaks were eluted in fractions 15 to 20 and 25 to 35, respectively. The second peak was eluted at the fraction containing 4-pyridoxic acid and showed a fluorescence spectrum identical with that of authentic 4-pyridoxic acid. On rechromatography with carrier amounts of 4-pyridoxic acid, the labeled material and the added carrier were eluted at the same effluent volume. The first peak of labeled material was not identified. The material which was not retained on the anion exchange column after washing the column with water was concentrated and fractionated on a column of Dowex 50 (Fig. 10). The main peak appearing at fractions 40 to 55 was identified as pyridoxine on the basis of its fluorescence spectrum, co-chromatography with carrier amounts of pyridoxine and high voltage electrophoresis. The isotope-containing peak appearing in fractions at 35 to 40 ml. had the same retention time as pyridoxal and was identified by the same technics used for pyridoxine.

COMMENTS

The purpose of the present study was to obtain initial information on the metabolism of labeled pyridoxine in human subjects. It was particularly important to secure a meticulous collection of urine samples over a long period of time. Therefore subjects with a personal interest in the project were chosen for the experiment. The results were comparable in all three subjects and the fact that two of them suffered from recurrent kidney stones, a disease in which a deficiency of vitamin B_6 has been implicated, presumably does not invalidate the general con-

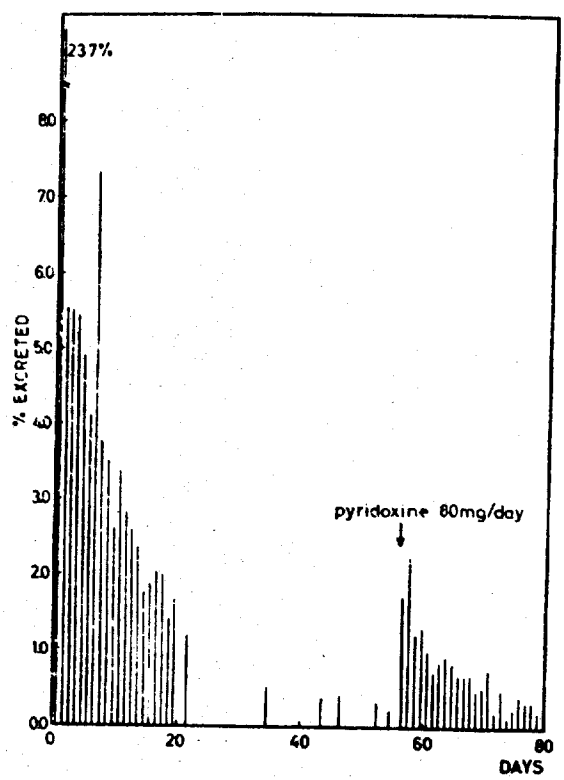

FIG. 8

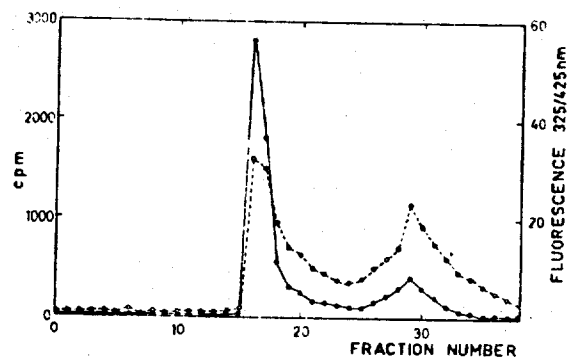

FIG. 9

FIG. 8. Daily urinary excretion of isotope in subject S. S. On day 56 supplementation of the diet with 80 mg. pyridoxine per day was started.

FIG. 9. Fractionation of a column of Dowex 2 in formate form of urine collected from subject S. S. during period 4 to 8 hours after administration of tritium-labeled pyridoxine. Fractions 25 to 35 contain 4-pyridoxic acid. The column was eluted with 0.6 M formic acid. ●———● denotes radioactivity; O- - -O denotes fluorescence.

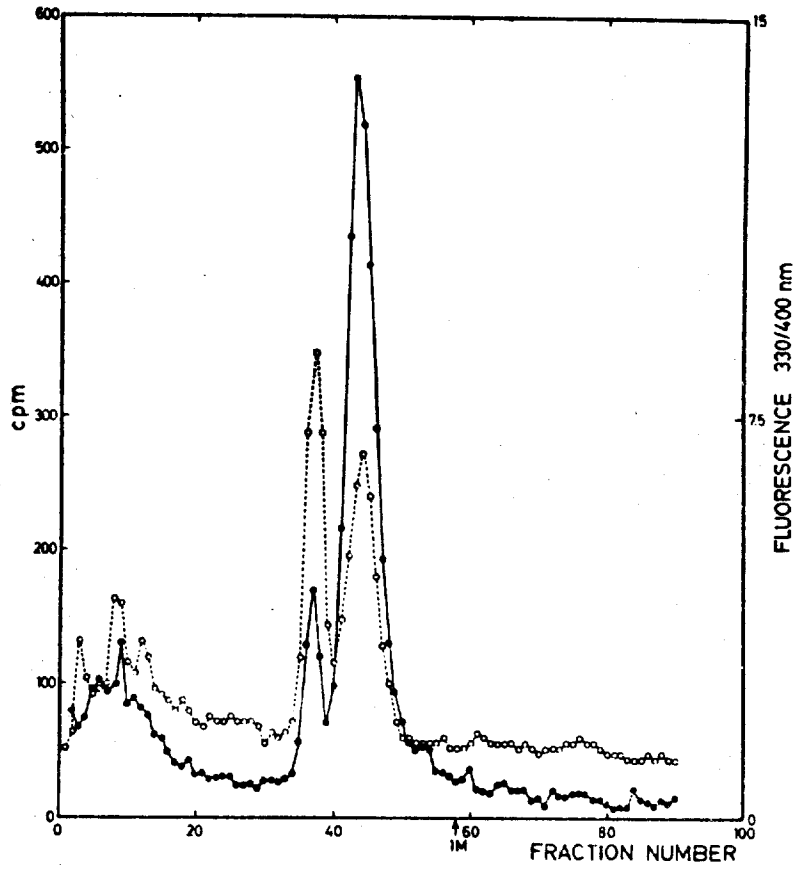

FIG. 10. Fractionation on a column of Dowex 50 of urinary metabolites of pyridoxine, which were not retained on a column of Dowex 2 in formate form. The urine was collected from subject S. S. during period 4 to 8 hours after administration of labeled pyridoxine. The labeled material eluted in fractions 35 to 40 has been identified as pyridoxal and the material in fractions 40 to 55 as pyridoxine. The column was eluted with 100 ml. of 0.25 M ammonium formate at pH 7.0 followed by 60 ml. of 1 M ammonium formate at pH 7.0. ●———● denotes radioactivity. O - - - O denotes fluorescence.

clusions drawn from this study. It should be mentioned that facilities were not available at this stage to keep the subjects on a diet with a constant intake of vitamin B_6.

After the intravenous or oral administration of tritium-labeled pyridoxine, the isotope-retention curves followed the same general pattern in the three subjects. They were characterized by a rapid initial fall due to the excretion of 15 to 20 per cent of the administered isotope within the first day, followed by a slow decline with a half-life of eighteen to thirty-eight days. In subjects S. S. and R. E. a deviation from the fit of the experimental points to the curve was noted at the end of the experiment, possibly due to the low counts obtained in the late samples. The isotope retention curves obtained in human subjects were similar to those obtained previously in rats,[27] in which the half-life of the second component of the curve ranged between fifty-nine and seventy-one days in nondeficient animals. It has been shown in experiments with animals[26] that the form of the first part of the retention curve is dependent on the amount of pyridoxine administered and the amount of pyridoxine in the diet, i.e., if larger amounts are given, a proportionally larger amount is excreted immediately. Similar observations have been made in human subjects by Brain and Booth,[28] who found that 50 per cent of an

oral dose of 1 mg. of pyridoxine was excreted within 24 hours if given simultaneously with an intravenous injection of 100 mg. of unlabeled pyridoxine, whereas only about 17 per cent was excreted when this flushing technic was not used.

We used a simple metabolic model for interpretation of the results and as a basis for the quantitative calculations attempted. The isotope retention curves had the form which would be obtained if the over-all metabolism of pyridoxine were represented by a compartment model, consisting of a storage compartment with a slow fractional turnover rate and a smaller compartment with a much faster fractional turnover rate from which excretion into the urine takes place. The two compartments should not be regarded as having any defined anatomical meaning, since the administered pyridoxine enters into a network of metabolic reactions and is transformed into pyridoxal, pyridoxamine and the phosphorylated forms of the vitamin.[29,30] These different forms of vitamin B_6 occur in different proportions and concentrations in the various tissues,[29,31–33] but at the present time little is known about the rate at which transformations between the different forms of vitamin B_6 occur or about the rate of distribution between tissues of the body. On the basis of the proposed model it was possible to calculate a value for the fractional turnover rate of the total amount of vitamin B_6 in the body, which in our study ranged from 2.2 to 4.4 per cent per day. This value includes the immediate dietary intake of the vitamin, and therefore it may be of greater interest to discuss the fractional rate of loss of vitamin B_6 from the body reservoir of the vitamin, i.e., from compartment B in our model. In our subjects this fractional rate of loss, $k_2 \cdot k_3 / (k_1 + k_3)$, was 2 to 3 per cent per day, which is somewhat higher than the value obtained in rats (1 per cent per day). It is not known if this value was influenced by the dietary level of pyridoxine, but it is of interest that no large difference in values was found between vitamin B_6-deficient rats and nondeficient rats.[27]

Rabinowitz and Snell[30] gave 82 mg. of pyridoxine to human subjects and determined urinary excretion of pyridoxine and its metabolities with microbiological methods. The amount recovered as additional pyridoxine excreted in the urine ranged from 38 to 45 per cent during the 24 hour period after administration, whereas after this period urinary excretion returned to the basic level. Other investigators[28] have also failed to account for the administered pyridoxine recovered as urinary metabolites. Therefore the possibilities of additional excretory pathways or unidentified metabolites have been considered. Although such explanations are not excluded by findings in the present study, it appears that the slow elimination of part of the administered pyridoxine may be one of the reasons for the findings reported previously. The fact that only 2 to 3 per cent of the body store of vitamin B_6 was lost per day agrees well with the clinical experience that symptoms of vitamin B_6 deficiency develop slowly in human subjects when a vitamin B_6-deficient diet is instituted or when medication with isonicotinic acid hydrazide is given.[34–36]

Data on the rate constants may be obtained from the isotope retention curve, but this curve does not make it possible to calculate the actual amounts of vitamin B_6 present in the compartments, transferred between them, or excreted in the urine. To determine these amounts it is necessary to know the specific radioactivity-time relation. Unfortunately, the concentration of the different forms of vitamin B_6, for example, in blood, is very low, which makes it difficult to isolate a sufficient quantity for the determination of specific radioactivity. Therefore we chose to determine the specific activity of 4-pyridoxic acid in urine. This acid is formed from pyridoxal and pyridoxal phosphate[37,38] and is considered to represent the main excretory product after the administration of pyridoxine to humans.[22,30] If the different forms of vitamin B_6 eventually reach approximately the same specific radioactivity after administration of labeled pyridoxine, and if this specific radioactivity is the same as that of the immediate excretory product, 4-pyridoxic acid, it should be possible to calculate the body pool of vitamin B_6 by the principle of isotope dilution. On day 10 urinary 4-pyridoxic acid in our three subjects contained $5 \cdot 10^{-2}$ (R. E.), $4 \cdot 10^{-2}$ (S. S.) and $9 \cdot 10^{-2}$ (W. W.)

per cent of the administered amount of isotope per μg. of urinary 4-pyridoxic acid. These values, however, cannot be used directly to calculate the amount of vitamin in the body, since the urinary 4-pyridoxic acid originating from the reservoir compartment is constantly diluted with 4-pyridoxic acid originating from the dietary pyridoxine. Consequently, a calculation based on the principle of isotope dilution would result in an overestimate of the total body pool. By use of the two-compartment kinetic model, however, it is possible to obtain a value for the total body pool of the vitamin. The fit of the experimental values to the calculated specific activity-time curves is not as good as for the isotope retention curves. Daily variations in dietary intake of vitamin B_6 cause day to day variation in the specific activity of 4-pyridoxic acid and the form of the first part of the curve is dependent on the value of γ_1. It has been shown by experiments in rats that the value of γ_1 is dependent on the route of administration of the labeled pyridoxine and on the time intervals chosen for the initial urinary collections, and also that the first part of the isotope retention curve probably can be resolved into several components. The uncertainty in the γ_1 value does not significantly affect the calculated pool sizes. It may also be noted that the if γ_1 is much larger than γ_2, approximate expressions may be used for the fractional turnover rate of the total amount of vitamin B_6 in the body and for the fractional rate of loss from compartment B, i.e., γ_2/N and γ_2, which are independent of the value of γ_1.

In the conversion of pyridoxine to 4-pyridoxic acid, two of eight hydrogens are lost which are located in positions such that they could have exchanged for tritium during the preparation of the labeled pyridoxine. No correction has been applied for this loss of hydrogens, which results in an overestimate of the amount of vitamin B_6 in the body.

The proposed model allows calculation of the transfer between the two compartments which varied between 3.7 and 22 mg. per day. These values are dependent on the value of γ_1 in equation 1 and therefore only suggest an equilibrium between the body reservoir of vitamin B_6 and more labile forms. In one subject it was found that the addition to the diet of 80 mg. of pyridoxine per day resulted in a considerable increase in the excretion of isotope, which may be interpreted as a mobilization of vitamin B_6 from a body reservoir.

The elimination of vitamin B_6 was calculated to be 1.7 to 3.6 mg. per day, which may be compared with the values obtained for the excretion of 4-pyridoxic acid determined by fluorometric methods (0.65 to 0.99 mg. per day). Thus 4-pyridoxic acid would account for 20 to 40 per cent of the vitamin B_6 eliminated daily. Similar values would be obtained if the isotope recovered in the 4-pyridoxic acid fraction by ion exchange chromatography was calculated as per cent of the total amount of isotope in the urine during days 4 to 14. In the urine samples collected immediately after the administration of the labeled pyridoxine there was proportionally less isotope in the 4-pyridoxic acid fraction, since in these samples a larger amount of isotope was found in the pyridoxine fraction. In experiments conducted by Rabinowitz and Snell[30] 4-pyridoxic acid accounted for about 91 per cent of the amount of pyridoxine metabolites recovered in the urine when subjects were given the basal diet, and for about 75 per cent when 82 mg. of pyridoxine had been administered; whereas Reddy and coworkers[39] found that subjects whose constant daily intake of vitamin B_6 was 8.3 to 18.6 μM excreted about 50 per cent as 4-pyridoxic acid.

SUMMARY

Tritium-labeled pyridoxine was administered orally to one subject and intravenously to two subjects. The urinary excretion of isotope was followed daily for up to twenty-five days, and the specific radioactivity of the urinary 4-pyridoxic acid was determined. During the first days after administration of the pyridoxine, 15 to 20 per cent of the administered isotope was excreted in the urine; the remainder that was eliminated had a half-life of eighteen to thirty-eight days. A simple model for estimating the over-all metabolism of pyridoxine in human subjects is proposed, which consists of a small compartment with a rapid turnover rate and a larger storage compartment. The rate of elimination of vitamin B_6 from the body

reservoir was estimated to be 2 to 3 per cent per day and the total turnover rate of the vitamin to be 2.2 to 4.4 per cent per day. Approximate values were calculated for the body content of the vitamin, 40 to 150 mg., and for the daily elimination, 1.7 to 3.6 mg. per day. The isotope-containing compounds in the urine were fractionated by ion exchange chromatography and it was found that 4-pyridoxic acid accounted for 20 to 40 per cent of the amount of isotope eliminated.

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THE EXCRETION OF PYRIDOXINE, "PSEUDOPYRIDOXINE," AND 4-PYRIDOXIC ACID IN THE URINE AND SWEAT OF NORMAL INDIVIDUALS*

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Snell, Guirard, and Williams (1) in 1942 showed that a metabolite of pyridoxine which they termed "pseudopyridoxine" was excreted in the urine. We have shown that this "pseudopyridoxine" occurs in sweat as well as in urine (2). "Pseudopyridoxine" was found to be inactive for the yeast, *Saccharomyces carlsbergensis*, used in the Atkin, Schultz, Williams, and Frey (3) assay method for pyridoxine, thus making it possible to determine pyridoxine in the presence of pseudopyridoxine. It has been shown (2, 4) that *Streptococcus lactis* R and *Lactobacillus casei* respond to pseudopyridoxine but not to pyridoxine, making possible a microbiological pseudopyridoxine assay, once a standard became available. In 1944 Harris, Heyl, and Folkers (5) synthesized "pyridoxal" and "pyridoxamine" from pyridoxine. Snell (6) showed that both of these compounds were active for the growth of *Streptococcus lactis* and that pyridoxal was active for the growth of *Lactobacillus casei*. Pyridoxal, therefore, appeared to be a better standard for pseudopyridoxine assay. This does not necessarily infer that pyridoxal is identical with pseudopyridoxine.

Huff and Perlzweig (7, 8) described, isolated, and characterized a third metabolite of pyridoxine, "4-pyridoxic acid" (2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine). The existence of this compound in human urine had been indicated by Singal and Sydenstricker (9) in 1941. A fluorometric method for the determination of this metabolite has been described by Huff and Perlzweig (8).

In this paper we wish to report the excretion of pyridoxine, pseudopyridoxine (as pyridoxal), and 4-pyridoxic acid in the sweat and urine of young men maintained in a "hot moist" environment on a "normal" diet with and without added pyridoxine.

EXPERIMENTAL

Four adult male subjects, age 21 to 28 years, were maintained for 8 hours per day under hot moist conditions for 5 days each week. The temperature

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Illinois.

of the experimental chamber was 37.8° and the relative humidity 70 per cent. The subjects were kept on a constant diet throughout the experiment. During Period II the diet was supplemented with 8 mg. of pyridoxine per day.

TABLE I

Average Daily Excretion of Pyridoxine and Its Metabolites

Period No.	Subject	Pyridoxine	"Pseudopyridoxine" (pyridoxal)	4-Pyridoxic acid	Total
		mg.	*mg.*	*mg.*	*mg.*
I. No dosage. Urine, per 24 hrs.	C	0.114	0.192	2.830	3.14
	D	0.187	0.375	3.022	3.58
	E	0.118	0.256	3.297	3.65
	F	0.131	0.181	3.367	3.68
Average		0.138	0.246	3.130	3.51
% of total		3.9	6.8	89.3	100
I. Skin excretion, per 8 hrs.	C	0.00	0.030	0.155	0.185
	D	0.00	0.030	0.193	0.222
	E	0.00	0.032	0.257	0.288
	F	0.00	0.035	0.189	0.224
Average		0.00	0.031	0.198	0.233
% of total		0	13.6	86.4	100
II. Dosage, 8 mg. pyridoxine per day. Urine, per 24 hrs.	C	0.354	0.460	7.540	8.35
	D	0.243	0.979	8.162	9.38
	E	0.409	0.716	6.220	7.34
	F	0.445	0.443	4.932	5.82
Average		0.363	0.650	6.713	7.72
% of total		4.6	8.4	87	100
II. Skin excretion, per 8 hrs.	C	0.0044	0.023	0.228	0.255
	D	0.0063	0.110	0.241	0.358
	E	0.0075	0.026	0.319	0.352
	F	0.0077	0.039	0.253	0.299
Average		0.0065	0.049	0.260	0.316
% of total		2.1	15.5	82.4	100

Complete 8 hour skin excretion collections and 24 hour urinary collections were made. The daily collections were composited for each 5 day experimental period. At the end of each experimental period, a 4 hour collection of undiluted sweat was made under the same environmental conditions.

The sweat and urine were analyzed for pyridoxine with a slight modification of the *Saccharomyces carlsbergensis* method of Atkin, Schultz, Williams,

and Frey (3). Pseudopyridoxine was determined with *Streptococcus lactis* R with the medium of Luckey, Briggs, and Elvehjem (10) for folic acid assay with the omission of pyridoxine and the addition of 80 γ of folic acid concentrate[1] and 10 cc. of Salts B (11). Pyridoxal[2] was used as a standard for this determination. 4-Pyridoxic acid[3] was determined by the fluorometric lactone method of Huff and Perlzweig (8).

The average daily excretion in the urine and through the skin of each of these three metabolites for the two experimental periods is given in Table I.

From Table I it is apparent that 4-pyridoxic acid is quantitatively the most important metabolite excreted, amounting to over 80 per cent of the

TABLE II

Concentration of Pyridoxine and Its Metabolites in Sweat

Post period No.	Subject	Pyridoxine	"Pseudopyridoxine" (pyridoxal)	4-Pyridoxic acid	Total
		γ per cc.	*γ per cc.*	*γ per cc.*	*γ per cc.*
I. No dosage	C	0.00055			
	D	0.00170	0.0825		
	E	0.00042	0.0043		
	F	0.00068	0.0105		
Average		0.00084	0.0324		
II. Dosage, 8 mg. pyridoxine per day	C	0.00049	0.0168	0.054	0.071
	D	0.0033	0.0145	0.048	0.066
	E	0.0016	0.0063	0.052	0.060
	F	0.0017	0.0090	0.055	0.066
Average		0.0018	0.0117	0.052	0.066
% of total		2.7	17.9	79.4	100

total pyridoxine and metabolite excretion. The daily excretion values for Period I seem high. However, these subjects had been receiving 2 mg. of pyridoxine daily in addition to their normal diet for several months prior to this experiment.

The sensitivity of the method for pyridoxine is such that the skin excretion for Period I may amount to 2 to 3 per cent of the total rather than zero.

In Table II are given the concentration data obtained on undiluted

[1] Dr. R. J. Williams, University of Texas, very kindly supplied the "folic acid" concentrate.

[2] Pyridoxal and pyridoxamine were generously supplied by Dr. Karl Folkers of Merck and Company, Inc., Rahway, New Jersey.

[3] 4-Pyridoxic acid was very kindly supplied by Dr. W. A. Perlzweig of Duke University.

sweat of the four subjects. These samples were collected during the 4 hour periods on the day following the conclusion of each experimental period.

The data in Table II indicate that the concentration ratio of the metabolites in sweat is about the same as in urine.

DISCUSSION

The average daily excretion value of pyridoxine and its metabolites under a continuous "hot moist" environment was 3.51 mg. in the urine, plus 0.69 (3 × 0.23 to convert from an 8 hour to a 24 hours basis)[4] through the skin, during Period I with no dosage. Thus 16.5 per cent of the pyridoxine plus metabolites was excreted through the skin. Similarly, for Period II, during which the diet was supplemented with 8 mg. of pyridoxine daily, the average total daily excretion was 8.67 mg., 11 per cent of this excretion occurring through the skin. While this 11 to 16.5 per cent excretion through the skin is a relatively small proportion of the total excretion, it cannot be definitely concluded that sweating conditions do not affect the pyridoxine requirement.

With the addition of 8 mg. of pyridoxine per day to the normal diet, the average increase in total excretion was 4.3 mg. or a 54 per cent recovery of the added pyridoxine. Of this increase, 5.5 per cent occurred as pyridoxine, 9.5 per cent as pseudopyridoxine, and 85 per cent as 4-pyridoxic acid, indicating that the added pyridoxine was metabolized in a similar manner to the pyridoxine present in the food.

Snell has shown (6) that pyridoxal and pyridoxine are approximately equally active for *Saccharomyces carlsbergensis*. Therefore, in order for pyridoxal and the pseudopyridoxine found in urine to be the same compound, the pyridoxine values would have to be higher in all cases than the pseudopyridoxine values obtained with *Streptococcus lactis*. In the work reported in this paper the pseudopyridoxine values were always considerably higher than the pyridoxine values. If, then, pyridoxal is not the same compound as pseudopyridoxine, the assumption that they are equally active for *Streptococcus lactis* may be possibly incorrect. Gunsalus, Bellamy, and Umbreit (12) have shown that a phosphorylated derivative of pyridoxal acts as the coenzyme of tyrosine decarboxylase. It may be that urinary pseudopyridoxine is such a conjugate of pyridoxal and is inactive for yeasts but retains its activity toward bacteria.

One of the questions arising from this study is the functional form of pyridoxine in the body. Scott *et al.* (13) have shown that the lactone of

[4] These would be maximal excretion values since they presuppose that there is no compensatory relationship between urinary excretion and skin excretion. Other work has indicated that this relationship frequently does exist.

"5-pyridoxic acid" (α-pyracin) is active in preventing anemia in the chick. From the work reported here it would appear that some oxidation product of pyridoxine is possibly the active form in the body. Further work must be done to determine whether the final completely oxidized compound excreted in such large amounts is the functional form in the body or rather the final end-product of metabolism.

SUMMARY

1. A study was made of the excretion of pyridoxine, pseudopyridoxine (as pyridoxal), and 4-pyridoxic acid in the urine and sweat of four men subjected to a hot moist environment.

2. Over 85 per cent of the total pyridoxine and metabolites excreted in the urine was in the form of 4-pyridoxic acid, 4 to 4.5 per cent was pyridoxine, and 7 to 8 per cent was pseudopyridoxine.

3. The percentage distribution of these three compounds in sweat was similar to that in urine.

4. The total amount of pyridoxine and its metabolites present in sweat appears to be too small to have any significant influence on the pyridoxine requirement of persons subjected to an environment inducing profuse sweating throughout the day.

5. When 8 mg. of pyridoxine per day were given orally, an average of over 50 per cent of the pyridoxine was recovered in the sweat and urine as pyridoxine and its metabolites.

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TREATMENT OF NEUROPSYCHIATRIC DISORDERS WITH VITAMINS

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During the past decade vitamin deficiency has been looked for and often found in many neuropsychiatric disorders. It must be emphasized, however, that merely finding a vitamin deficiency concurrent with a disease does not indicate that this deficiency is the cause of the original disease. Secondary or "conditioned" vitamin deficiencies should be clearly distinguished from the original or primary disease, else utter confusion will result.

Almost every vitamin has also been credited with playing a role in the maintenance of a normal nervous system. The more important of the vitamins thus accredited are thiamine hydrochloride, nicotinic acid, riboflavin, pyridoxine and alpha-tocopherol. Only thiamine hydrochloride and nicotinic acid, however, have been related clinically in a definite causal role to neuropsychiatric syndromes in man.

The sequence of events in a vitamin deficiency disease begins with an inadequate vitamin supply, due to failure to ingest, absorb or utilize, followed in succession by tissue depletion, biochemical "lesions,"[1] reversible anatomic changes and finally irreversible anatomic lesions. Adequate treatment initiates the sequence of tissue replenishment, early disappearance of the biochemical "lesions" and less rapid disappearance of the reversible anatomic lesions. The importance of early diagnosis and treatment is obvious.

The neuropsychiatric conditions in which there is substantial evidence that a vitamin deficiency may play an etiologic role are listed in table 1. This table includes the vitamin or vitamins predominantly concerned, an evaluation of vitamin therapy and references to the literature for each of the conditions listed. The evaluation of therapy concerns only the primary disease and is made without prejudice to the value of vitamins in the prevention or treatment of a secondary deficiency. This table does not include a number of neuropsychiatric syndromes, such as sciatica, eighth nerve deafness and other single nerve neuropathies, herpes zoster, hysteria, impotence and psychogenic pain, for which vitamins have been recommended because of their drug action or for use as a placebo, in which conditions the rationale of their use is dubious or the results claimed can be explained equally well or better as fortuitous.

A neurasthenic syndrome is probably the earliest and most frequent manifestation of both thiamine deficiency[2]

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and pellagra.[3] As in neurasthenic syndromes of any origin, the symptomatology is varied but the outstanding characteristics are anorexia, fatigue and insomnia. It would not be inferred that all neurasthenia is based on vitamin deficiency. Such is definitely not the case.

The polyneuropathies of thiamine deficiency,[4] whether mild or severe, are bilateral and symmetrical and characteristically involve first and predominantly the lower extremities. Peripheral neuropathy that involves a single nerve, that is not bilateral and symmetrical or that does not involve first and predominantly the lower extremities is not, in my experience, due to thiamine deficiency alone. The polyneuropathies fulfilling the foregoing criteria occurring in association with beriberi, alcoholism, pellagra, pregnancy, cachexia, diabetes, hyperthyroidism and chronic diarrheal diseases are usually a manifestation of thiamine deficiency. There is often coexisting latent or manifest deficiency of other vitamins.

Wernicke's disease [5] occurs most often, but not exclusively, in association with chronic alcoholism. It is characterized clinically by ophthalmoplegia, polyneuropathy, ataxia and progressive clouding of consciousness. It is usually associated with multiple vitamin deficiencies. Thiamine, when administered in adequate amounts, relieves the ophthalmoplegia within forty-eight to ninety-six hours.[6] The mental status is frequently but not consistently improved by thiamine therapy.

The mental symptoms most commonly seen in pellagra [7] are those usually seen in any organic psychosis. These include memory defects, disorientation, confusion and confabulation. Periods of excitement, depression, mania, delirium and paranoia occur not infrequently. These cerebral disorders may appear before other signs of pellagra are evident. Response to nicotinic acid is usually prompt, especially when the psychosis is of short duration. In the psychoses of longer duration the response is by no means spectacular, and frequently specific therapy does not help at all. This means that in the latter cases the processes may have advanced to the irreversible anatomic stage. It must be emphasized, however, that pellagrins lack other dietary factors necessary for normal brain metabolism, and these deficiencies may contribute to the cerebral manifestations. Adequate amounts of all other nutritive essentials should therefore be given in order to obtain a maximal therapeutic response.

Nicotinic acid deficiency encephalopathy is a term used by us [8] to designate a syndrome characterized by sucking and grasping reflexes, changing cogwheel rigidities and progressive clouding of consciousness. This syndrome [9] is seen most frequently in association with chronic alcoholism, endemic pellagra, senility and cirrhosis of the liver. In our study of 150 cases the mortality was reduced from above 90 per cent to 15 per cent by nicotinic acid therapy. It should be emphasized, however, that these patients are usually left with organic memory defects. Most of these subjects are in the elder age group and their conditions are probably complicated by cerebral arteriosclerosis.

A relationship of thiamine, nicotinic acid and ascorbic acid to infantile paralysis,[10] delirium tremens [11] or Korsakoff's psychosis [12] has been suggested but, in my opinion, has not been proved. In all these, as in many other diseases, an associated or secondary vitamin deficiency may occur. In delirium tremens the decided increase in total metabolism raises the vitamin requirements to such an extent that an acute deficiency may be precipitated from which the patient may die unless attention is given to insuring adequate nutrition.

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[Footnote 7 continued in next column]

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12. Bowman, K. M.; Goodhart, Robert, and Jolliffe, Norman: Observations on the Role of Vitamin B_1 in the Etiology and Treatment of Korsakoff Psychosis, J. Nerv. & Ment. Dis. **90:** 569 (Nov.) 1939. Jolliffe, Wortis and Fein.[6]

sclerosis and paralysis agitans has recently been the subject of conflicting reports.[13] In all disorders which may be viewed as syndromes rather than as disease entities a positive response to treatment in but few patients may be significant, for it may indicate a curable disease entity within a syndrome previously considered irreversible. It seems reasonable, therefore, and in view of the conflicting reports and of the progressive nature of these syndromes, to give the vitamins on an experimental basis under carefully controlled conditions.

Syndromes collectively labeled paralysis agitans, while not directly fatal, usually follow a progressive course, the victims eventually becoming helpless. Since muscular rigidity and weakness are characteristic of paralysis agitans, and since pyridoxine is involved in muscle metabolism, it seemed worth while to test its effect in this syndrome. I therefore selected 15 patients having paralysis agitans, all of whom were chairfast or bedfast, 10 for more than three years, and administered to each 50 or 100 mg. of pyridoxine parenterally either daily or every other day for at least four weeks. Of the 15 patients 4 showed definite objective improvement. These results were reported in April 1940 before the Minnesota State Medical Association.[14] Similar results were reported a short time later by Spies Hightower and Hubbard.[15] Two of the 4 patients who showed objective improvement have since died, one following a fracture of the femur and the other following a myocardial infarction; the improvement in these 2 patients was maintained until the onset of the terminal illness. The improvement in the third patient has now been maintained for more than fourteen months, although he must receive pyridoxine parenterally three times a week for which saline, thiamine or nicotinamide cannot be substituted. The fourth patient has had a regression, so that now the tremors and rigidities are about the same as they were at the start of the treatment.

In reporting on these 15 helpless patients I noted that "thus far objective beneficial results . . . have been limited to the lessening of rigidities and increase in strength in patients whose complete disability was of less than three years' duration." Since then I have treated 46 ambulatory patients having paralysis agitans with pyridoxine, without selection as to age, type or degree of disability within ambulatory limits. Fourteen patients, all of whom showed no improvement, abandoned the treatment in less than three weeks. Shorter trials of therapy, such as recently reported by Zeligs,[16]

TABLE 1.—*Neuropsychiatric Disorders for Which There Is Substantial Evidence That Vitamin Deficiency May Play an Etiologic Role*

Disease or Syndrome	Predominant Vitamin Deficiency	Value in Therapy	References to Literature
A neurasthenic syndrome	(a) Thiamine	Yes	Williams, Mason and Smith.[2] Williams, Mason, Wilder and Smith.[2] Jolliffe, Goodhart, Gennis and Cline.[2] Jolliffe.[2]
	(b) Nicotinic acid	Yes	Spies, Bean and Stone.[3] Spies, Aring, Gelperin and Bean.[3] McLester, Spies and Aring.[3] Sydenstricker.[9]
Polyneuropathy	Thiamine	Yes	Spies and Aring.[3] Jolliffe, Colbert and Joffe.[4] Jolliffe and Colbert.[4] Goodhart and Jolliffe.[4] Jolliffe and Goodhart.[4] Jolliffe and Rosenblum.[4] Jolliffe.[4] Fein, Ralli and Jolliffe.[4] Wechsler.[4] Vorhaus, Williams and Waterman.[4] Borson.[4] Fantus.[4] Meiklejohn.[4] Brown.[4]
Ophthalmoplegia of Wernicke's syndrome	Thiamine	Yes	Campbell and Biggart.[5] Alexander.[5] Jolliffe, Wortis and Fein.[5] Jolliffe.[5]
Pellagra psychosis	Nicotinic acid	Yes	Matthews.[7] Sydenstricker, Schmidt, Fulton, New and Geeslin.[7] Spies, Aring, Gelperin and Bean.[3] Spies, Grant, Stone and McLester.[7] Spies, Vilter and Ashe.[7] Jolliffe (Recent Advances; Newer Knowledge).[8]
Nicotinic acid deficiency, encephalopathy	Nicotinic acid	Yes	Jolliffe, Bowman, Rosenblum and Fein.[8] Jolliffe.[9] Cleckley, Sydenstricker and Geeslin.[9] Sydenstricker.[9]
Infantile paralysis	(a) Ascorbic acid	No	Heaslip.[10] Jungeblut.[10]
	(b) Thiamine	No	McCormick, W. J.: Poliomyelitis, Canad. M. A. J. 38: 200 (March) 1938. Hamburger, F.: Bi-Vitaminmangel zur Verhütung der Poliomyelitis, Wien. klin. Wchnschr. 51: 825 (Aug. 5) 1938. Jungeblut.[10]
Delirium tremens	(a) Thiamine	No	Wortis.[11] Rosenbaum, Piker and Lederer.[11] Jolliffe and Wortis.[11] Wortis and Jolliffe.[11] Spies, Aring, Gelperin and Bean.[3]
	(b) Nicotinic acid	No	Rosenbaum, Piker and Lederer.[11] Jolliffe and Wortis.[11] Wortis and Jolliffe.[11] Spies, Aring, Gelperin and Bean.[3]
	(c) Ascorbic acid	No	Wortis, Wortis and Marsh.[11] Alexander, Pijoan, Schube and Moore.[11]
Korsakoff psychosis	Thiamine	No	Bowman, Goodhart and Jolliffe.[12] Jolliffe, Wortis and Fein.[6]
Muscular dystrophy (some types)	(a) Alpha-tocopherol	Exp.[a]	Bicknell.[13] Stone.[13] Spies and Vilter.[13] Donovan.[13] Shelden, Butt and Woltman.[13] Ferrebee, Klingman and Frantz.[13]
	(b) Pyridoxine	Exp.	Ferrebee, Klingman and Frantz.[13] Antopol and Schotland.[13] Bakwin.[13]
Amyotrophic lateral sclerosis (some types)	(a) Alpha-tocopherol	Exp.	Bicknell.[13] Spies and Vilter.[13] Ferrebee, Klingman and Frantz.[13] Wechsler.[13] Denker and Scheinman.[13]
	(b) Pyridoxine	Exp.	Shelden, Butt and Woltman.[13]
Paralysis agitans (some types)	Pyridoxine	Exp.	Jolliffe.[14] Spies, Hightower and Hubbard.[15] Zeligs.[16] Wortis.[17] Baker.[13]

[a] Exp. indicates experimental.

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16. Zeligs, M. A.: Use of Pyridoxine Hydrochloride (Vitamin B_6) in Parkinsonism, J. A. M. A. **116**: 2148 (May 10) 1941.

who gave "daily doses of 50 to 100 mg. (repeated one to three times)" intravenously, are entirely inadequate for judging this form of therapy. The group of 32 patients who continued the treatment longer than three weeks, of whom 23 were economic cripples, has

TABLE 2.—*Ambulatory Patients with Paralysis Agitans: Pyridoxine Treatment for Three Weeks or More*

Group	Number	Improvement: None	Improvement: Subjective	Improvement: Objective	Improvement: Return to Work	Continuing Therapy
Arteriosclerotic	6	5	1	0	0	1
Postencephalitic	10	4	6	4	2	6
Idiopathic	16	10	6	2	1	7
Total	32	19	13	6	3	14

shown, I believe, worth while results (table 2). The most conservative estimate of improvement would be to take as a criterion the 10 per cent who showed economic rehabilitation (one as a farm hand, one as a clerk in a vegetable market, the third as an insurance broker). A less conservative estimate would be the 30 per cent who showed objective improvement. A far too liberal estimate would be the 40 per cent who show subjective improvement and are still willing to continue treatment that involves travel and parenteral administration. One of my associates[17] has studied 12 cases of long-standing parkinsonism (average duration ten and one-half years) and noted no beneficial results from adequate pyridoxine therapy.

The fact that the improvement in helpless patients previously reported on was limited to those with the idiopathic and arteriosclerotic types of the condition, while in the ambulatory series reported here improvement seems more frequent in the postencephalitic group, indicates that the entire series is still too small to allow definite conclusions to be drawn. It seems justifiable to say, however, that the syndrome of paralysis agitans, if not of too long duration, appears to include cases which are helped by pyridoxine.

Nutritional treatment of neuropsychiatric disorders consists of (1) general measures applicable to all and the specific therapy. The principles on which the general treatment is based are (*a*) that deficiency disease in man is usually not single but multiple and (*b*) that adequate treatment does not consist of the administration of specific chemicals (crystalline vitamins), whether singly or in combination. All neuropsychiatric patients to whom specific therapy is to be given are therefore placed on the following regimen:

1. A full, nutritious diet of natural unrefined foods. To accomplish this, all vitamin-free or vitamin-poor foods are excluded from the diet. (These include sugar, candy, jellies, jams, white flour products, refined cereals, polished rice and alcoholic drinks). The patient is instructed to eat each day citrus fruit juices or tomato juice 360 cc., milk 720 cc., meat (including frequent servings of liver or pork muscle) 250 Gm., eggs 2, butter 45 Gm., salad (raw vegetables and fruit) 250 Gm., salad oil 15 cc. and potatoes 250 Gm. The balance of the diet is selected by the patient from cooked or raw vegetables, cooked or raw fruits, whole grain cereals and whole grain bread.

2. Vitamin supplements by mouth. Routinely the patients are given a fish liver oil in an amount containing 10,000 units of vitamin A and a source of the entire vitamin B complex. Such products as 30 Gm. of brewers' yeast, 20 Gm. of vegex, 45 cc. of aqueous liver extract or 60 Gm. of wheat germ cereal are preferred to synthetic products in capsule form from which some factors as yet impossible to encapsulate are likely to be missing.

TABLE 3.—*Specific Treatment of Neuropsychiatric Disorders with Vitamins*

Disease or Syndrome	Vitamin	Amount in Mg.	Route	Frequency	Comment
Neurasthenic	Thiamine	10 to 30	Parenteral	Daily	Trial of at least 3 weeks if less than 3 months' duration, if of longer duration trial of at least 3 months
	Nicotinic acid amide	100 to 300	Mouth	Daily	
Polyneuropathy					
Mild	Thiamine	10 to 30	Parenteral	Daily	10 days to 3 weeks
Moderate	Thiamine	20 to 50	Parenteral	2 times daily	3 to 6 weeks
Severe	Thiamine	20 to 100	Parenteral	2 times daily	3 to 6 weeks then in smaller doses until well after improvement ceases
Ophthalmoplegia of Wernicke's syndrome	Thiamine	50 to 100	Parenteral	3 times daily	2 to 7 days
Pellagra psychosis	Nicotinic acid	500 to 1,000	Mouth	Daily	1 to 4 weeks then continue smaller doses until complete recovery
Nicotinic acid deficiency, encephalopathy	Nicotinic acid	500 to 1,000	Mouth	Daily	3 to 7 days
	Nicotinic acid amide	100 to 200	Parenteral	Daily	3 to 7 days then continue smaller doses by mouth through convalescence
Muscular dystrophies	Pyridoxine	50 to 100	Parenteral	Daily	Trial of at least 3 months
Paralysis agitans	Pyridoxine	50 to 100	Parenteral	Daily	Trial of at least 1 month
Amyotrophic lateral sclerosis	Alpha-tocopherol	150 to 200	Mouth	Daily	Trial of at least 3 months
		100 to 150	Parenteral	Daily	

3. Parenteral administration of the entire vitamin B complex. This practice is based on the empirical observation that these patients seem to do better when at least a quantity of the entire B complex is given parenterally. It is especially important when absorption or utilization of the vitamins may be impaired. I use as a routine 2 or 3 cc. of crude liver extract or liver residue as a vehicle for the specific vitamin prescribed and continue it as long as vitamins are administered parenterally. If injections are long continued local soreness may make it advisable to substitute for liver extract synthetic vitamins that can be given either intravenously or intramuscularly. For this purpose I use thiamine hydrochloride 10 mg., riboflavin 1 mg. and nicotinic acid amide 100 mg.

These disorders are complicated at times by gastrointestinal or other disturbances which prevent this routine from being followed. The diet may then be modified to meet the individual requirement, as much as possible of its nutritive qualities being retained. The vitamin A is increased to 20,000 units; in place of the bulky sources of the B complex crystalline vitamins are given twice daily in the following amounts: thiamine 2.5 mg., riboflavin 3.75 mg., nicotinic acid amide 25 mg., and 200 mg. of ascorbic acid is given in two doses.

17. Herman: Personal communication to the author.

Specific treatments recommended in table 3 are based on the principle that when biochemical or reversible anatomic lesions are present it is safer to err on the side of wasting the vitamin than to give too little for too short a period of time. If the patient has liver disease or diarrhea or is an alcoholic addict, the larger doses recommended in the table should be given. Clinical as well as experimental evidence indicates that in these conditions much larger doses of vitamins are required to produce maximal response than when absorption is unimpaired and the liver normal.

In view of recent expressions concerning toxic effects of vitamins, I believe it pertinent to state that in treating more than 3,000 patients with clinically manifest deficiency disease I have not observed any toxic effects when the vitamins were administered as a crystalline vitamin dissolved in saline solution without added preservatives, in the doses described, and while the patient was maintained with a good diet and the entire vitamin B complex (except, of course, the vasomotor phenomena of nicotinic acid). Drs. Borsook, McLester, Sebrell, Spies and Sydenstricker have informed me that their experiences have been similar. Dr. Borsook has administered by intravenous injection to each of 70 patients for at least three years 100 mg. of thiamine hydrochloride daily (except on Sundays and holidays) without observing any signs of toxicity. I have observed, however, local and general sensitivity to solutions of thiamine containing added preservatives. Cutaneous tests performed on these patients demonstrated sensitivity to the preservative and not to the thiamine. Subsequent administration of crystalline thiamine dissolved in saline solution was in no instance followed by either local or general sensitivity in the same patients. Two patients who were maintained with dextrose, saline solution and large doses of nicotinic acid and 4 patients who were maintained with a poor diet plus dextrose, saline solution, crystalline B vitamins in maintenance doses and large doses of nicotinic acid may possibly have manifested signs of nicotinic acid toxicity. I believe, however, that in these subjects deficiency of other vitamins is the more likely explanation.

CONCLUSION

No untoward effects have been observed after the therapeutic administration of any of the crystalline B vitamins to more than 3,000 patients.

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THE TOXICITY OF VITAMIN B_6, 4-DESOXYPYRIDOXINE, AND 4-METHOXYMETHYLPYRIDOXINE, ALONE AND IN COMBINATION, TO THE CHICK EMBRYO*

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Ott (1, 2) has shown that desoxypyridoxine (2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine) and methoxypyridoxine (2-methyl-3-hydroxy-4-methoxymethyl-5-hydroxymethylpyridine), analogues of pyridoxine, act as pyridoxine antagonists in the chick. Because of our interest in the effects of metabolic antagonists on the development of the chick embryo (3), and on the behavior of mouse tumors growing on the chorioallantoic membrane of the chick embryo (4), these substances, and various forms of vitamin B_6, were studied in the fertile chick egg.

Materials and Methods

Fertile white Leghorn eggs were obtained from a commercial source. The eggs were incubated at 38° and 75 per cent relative humidity. Pyridoxine hydrochloride, pyridoxamine dihydrochloride, pyridoxal hydrochloride and its phosphate, and methoxy- and desoxypyridoxine hydrochloride were used in this study. These chemicals were dissolved in saline just before use, and introduced into the yolk sac through a hole drilled in the blunt end of the egg. The opening was then sealed with a drop of paraffin. The volume of solution injected into each egg ranged between 0.05 to 0.2 cc. Eggs varying from 0 to 13 days of incubation were used. Following injection, the eggs were candled daily, and the dead embryos were weighed and examined for gross developmental abnormalities. Observations were usually made for 10 days after injection, and embryos surviving beyond this period were often sacrificed. More than 1850 embryos were used in this study. The number of embryos used at each dosage of the injected drugs varied from six to twenty, and at critical dosages several experiments were run. It was not feasible to make accurate LD_{50} determinations for each compound, or combinations of compounds, in embryos of various ages, and only approximate LD_{50} values, sufficient to characterize

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each situation, were estimated by inspecting a graph of the pertinent data. In studying the protective value of various forms of vitamin B_6 against the antagonists, the ratio permitting 50 per cent survival of embryos was approximated.

Results

Toxicity of Vitamin B_6 and Related Compounds to Chick Embryo

The approximate LD_{50} values of the compounds tested in the chick embryo after various periods of incubation are summarized in Table 1.

Cravens and Snell (5) tested some of these compounds in the 6 day embryo, and our values are in general accord. They found that pyridoxine was toxic between 5 and 10 mg. per egg, pyridoxal hydrochloride at 5 mg., desoxypyridoxine at 5 mg., and pyridoxamine was non-toxic at 10 mg. per egg, the highest dose tested. When injected at 0 days, desoxypyridoxine produced 100 per cent deaths at doses of 0.5 to 1.0 mg. per egg, a somewhat smaller dose than proved toxic in our experiments.

The most interesting toxicity data were obtained with methoxypyridoxine, and this compound was consequently studied in considerable detail. Only 0.04 mg. per egg of methoxypyridoxine was required to produce 50 per cent mortality at 0 days, but as the embryo became older, this amount became progressively greater, so that at 13 days it was 3.5 mg. per egg. This rise in the LD_{50} dose roughly paralleled the increase in mass of the chick embryo. Embryonic deaths usually occurred within 2 to 5 days after the injection of the drug; the older embryos receiving a fatal dose tended to survive longer than the younger ones.

Embryonic abnormalities were not consistently found with these agents, but occasionally gross malformations were seen in embryos surviving for long periods, particularly following the injection of desoxypyridoxine, pyridoxal hydrochloride, or pyridoxal phosphate. In embryos intoxicated by methoxypyridoxine, apparently normal growth and development proceeded for the several days before they succumbed. The mechanism whereby methoxypyridoxine caused the death of the embryo is not known, but it did not appear to interfere with growth.

Counteraction of Effects of Antagonists by Vitamin B_6

Pyridoxine, pyridoxamine, pyridoxal hydrochloride, and pyridoxal phosphate gave clear cut protection to the 4 day chick embryo against methoxypyridoxine. The studies on the protective activity of each form of vitamin B_6 are described separately, and the results are summarized in Fig. 1.

Pyridoxine—The 4 day embryo was used in most of the experiments, but a few studies were carried out with 0 and 13 day embryos. In the 4

day embryo, 0.07 mg. per egg of pyridoxine protected 50 per cent of the embryos against 0.5 mg. per egg of methoxypyridoxine (3 × LD_{50}). As the dose of methoxypyridoxine was raised, a much smaller increase in pyridoxine was necessary to provide protection; at the highest dose of methoxypyridoxine tested, 40 mg. per egg (240 × LD_{50}), 0.5 mg. of

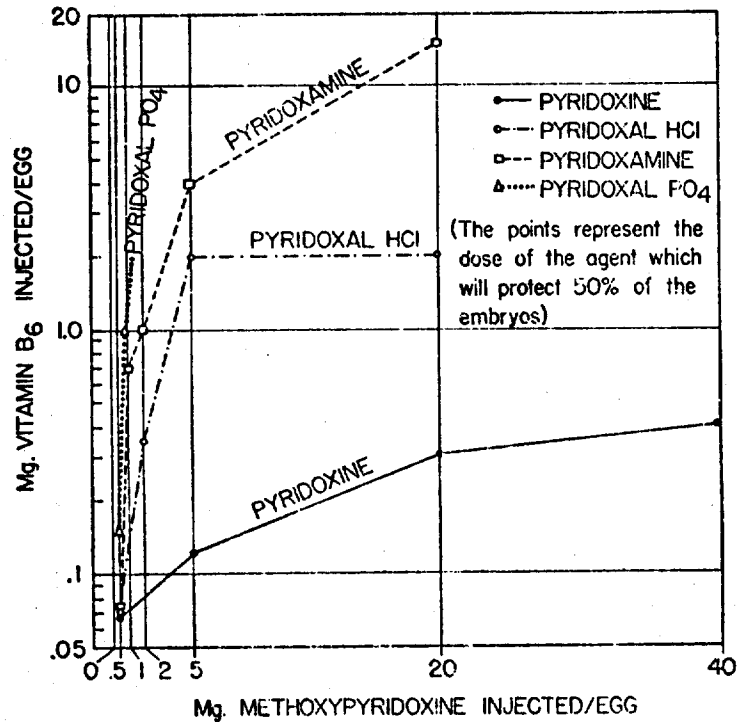

Fig. 1. The protective activity of various amounts of pyridoxine, pyridoxal hydrochloride, pyridoxamine, and pyridoxal phosphate against a range of doses of methoxypyridoxine in the 4 day chick embryo. The toxicity estimates are based on embryonic deaths occurring during the period of 2 to 7 days after injection. The estimated LD_{50}, as charted, is the amount of the vitamin B_6 compound protecting 50 per cent of the embryos against a given dose of methoxypyridoxine. Ten to 90 embryos (average of forty) were used to obtain each point on the chart. The abscissa is plotted on a logarithmic scale.

pyridoxine protected 70 per cent of the embryos. It is of interest that, in the presence of pyridoxine, methoxypyridoxine has a lower intrinsic toxicity (>40 mg. per egg) than pyridoxine, pyridoxal hydrochloride, or pyridoxal phosphate.

Scattered observations on embryos of different ages suggest that the protective ratio of pyridoxine to methoxypyridoxine is more closely re-

lated to the LD_{50} dose of methoxypyridoxine, rather than to the absolute dose of the drug. For example, 0.2 mg. per egg of pyridoxine failed to protect 0 and 4 day embryos against 5 and 20 mg. per egg, respectively (120 × LD_{50}) of methoxypyridoxine, whereas it protected 50 per cent of 4 day embryos against 5 mg. (30 × LD_{50}), and 0.1 mg. of pyridoxine protected 50 per cent of 13 day embryos against 20 mg. (6 × LD_{50}) of methoxypyridoxine.

Pyridoxine, injected several days prior to methoxypyridoxine, was still capable of protecting against the antagonist. Thus, when 0.5 mg. of pyridoxine was injected into 2 day embryos, it protected them against the injection of 5 mg. of methoxypyridoxine made 2 days later. Similarly, the injection of 1 mg. of pyridoxine at 4 days of incubation protected against 20 mg. of methoxypyridoxine injected at 10 days. Thus pyridoxine remains active for at least 6 days after injection into the egg.

The ability of pyridoxine to reverse the toxicity of methoxypyridoxine was tested in the 4 day embryo. When 5 mg. of methoxypyridoxine were followed 1, 2, 4, 8, 24, or 48 hours later with 0.5 mg. of pyridoxine, those embryos receiving pyridoxine within 24 hours showed appreciable protection, whereas all embryos injected at 48 hours died. The effects of a large dose of methoxypyridoxine (30 × LD_{50}) thus may be reversed by pyridoxine within 24 hours, but not at 48 hours. The great majority of deaths in the 4 day embryos treated with methoxypyridoxine occurred between 48 and 72 hours, so the failure of pyridoxine to reverse the toxicity of methoxypyridoxine at 48 hours is not surprising.

Pyridoxal Hydrochloride and Pyridoxamine—These compounds appeared to be as active as pyridoxine in counteracting the toxic effects of 0.5 mg. per egg of methoxypyridoxine. As the dosage of methoxypyridoxine was increased, the amounts of these agents necessary to provide protection rose promptly to higher levels, and pyridoxal hydrochloride showed considerably more protective activity than pyridoxamine (Fig. 1).

Pyridoxal Phosphate—This agent was found to have weak protective activity against methoxypyridoxine. It provided protection against 0.5 mg. per egg of methoxypyridoxine, but when the dose of the antagonist was increased above 1 mg. per egg, pyridoxal phosphate, given in doses up to its limiting intrinsic toxicity (3 to 5 mg. per egg), failed to protect the embryos.

Desoxypyridoxine—Cravens and Snell (5) found that vitamin B_6 would protect 0 day embryos against desoxypyridoxine, but in the 4 to 6 day embryo vitamin B_6 could not diminish the toxicity of desoxypyridoxine. Similarly, in our experiments with the 4 day embryo, desoxypyridoxine did not show any vitamin B_6 or antivitamin B_6 activity. The LD_{50} of desoxypyridoxine in the 4 day embryo is 4 mg. per egg. At 5 mg. of

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desoxypyridoxine, 5 and 3 mg. of pyridoxine and pyridoxal phosphate, respectively, did not provide any protection. Conversely, 2 mg. of desoxypyridoxine did not protect the chick embryo against 0.5 mg. of methoxypyridoxine.

DISCUSSION

A number of interesting differences between the chemically closely related compounds studied have been brought out by the use of the chick embryo. If the antivitamin B_6 activity of methoxypyridoxine is eliminated by adding pyridoxine, these agents show the following order of toxicity in the 4 day embryo: methoxypyridoxine, not toxic at 40 mg. per egg, the highest level tested, in the presence of a protective amount of pyridoxine; pyridoxamine, not toxic at 20 mg. per egg, the highest level

TABLE I

Approximate LD_{50} Doses of Vitamin B_6 and Related Compounds for Chick Embryos of Different Ages

The values are expressed as mg. per egg.

Age of embryo when injected	Pyridoxine	Pyridoxamine	Pyridoxal	Pyridoxal phosphate	Desoxy-pyridoxine	Methoxy-pyridoxine
days						
0			>3.0		1.0-2.0	0.04
2						0.07
4	12.0	>20.0	5.0	3.0-5.0	4.0	0.17
6						0.25
8						0.80
10						0.90
13	>20.0	>20.0	5.0		12.0	3.50

tested; pyridoxine, approximate LD_{50}, 12 mg. per egg; pyridoxal hydrochloride and pyridoxal phosphate, LD_{50} range, 3 to 5 mg. per egg; desoxypyridoxine, approximate LD_{50}, 4 mg. per egg.

These limited data, and those contained in Table I, suggest that the toxicity of these agents, in the presence of vitamin B_6, shows relatively little change in the chick embryo from 0 to 13 days of age, particularly as contrasted with the marked decrease in the toxicity of methoxypyridoxine with the increase in the age of unprotected embryos. The mechanisms of the lethal action of these agents, as apparently dissociated from their vitamin B_6 activity of antagonism, present an interesting problem.

The embryo can be protected against the toxic effects of methoxypyridoxine (and this was studied particularly in the 4 day embryo) by vitamin B_6, and considerable differences were found in the protective activity of the different vitamin B_6 compounds. The protective dose of pyridoxine

was shown to increase relatively little with large increases in methoxypyridoxine. Pyridoxal hydrochloride and pyridoxamine showed protective activity approximately equal to that of pyridoxine at 3 × LD_{50} doses of methoxypyridoxine, but as the dose of methoxypyridoxine was increased, these compounds appeared less active than pyridoxine, and pyridoxamine was less active than pyridoxal hydrochloride. Pyridoxal phosphate was the least active of the vitamin B_6 compounds tested against 3 × LD_{50} doses of methoxypyridoxine, and it did not protect against larger doses. On the basis of the relative protective plateaus in dosage that these agents (excluding pyridoxal phosphate) achieve against 5 mg and higher doses of methoxypyridoxine, their relative protective activities against methoxypyridoxine are estimated as 30 (pyridoxine) to 3 (pyridoxal hydrochloride) to 1 (pyridoxamine) (Fig. 1).

Our results with desoxypyridoxine are in accord with those of Cravens and Snell (5). In their experiments with the 0 day eggs, desoxypyridoxine was lethal at doses of 1.0 mg. per egg. 50 per cent of the embryos was protected against this dose by approximately 0.01 mg., 0.02 mg., and 0.05 mg. of pyridoxine, pyridoxamine, and pyridoxal hydrochloride, respectively. In 4 and 6 day embryos, however, larger amounts of desoxypyridoxine were required to show toxicity, and none of these agents protected against these increased amounts of desoxypyridoxine; they conclude that this toxic action is not due to the antivitamin B_6 activity of desoxypyridoxine. Our data support this view, and it seems probable that desoxypyridoxine can intoxicate the chick embryo by two different mechanisms. The first mechanism is unrelated to its antivitamin B_6 activity, and may be referred to as its intrinsic toxicity. The intrinsic toxicity of desoxypyridoxine rises from about 2 mg. per egg at 0 day to 12 mg. per egg at 13 days, and this toxic activity may be related to those of pyridoxine, pyridoxal hydrochloride, and pyridoxal phosphate (Table I).

The second mechanism by which desoxypyridoxine is toxic is due to its antivitamin B_6 activity. At 0 day, desoxypyridoxine is toxic at 1.0 mg. per egg, and this action can be prevented by vitamin B_6. As the embryo becomes older, a larger amount of antivitamin B_6 activity is necessary to produce embryonic death. For example, the LD_{50} dose of methoxypyridoxine increases from 0.04 mg. per egg at 0 day to 0.17 mg. per egg at 4 days, a 4-fold increase. If it is assumed that the dose of desoxypyridoxine must be increased proportionally in order to produce a lethal antivitamin B_6 effect in the 4 day embryo, the estimated dose of desoxypyridoxine necessary will produce death because of its intrinsic toxicity. Methoxypyridoxine is, thus, far superior to desoxypyridoxine as an antagonist of vitamin B_6 in the chick embryo, since it has a much lower in-

trinsic toxicity, and from the above considerations it is estimated to be at least 25 times more active than desoxypyridoxine as a vitamin B_6 antagonist.

The resistance of the embryo to methoxypyridoxine as it increases in age prompts speculation. This may mean that the embryo, as it grows, is forming vitamin B_6, or, as suggested by Cravens and Snell (5), that the vitamin B_6-dependent systems, as they develop, become resistant to the antagonist. The suggestion, in our data, that the dose of pyridoxine protecting embryos against methoxypyridoxine is more closely related to the LD_{50} dose of the antagonist at a given age than to the absolute amount of the drug does not clarify this problem. The vitamin B_6 content of the egg must be considered as a factor in these experiments, particularly since Rabinowitz and Snell (6) have reported that each gm. of dried egg contains 0.0056 mg. of pyridoxal hydrochloride, 0.0012 mg. of pyridoxamine, and a negligible amount of pyridoxine. This may be roughly estimated as about 0.08 mg. of vitamin B_6 per whole egg, the significance of which in relation to our observations is not clear.

The mechanism whereby methoxypyridoxine antagonizes vitamin B_6 is unknown. Umbreit and Waddell (7) have studied the antagonistic action of desoxypyridoxine *in vitro*. They conclude that desoxypyridoxine is phosphorylated and competes with pyridoxal phosphate, the active form of vitamin B_6. In the chick embryo, however, the facts that pyridoxine, pyridoxal hydrochloride, pyridoxamine, and pyridoxal phosphate are effective against methoxypyridoxine in a descending order, and that pyridoxine, once it achieves a certain level, will protect against increasing doses of methoxypyridoxine, suggest that pyridoxine is the most active form of vitamin B_6 of those tested.

The exact rôle of vitamin B_6 in the metabolism of birds and mammals has not been demonstrated. It is known, however, to play an important rôle as a coenzyme in transamination, decarboxylation, and possibly in carboxylation reactions, and Bonner and Bonner (8) have stated that, "In its possible rôle as a coenzyme in transamination, in particular, it may occupy a key position in the synthesis... of the proteins of the plant." It is of interest, therefore, that chick embryos treated with lethal doses of methoxypyridoxine continue to differentiate and enlarge for the 2 to 5 day period before death, presumably due to the continued synthesis of protein and the formation of new cells. It appears likely that the vitamin B_6 deficiency induced by methoxypyridoxine in the chick embryo produces death by means of a physiological derangement, not immediately related to the processes of tissue growth, or by the accumulation, in the closed system of the egg, of lethal concentrations of the by-products of a vitamin B_6 deficiency. One of these limiting factors, in producing early embryonic

death, may mask the development of more complete or profound evidences of a vitamin B_6 deficiency. At the moment, it can only be concluded that the effects of methoxypyridoxine in the chick embryo may permit a further exploration of the mechanism of action of vitamin B_6 and supply a new method for assaying compounds for vitamin B_6 activity.

SUMMARY

Methoxypyridoxine is toxic to the chick embryo because of its antivitamin B_6 activity. Its toxicity decreases with the age of the embryo, the LD_{50} increasing from 0.04 mg. per egg at 0 day to 0.17 mg. per egg at 4 days and 3.5 mg. at 13 days. The lethally intoxicated embryos may survive for several days and continue to show grossly normal growth and development. Pyridoxine, pyridoxal hydrochloride, pyridoxamine, and pyridoxal phosphate will protect the chick embryo against methoxypyridoxine, and their activity decreases in the order listed. A relatively constant amount of pyridoxine will protect the embryo against a wide range of concentrations of methoxypyridoxine, whereas, at the other extreme, pyridoxal phosphate protects against only a small amount of methoxypyridoxine.

Desoxypyridoxine is a weak vitamin B_6 antagonist in the 0 day chick embryo, as shown by Cravens and Snell (5). In older embryos its intrinsic toxicity proves lethal at levels which do not apparently produce a severe vitamin B_6 deficiency, as evidenced by the failure of vitamin B_6 to protect the embryo against its action. It is estimated that methoxypyridoxine is at least 25 times more active than desoxypyridoxine as a vitamin B_6 antagonist in the 0 to 4 day chick embryo.

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Kato-'65
Bib No. 1288

Kato, H.: THE EFFECT OF B VITAMINS ON THE BIOSYNTHESIS OF PROTEIN-BOUND LIPOIC ACID. III. INFLUENCE OF THE OVERDOSE OF B VITAMINS ON THE BIOSYNTHESIS OF PROTEIN-BOUND LIPOIC ACID. Bitamin, Vol. 31 (4), pp. 309-314, 1965. Department of Pediatrics, Kyoto Prefectural University of Medicine, Kyoto.

With increasing use of vitamin preparations, particularly active vitamin preparations, the problems due to massive vitamin intake has recently drawn attention. One of the problems that require attention is their effect on the metabolism of other vitamins. In 1940, Sydenstricker et al. (1) observed the aggravation of B_1 and B_2 deficiency syndrome in pellagrins given massive doses of nicotinic acid, and Leitner et al. (2) also called attention to a similar phenomenon. In Japan, Inoue et al. (3) continuously administered large doses of TPD (thiamine propyldisulfide), a derivative of vitamin B_1, and noted vitamin B_2 deficiency syndrome in the patients, but Miyagawa et al. (4,5) failed to observe any apparent change in the organs or any disorder due to large dose administration of B_1. In the previous 2 papers, the author discussed the effect of B_1, B_2, and B_6 deficiencies on the biosynthesis of protein bound LiA (lipoic acid). In thepresent experiment, he studied whether or not massive administration of B_1, B_2, and B_6 could influence the LiA metabolism, particularly the biosynthesis of bound LiA.

EXPERIMENTAL PROCEDURE

(1) EXPERIMENTAL ANIMALS AND FEED

Forty days old male Wistar white rats weighing about 50 g were raised on the standard solid feed for 4 - 6 days, and divided into 9 groups. The feeds given to these groups contained B_1-HCl at 1 mg (group II), 10 mg (group III), and 50 mg (group IV) per kg of body weight, TTFD (thiamine tetrahydrofurfuryldisulfide) at 1, 10, and 50 mg per kg b.w. (V, VI, and VII groups, respectively), B_2 at 1 mg per kg of b.w. (group VIII), and B_6 at 1 mg per kg of b.w. (group IX). After the animals were raised on the feeds for a specific period of time, they were killed by decapitation, and examined. The amount of B_1, B_2, and B_6 contained in the standard solid feed was 0.2 (mg%). The amount of feed, given upon demand, was 10 - 20 g per animal daily.

(2) DETERMINATION OF THE BIOSYNTHETIC LEVEL OF PROTEIN BOUND LiA.

As in the previous experiment, Nose's method was employed (6). The nitrogen level of liver Mit (mitochondria) was measured by micro-Kjeldahl method.

(3) DETERMINATION OF LIVER GOT ACTIVITY

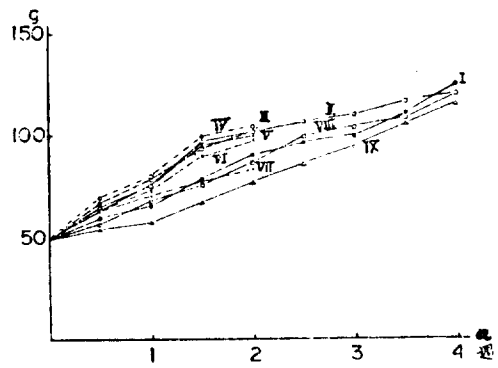
Reitman-Frankel's method was employed (7). Ground liver solutions diluted 1000 and 500 fold were prepared with 0.1 M phosphate buffer at pH 7.4, and permitted to react at 37°C for 60 minutes. The activity was examined in terms of the amount of the resultant oxaloacetic acid.

EXPERIMENTAL RESULTS

(1) GROWTH AND BODY WEIGHT

As shown in Figure 1, the control group (group I) indicated a linear growth curve, and the massive B_2 group (VIII), a similar pattern of growth. The growth of the massive B_1-HCl groups (II, III, and IV) was more favorable than the control group for the first 2 weeks, and the TTFD groups, V and VI, also showed a favorable growth pattern, their results being close to that of the B_1-HCl groups. However, group VII which received an extremely large dose of TTFD, at 50 mg/kg, showed suppressed growth. The massive B_6 group (IX) exhibited a pattern different from the B_1 massive dose groups, with a temporary drop in weight gain. It was noted, however, that groups I, II, VIII, and IX which remained under observation for longer periods exhibited gradual diminution of the effect on body weight, with their body weight returning to the control level in 4 weeks.

(2) BIOSYNTHESIS OF PROTEIN BOUND LiA.



KEY: a, weeks

Figure 1. Growth Curves

TABLE 1. BIOSYNTHETIC ACTIVITY OF PROTEIN BOUND LiA (cpm/mg N)

a		1	2	3	5
c 对照		3914±175*	3625±215	4012±213	3955±302
B_1-HCl	1	5137±309	5802±210	[illegible]	[illegible]
B_1-HCl	10	—	[illegible]±415	—	
B_1-HCl	50	—	5423±303		
TTFD	1	—	5575±196	—	—
TTFD	10	—	5300±314	—	—
TTFD	50	—	3625±511	—	—
B_2	1	4057±237	3874±219	4123±251	4087±306
B_6	1	3908±285	2687±212	3578±262	3811±251

d * 标准偏差

KEY: a, dose (mg/kg); b, duration (weeks); c, control; d, standard deviation

As of 2 weeks from the onset of experiment, the massive B_1-HCl groups (II, III, and IV) and the TTFD massive dose groups (V, VI) exhibited the highest protein bound LiA forming capacity of the liver MiT, but the B_2 massive dose group (VIII) showed no difference from the control as shown in Table 1 and Figures 2 and 3. The values of the TTFD-50 mg/kg group (VII) were slightly lower than the control level. The biosynthesis of LiA in the B_6 group (IX) was clearly suppressed. In the 4th week, however, the groups given 1 mg/kg of B_1, B_2, and B_6 (groups II, VIII, and IX) showed no difference.

(3) LIVER GOT ACTIVITY

The determination of liver GOT activity revealed no apparent difference between the groups.

(4) CHANGES IN THE BOUND LiA OF LIVER MiT IN RELATION TO TIME FOLLOWING THE ADMINISTRATION OF LiA-^{35}S.

LiA-^{35}S was intraperitoneally administered to groups II and IX at 20 mg/kg in the 2nd week after the onset of administration, and the incorporation of the substance into the liver MiT protein was studied in relation to time. The results are illustrated in Table 3 and Figure 4. As noted in the graph, the B_1 groups gave the same results as the control as of 30 minutes later, but the B_6 group showed a value 70% lower than the control. In either case, the value dropped rapidly, with no difference among the groups in 6 hours.

(5) EFFECT OF OTHER COMPOUNDS

B_1, CoC (cocarboxylase), B_6, and PAL-P (pyridoxal phosphate) were added to liver MiT of the control group *in vitro*, and their effect on the biosynthesis was studied. As shown in Table 4, the compounds produced no significant effect.

(6) NITROGEN LEVEL IN LIVER MiT

As shown in Figure 5, no significant difference between the groups was noted.

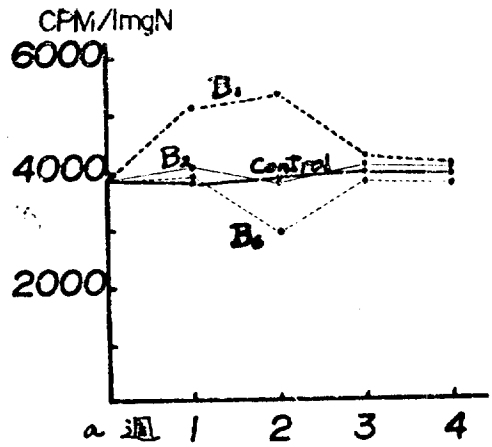


Figure 2. Synthetic Activity of Protein-Bound LiA.
(KEY: a, weeks)

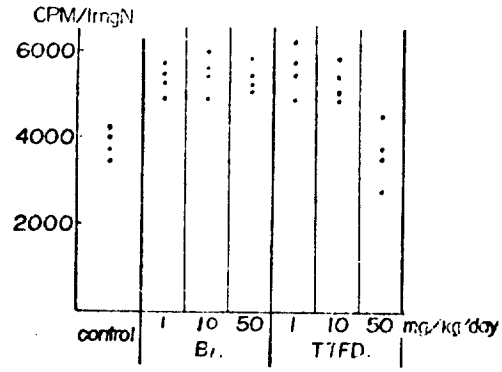


Figure 3. Synthetic Acitvity of Protein Bound LiA.

TABLE 2. LIVER GOT ACTIVITIES IN THE WHITE RATS GIVEN LARGE DOSES OF VITAMINS (pyruvic acid u mol/mg/hr)

a 投与量(mg/kg)		b 飼育期間(週) 1	2	3	4
c 対照		1.21	1.13	1.24	1.18
B_1-HCl	1	1.17	1.19	1.11	1.22
B_1-HCl	10	--	1.25	—	-
B_1-HCl	50	—	1.01	—	—
TTFD	1	—	1.18	-	—
TTFD	10	—	1.21	—	-
TTFD	50	—	1.06	—	—
B_2	1	1.18	1.23	1.22	1.10
B_6	1	1.23	1.14	1.17	1.14

KEY: a, dose (mg/kg); b, duration (week)

TABLE 3. INCORPORATION OF LiA-^{35}S INTO LIVER MiT PROTEIN (in vivo)

a 投与後時間(分)	b 対照	c B_1大量(II)	d B_6大量(IV)	e 投与後時間(分)	f 対照	g B_1大量(II)	h B_6大量(IV)
30	3156 2550	2992 2430	2240 1728	120	681 516	870 628	418 260
f 平均	2853	2711	1984	f 平均	600	749	354
60	2128 1582	2171 1737	1251 1053	360	306 124	471 231	157 99
f 平均	1855	1954	1102	f 平均	215	351	123

g 単位 = cpm/mg N

KEY: a, time (min) after administration; b, control; c, B_1-large dose (II); d, B_6-large dose (IV); e, time (min) after administration; f, average; g, unit of measurement

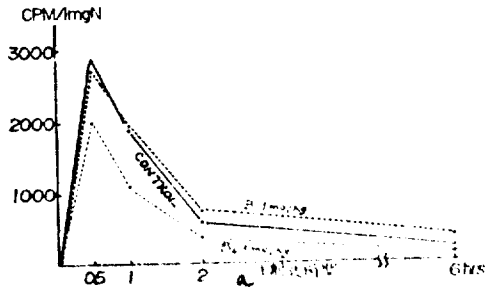


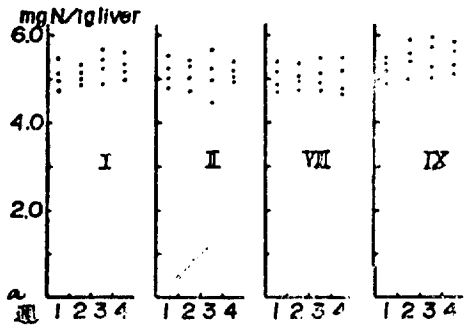
Figure 4. Uptake of LiA-^{35}S into Liver MiT Protein. (in vivo)
(KEY: a, time after injection)

TABLE 4. EFFECTS OF B_1, CoC, B_6, and PAL-P on THE BIOSYNTHESIS OF PROTEIN BOUND LiA.

a 添加	cpm/mg N
—	3950
B_1	3797
CoC	3885
B_6	3416
PAL-P	4070

B_1, CoC $2\times10^{-5}M$ B_6, PAL-P $4\times10^{-5}M$

KEY: a, Agent added.



KEY: a, weeks

Figure 5. Nitrogen Levels in Liver MiT.

DISCUSSION

With the recent progress in vitaminology, particularly in conjunction with the advances in the research on the coenzyme action and physiologic activity of vitamin B compounds, various preparations, such as those called "active", "coenzymic", or "internally retainable", have been produced and administered at massive doses in various clinical fields, primarily for their pharmacologic action rather than their inherent actions as vitamins. The research efforts and achievements centering around B_1 in the biochemical, pharmacologic, and clinologic disciplines, in Japan have been remarkable, and in our pediatric field, the use of B_1 and its derivatives at massive doses has been expanded to such areas as neurologic, muscular, and nutritional disturbances, rheumatic disorder, and neurotonia. On the other hand, however, some investigators assert that the massive administration of B_1 increases the excretion of other vitamin B compounds or disrupts their phosphorylation. Thus, it is deemed imperative to thoroughly investigate the degree and mechanism of disturbance in the utilization of other vitamins and the biochemical side effects due to massive intake of a specific form of vitamin.

The authors conducted the above experiment, and obtained a series of data. When B_1-HCl was orally administered to young white rats at 1, 10, and 50 mg per kg of body weight daily for 4 weeks, their weight gain during the first 1 - 2 weeks surpassed that of the control which had been given B_1 at a daily dose of 20 - 40 u, with clear signs of stimulated biosynthesis of protein bound LiA in the liver Mit. However, in 3 - 4 weeks, both the body weight and the biosynthesis of bound LiA were reduced to the control level despite the continued massive B_1 administration. When TTFD was given at large doses, i.e., at the molar equivalent to the dose of B_1-HCl, both factors indicated a rise as in the administration of B_1-HCl, at doses corresponding to 1 and 10 mg/kg of B_1HCl, but at the dose corresponding to 50 mg/kg, the weight gain dropped below the control level in 1 - 2 weeks, and the bound LiA synthetizing activity was no more favorable than that of the control. On the other hand, the administration of B_2 at 1 mg per kg of body weight daily produced no change in the growth curve, or the biosynthesis of bound LiA, while the same amount of B_6 caused the weight gain to drop

below the control level, with suppressed biosynthesis of bound LiA in 1 to 3 weeks. However, these effects of B_6 diminished in the 4th week despite the continued administration of the agent, with the weight gain and LiA biosynthesis being at the same levels as those of the control.

Summarizing the experimental results, B_2 exerted no significant effect, but the massive dose of B_1 or B_6 produced changes in body weight and the synthetic activity of LiA in a parallel manner. These changes, however, subsequently diminished despite continued intake of B_1 or B_6. These results can be interpreted as that, following the massive administration of B_1 or B_6, the growth and metabolism of a living organism is subjected to a wide range of effect including an influence on weight gain, and the stimulatory or inhibitory effect on the biosynthesis of bound LiA is secondary to the change in the growth rate and metabolism. It is also postulated that, while a living organism continues to receive massive dose of the vitamin, it reajusts its metabolic activity, or manifests its ability to eliminate the changes by producing a B_1 or B_6 combating mechanism or an adaptable enzyme system. The change occurring in the biosynthesis of bound LiA is not a direct effect of B_1, CoC, B_6, or PAL-P as is evidenced by the fact that the direct addition of these compounds to the liver MiT of the control animals produced no specific change.

With regard to the nature of the metabolic change produced by massive B_1 administration, no specific discussion can be made at this point due to lack of detailed investigation. As stated in the beginning in relation to Klopp's report (8), massive dose of B_1 results in an increased excretion of B_2 several hours later. Inoue (3) also observed that TPD-treated patients developed vitamin B_2 deficiency symptoms. According to Shinagawa (9), large dose administration of B_1 and TAD (thiamine allyldisulfide) was followed by an increased urinary excretion of nicotinic acid and B_2. Several years earlier, Cherillard et al. (10) stated that the phosphorylation reaction of B_1 is disrupted by B_2 and B_6, asserting that a molar equivalent of B_6 completely disrupts the esterification of B_1 while in the presence of a large amount of B_1, the phosphorylation of B_6 is disturbed. Moreover, Richards (11) reported that young white rats developed vitamin B_6 deficiency symptoms upon massive administration of vitamin B_1, and other vitamin deficiencies subsequently. These experimental observations suggest the complex competitive phenomena among B_1, B_2, and B_6 in relation to their phosphorylation reaction. In Murata's experiment in which B_1 was given to laboratory animals at various doses, no change in organ B_2 level due to the B_1 was noted. Although these studies are the major research efforts concerning the metabolism of massive B_1 intake, the results do not seem to provide any direct clue to the interpretation of our experimental results.

Mibu of this faculty (12) previously held that the composition of amino acids or proteins in the diet exerts a significant effect on the biosynthesis of bound LiA. Thence, we assumed that the massive dose of B_1 may influence the utilization of the proteins in the diet, and the biosynthesis of bound LiA, subsequently. However, the nitrogen level in the liver Mit in this experiment indicated no appreciable difference among the groups, failing to provide any substantial ground for

the above assumption. When the dose was increased to 50 mg/kg, the effect of B_1 differred from that of TTFD. There is no simple explanation to this phenomenon. TTFD can be absorbed through the intestinal tube more readily than B_1-HCl. Therefore, its concentration gradient at the time of internal uptake should naturally be higher than that of B_1-HCl. Thus, it is yet to be investigated whether the successive accumulation of such differences resulted in the kind of outcome the author obtained from the present investigation, whether there is an optimum range of dose for B_1 with regard to its effect on the body weight and biosynthesis of bound LiA, or whether the structural difference between B_1 and TTFD produced the difference in pharmacologic effect as a result of the massive dose.

The adverse effect of massive B_6 administration on the growth and biosynthesis of bound LiA has already been discussed. Which of the metabolic systems is subject to theprimary change is yet to be clarified. Yamada (13) intravenously injected B_1, B_6, and their derivatives at 50 mg, and observed the depression of the blood sugar level and the elevation of the blood insulin activity level by TTFD in 2 hours, and the elevation of the blood insulin activity level by B_6 and PAL-P. He also found that intraperitoneal injections of TTFD, B_6, and PAL-P to hypophysectomized and adrenalectomized white rats caused a drop in blood sugar. Moreover, he administered B_6 and TTFD to humans by the intravenous route at 100 mg, and observed an increased excretion of 17-OHCS into the urine 1 hour later. These results suggest that massive administration of B_6 influences various endocrine systems such as the adrenals and pancreas, and these general effects produced secondary changes such as a change in body weight and activation of LiA, but a closer study is yet to be made.

LiA-^{35}S was injected to white rats which had received massive doses of B_6 for the preceding 2 weeks, and the formation of bound LiA <u>in vivo</u> was investigated. As illustrated in Table 3 and Figure 4, the values were significantly lower than the control level. As has been maintained by Cherillard et al., the internal phosphorylation of B_6 may be competing with the reaction depending upon ATP which is also required for the biosynthesis of bound LiA. Examining the results of this study, it is found that such an effect is temporary, and the prolongation of the administration of B_6 is of no significance.

In a series of 3 papers, the author has discussed the correlation between various water soluble vitamins and the formation and maintenance of bound LiA. The results of the experiment varied according to the type and dose of the vitamins and were highly characteristic. Some of the results are yet to be analyzed and explained, but since these experimental facts are immediately related to our clinical practice, they should be used as the foundation for the elucidation of their action mechanism and proper clinical application.

CONCLUSION

B_1-HCl, TTFD, B_2 and B_6 were orally administered to young white rats at massive doses and the following results were obtained.

1. The administration of B_1-HCl stimulated the biosynthesis of protein bound LiA regardless of its dose.

2. TTFD at 1 and 10 mg/kg per body weight produced the same effect as B_1-HCl, but at 50 mg/kg, the biosynthesis of bound LiA was reduced.

Massive dose of B_2 exerted no effect on the biosynthesis of protein bound LiA.

4. Massive administration of B_6 temporarily depressed the biosynthetic activity of protein bound LiA.

The summary of this paper was presented at the 16th meeting of the Society of Vitaminology, and the 153rd meeting of the Committee for the Study of Vitamin B.

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蛋白結合型リポ酸の生成におよぼすB群ビタミンの影響

(III) ビタミンB_1, B_2, B_6大量投与時における蛋白結合型リポ酸の生成

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加　藤　英　彦

THE EFFECT OF B VITAMINS ON THE BIOSYNTHESIS OF PROTEIN-BOUND LIPOIC ACID

(III) INFLUENCE OF THE OVER-DOSE OF B VITAMINS ON THE BIOSYNTHESIS OF PROTEIN-BOUND LIPOIC ACID

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Influences of daily administration of a large amount of thiamine, thiamine tetrahydrofurfuryl disulfide (TTFD), riboflavin and pyridoxine to rats for a month were investigated by observations on the growth of rats, the biosynthesis of protein-bound lipoic acid in liver mitochondria and the activity of glutamic oxalacetic transaminase in liver homogenate. Over-dose of thiamine (1,10 and 50mg/kg) and TTFD (1 and 10mg/kg) induced an increased biosynthetic activity of protein-bound lipoic acid, but over-dose of TTFD (50mg/kg) and pyridoxine induced a decreased biosynthetic activity. Over-dose of riboflavin did not effect the biosynthetic activity of protein-bound lipoic acid. The activity of glutamic oxalacetic transaminase was not affected in those states.

近年ビタミン剤，とくにいわゆる活性型ビタミン剤の普及につれてビタミンの大量投与にかんする問題がとりあげられるようになつたが，このばあいに他種のビタミン代謝にたいしてなんらかの影響をおよぼす可能性は当然注目されるところである．すなわち，すでに1940年に Sydenstricker ら[1] はペラグラの患者にニコチン酸の大量投与を行なつたさい，B_1, B_2 欠乏症候がかえつて増悪することを認めたのをはじめとして，Leitner ら[2] も同様のことを指摘している．わが国においても井上ら[3] はB_1の誘導体である TPD*1 の大量を患者に連用したばあいにB_2欠乏症候のおこることを認めているが，宮川ら[4,5] はB_1大量投与により臓器内のB_2量に著変はみられず大量投与による障害はなかつたと報告し一定した結論は得られていない．前2報で著者はB_1, B_2, B_6の欠乏が蛋白結合型 LiA*2 の合成能におよぼす影響について報告したが，今回はB_1, B_2, B_6の大量投与により LiA 代謝ことに結合型 LiA の合成能が影響を受けるものであるか否かについて検討したのでその結果を報告する．

実　験　方　法

(1) 実験動物および飼料

実験動物としては生後40日目，体重50g前後の Wistar 系雄シロネズミを用いた．まず標準固型飼料で4～6日間飼育したのち9群にわけそれぞれ標準固型飼料に体重1kgあたりB_1-HCl を1mg (II群)，10mg (III群)，50mg (IV群)，TTFD*3 を1mg (V群)，10mg (VI群)，50mg (VII群)，B_2を1mg (VIII群)，B_6を1mg ((IX群) を毎日添加し一定期間飼育後に断頭出血死せしめ実験に供した．なお標準固型飼料の含量(mg%)は$B_1$0.2, $B_2$0.2, $B_6$0.2であつた．飼料は1日1匹あたり10～20gを自由に摂取させた．

(2) 蛋白結合型 LiA の合成能の測定

前報と同様に能勢[6] の方法に従つて測定した．なお肝 Mit*4 窒素量は Micro-Kjeldahl 法によつて測定した．

*1) TPD = Thiamine propyldisulfide　　*2) LiA = Lipoic acid
*3) TTFD = Thiamine tetrahydrofurfuryldisulfide　　*4) Mit = Mitochondria

（3） 肝 GOT 活性の測定

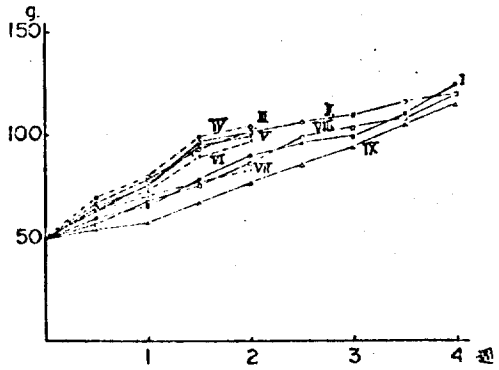
Reitman・Frankel 法[7] に準じて施行した．すなわち 0.1*M*, pH7.4 のリン酸緩衝液で肝の1000および500倍磨砕液をつくり 37°C, 60分間反応させたのち生成したオキザロ酢酸の量をもつてその活性の指標とした．

実　験　成　績

（1） 発育状態および体重

図1に示すように対照群（I 群）では体重はほぼ直線的に増加し，B_2大量投与群（VIII）もほぼこれと平行していた．一方，B_1-HCl 大量投与群（II, III, IV）では飼育2週目までは I 群より発育は良好で，TTFD を投与した V, VI 群も発育がよく B_1-HCl 投与群とほぼ同等の結果が得られた．しかし 50mg/kg というきわめて大量に投与した VII 群では発育はかえつて抑制される傾向が認められた．B_6大量投与群（IX）ではB_1大量投与群とは異なつた態度をしめし，体重増加は一時的に抑制された．しかし大量投与の体重におよぼす影響はさらに投与を続け観察した群（I, II, VIII, IX）ではしだいに軽度となり，4週目にはそれぞれ対照と同等の体重になつた．

図1　体重増加曲線



(2)　蛋白結合型 LiA の生成

表1　蛋白結合型 LiA 合成能（*cpm*/mg N）

投与量(mg/kg) ＼ 飼育期間(週)		1	2	3	5
対　照		3914±175*	3825±245	4012±213	3985±303
B_1-HCl	1	5137±109	5362±213	4308±381	4125±363
B_1-HCl	10	—	5500±445	—	—
B_1-HCl	50	—	5423±303	—	—
TTFD	1	—	5575±496	—	—
TTFD	10	—	5300±314	—	—
TTFD	50	—	3625±514	—	—
B_2	1	4057±237	3874±219	4123±251	4087±306
B_6	1	3908±285	2687±212	3578±262	3811±251

* 標　準　偏　差

図2　蛋白結合型 LiA 合成能

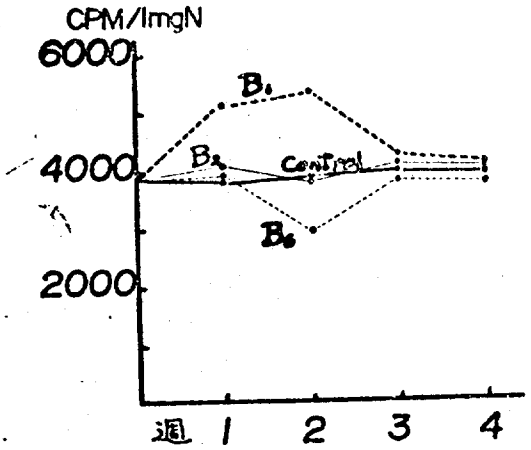
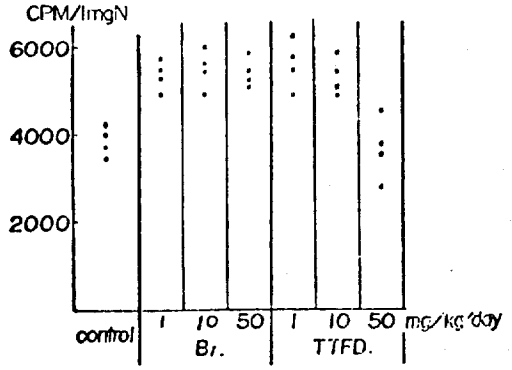


図3　蛋白結合型 LiA の合成能



飼育2週後の肝 Mit における蛋白結合型 LiA の合成能をみると表1, 図2, 3に示すように B_1-HCl 大量

投与群(II, III, IV)およびTTFD大量投与群(V, VI)がもつとも高く，B_2大量投与群(VII)は対照と差がなかつた。TTFD 50mg/kg投与群(VIII)では対照よりやや低い結果を得た。B_6投与群(IX)では合成能は著明に低下していた。しかしながらB_1, B_2, B_6を1mg/kg投与した群(II, VII, IX)について，1, 2, 3, 4週と経過を追つてみると，いずれも4週後にはほとんど差が認められなくなつた。

(3) 肝GOT活性

各群について肝GOT活性を測定したが表2にみるように著明な差は認められなかつた。

(4) LiA-^{35}S投与後の肝Mit内結合型LiAの経時的変化

大量投与2週目のII, IXにLiA-^{35}Sを20mg/kg腹腔内に投与し肝Mit蛋白へのとりこみを経時的にみた結果表3, 図4に示すように30分後ではB_1群は対照とほぼひとしいとりこみをみたが，B_6群は対照に比して70%にすぎなかつた。しかしいずれも急速に減少し6時間後にはほとんど対照との差が認められなくなつた。

表2　ビタミン大量投与シロネズミの肝GOT活性(ピルビン酸μモル/mg/hr)

投与量(mg/kg) ＼ 飼育期間(週)		1	2	3	4
対　照		1.21	1.13	1.24	1.18
B_1-HCl	1	1.17	1.19	1.11	1.22
B_1-HCl	10	—	1.25	—	—
B_1-HCl	50	—	1.01	—	—
TTFD	1	—	1.18	—	—
TTFD	10	—	1.21	—	—
TTFD	50	—	1.06	—	—
B_2	1	1.18	1.23	1.22	1.10
B_6	1	1.23	1.14	1.17	1.14

表3　LiA-^{35}Sの肝Mit蛋白へのとりこみ(*in vivo*)

投与後時間(分)	対　照	B_1大量(II)	B_6大量(IV)	投与後時間(分)	対　照	B_1大量(II)	B_6大量(IV)
30	3156 2550	2992 2430	2240 1728	120	684 516	870 628	448 260
平　均	2853	2711	1984	平　均	600	749	354
60	2128 1582	2171 1737	1251 1053	360	306 124	471 231	157 99
平　均	1855	1954	1102	平　均	215	351	123

単位＝*cpm*/mg N

図4　LiA-^{35}Sの肝Mit蛋白への取りこみ(*in vivo*)

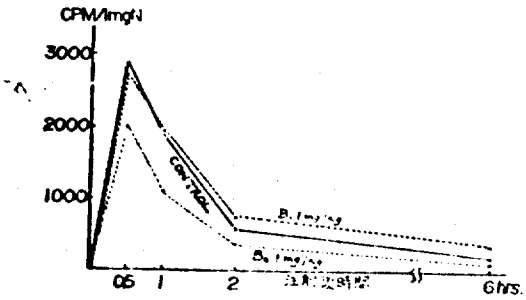


表4　蛋白結合型LiA合成能へのB1, CoC, B6, PAL-P添加の影響

添　加	*cpm*/mg N
—	3950
B_1	3797
CoC	3885
B_6	3416
PAL-P	4070

B_1, CoC　$2\times10^{-5}M$　　B_6, PAL-P　$4\times10^{-7}M$

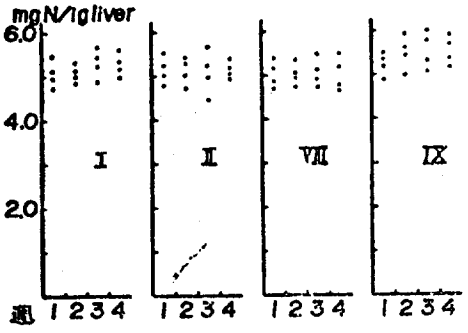
(5) 添加実験

対照群の肝 Mit に B_1, CoC*5), B_6, PAL-P*6) を *in vitro* で添加しその合成能にたいする影響をみたが表4に示すように，いずれも無添加のものと有意の差はなかつた．

(6) 肝 Mit 窒素量

図5にみるように各群とも有意の差は認められなかつた．

図5　肝 Mit-N 量



考　　察

最近のビタミン化学の進歩，とくにB群化合物の生理作用や補酵素作用にかんする研究の発展とあいまつて，いわゆる"活性型"，"補酵素型"または"体内維持型"，"吸収促進型"と称する種々の製剤をうみだし，しかも本来のビタミンとしての機能よりも薬理的効果を期待して大量の投与が各臨床分野で行なわれるようになつた．とくにB_1にかんしてわが国の生化学，薬学，臨床医学における研鑽はめざましくわれわれ小児科学の領域でも各種神経・筋疾患，栄養障害，リューマチ性疾患，自律神経不安定症候群などB_1あるいはその誘導体の大量投与の適応の範囲はいちじるしく拡大している．しかし一方ではわが国の研究者により，とくにB_1の大量投与時にB_6をはじめとする他のB群ビタミンの排泄増加や燐リン障害を来すとの主張もあり，このようなある特定のビタミンの大量投与により他のビタミンの利用障害その他生化学的副作用というべき現象がどの程度，またどのような機転でいかなる面におこつてくるのか，できるだけ早く充分検討することが必要である．

著者はこの線にそつて上述のような実験を行ない一定の成績を得た．すなわちB_1-HCl を幼シロネズミに1日体重1kgあたり1,10および50mg を4週間経口投与したところ1～2週間は1日 20～40μg のB_1を与えた対照に比べて体重増加，肝 Mit における蛋白結合型 LiA の生成ともに明らかに促進されたが3～4週目になると大量投与を続けているにもかかわらず体重，結合型 LiA 合成能ともに対照と同等となつた．TTFD を上記B_1-HCl と等モルになるように大量投与したばあい1mg，10mg/kg B_1-HCl 相当量の投与では B_1-HCl と同様に体重増加，結合型LiA合成能ともにあきらかに促進されたが50mg/kg を投与すると1～2週間で体重増加は対照よりわるくなり，かつ結合型 LiA 合成能は対照とほとんど変わらなかつた．他方，B_2の1日体重1kgあたり1mg の投与では4週間目まで体重曲線，結合型LiA合成能ともに影響がなく，B_6の同量投与では体重増加は対照よりわるく結合型LiAの生成は1～3週間で低下することが観察された．しかしB_6のこのような影響も連続投与しても4週目ではほとんど消失し，体重，結合型 LiA 合成能は対照と変わらない程度まで回復した．

以上の成績を総括的にながめると影響のまつたくみられなかつたB_2はべつとしてB_1またはB_6の大量投与はともに体重と結合型 LiA 合成能を平行して変化させたことが注目される．のみならずこれらの変化はB_1またはB_6をひき続いて投与してもまもなく消失していつたから B_1, B_6の大量投与により生体の発育，代謝系は体重増加にまで変化のおよぶかなり広範囲な影響をうけ二次的に結合型 LiA 合成能に促進または阻害の効果が現われたものと考えるべきであるが，その投与を続けていくうちに生体は影響された代謝の立て直しを行なうか，または大量の B_1, B_6を処理する機構たとえば適応酵素系の生成などによつてこれらの変化を消失せしめる可能性も推測することができる．なお結合型LiA の生成にあらわれた変化が B_1, CoC, B_6, PAL-P などの直接効果でないことは対照動物の肝Mitにこれらを直接添加してもなんらの影響をしめさなかつた事実より明らかである．

さてB_1大量投与によつて生体内でどのような代謝上の変化がおこつたかは詳細に検討されていないのでここでは具体的に議論することはできない．はじめに述べたようにB_1を大量に与えると数時間後にB_6の排泄増加の起こることが Klopp[8] により認められ，これと前

*5) CoC = Cocarboxylase　　*6) PAL-P = Pyridoxal phosphate

後して井上[3]は臨床的に TPD を使用した患者にB_2欠乏症状のあらわれることを，品川[9]はB_1, TAD*7)の大量投与により尿中B_2，ニコチン酸の著明な排泄増加のおこることを経験している．またこれより数年早くCherillardら[10]はB_1の附リン反応がB_2やB_6で阻害されることを述べ等量のB_6はB_1のエステル化を完全に阻害するとし一方，大量のB_1の存在下では逆にB_6の附リンが障害されることをみている．さらにRichards[11]は幼シロネズミにB_1の大量を投与するとB_6の欠乏症状が発現し，ついで他のビタミン不足がおこることを記載しており，これら諸家の成績は附リンの反応をめぐつてB_1, B_2, B_6のあいだに複雑な競合現象のあることを示唆しているものと考えられる．しかしB_1の投与量を種々変えて動物実験を行なつた村田らの実験ではB_1を大量摂取しても臓器内B_2量に大きな変化が認められていない．しかしいずれにせよこれらの諸説はB_1大量投与時の代謝にかんする研究の主要なものであるが著者が本報に述べた実験結果の解釈にたいする直接のてがかりを与えるものとは考えられない．

さきに教室の壬生[12]は食餌中のアミノ酸または蛋白の組成が結合型 LiA の生成に大きな影響を与えることを報告した．したがつてB_1の大量投与が食餌中の蛋白利用に影響し，このことを通じて結合型LiAの生成を左右する可能性を考えたが，著者の測定した肝 Mit 窒素量では各群間に特別の差はなく積極的な根拠を見出すことはできなかつた．なお投与量を 50mg/kg としたばあいにB_1と TTFD とでは異なる効果を示したことも明らかな事実であるが，その説明は困難である．TTFDの腸管よりの吸収はB_1-HCl よりも良好であるから体内にとりいれられるときの濃度勾配は当然B_1-HCl より高いはずである．このような差があいついだばあい著者のような成績の差となつて現われるのか，あるいは体重，結合型 LiA 合成能におよぼす効果にたいしてB_1投与量に至適範囲があるのか，それともB_1, TTFDの構造上の違いが大量の投与によつて上述のような薬理効果に差をきたしたものかは今後にのこされた課題である．

最後にB_6大量投与時に体重増加，結合型 LiA 合成能のいずれにもよくない影響があつたことは上述のようであるが，このばあい生体内のいかなる代謝系に一次的に変化がおこるかという点はまだ詳細な報告がない．さきに山田[13]はB_1, B_6およびそれらの誘導体50mgを静注すると TTFD では2時間後に血中インシュリン活性の上昇，血糖値の低下を，B_6, PAL-P でも血中インシュリン活性の上昇をきたすことを認めており，また下垂体，副腎摘出シロネズミにたいしてもTTFD，B_6, PAL-Pの腹腔内注射時は血糖下降をきたすことを見出している．さらに山田はヒトに100mgの TTFD またはB_6を静注し1時間後に尿中に 17-OHCS の排泄量がふえることを報告している．このような成績からみるとB_6大量投与は副腎，膵その他の種々の内分泌系にたいして影響を与えているようで，このような全身的影響が体重の面や LiA の活性化反応などにも二次的におよぶことは，いちおう考慮に値することであるが詳細は今後にのこされた問題である．

またB_6大量投与2週間目のシロネズミに LiA-^{35}S を注射し *in vivo* における結合型 LiA 生成をみた結果，対照より著明な低値をしめしたことは表 3, 図4 の通りである．このばあいさきに述べた Cherillard らの主張のように体内でのB_6のリン酸化が結合型 LiA 生成に必要な ATP 依存の反応と競合している可能性も考えられる．なお本報で述べたB_1, B_6投与の成績をみると，その効果はいずれにしても一時的であつて臨床応用に徒らな長期間の連用は無意味のばあいもあることを示唆するものであろう．

以上3報を通じて著者は各種水溶性ビタミンの存在と結合型 LiA の生成，維持との相互関係を検討したが対象となるビタミンの種類，投与量によつて種々異なつた，しかも特徴ある結果の得られることを知つた．このような結果のよつてくるところはまだ不明の点が多いが，その実験事実はわれわれの臨床にあたつていまだちにつながりをもつものであるから，これをもとにしてさらに詳細な機構の解明と臨床面への正しい適応に心がけなければならないと思う．

結　　論

幼シロネズミにB_1-HCl, TTFD, B_2, B_6の大量経口投与を行ないつぎの成績を得た．

1) B_1-HCl 投与時にはその量に関係なく蛋白結合型 LiA 合成能の増大を認めた．

2) TTFDのばあい体重あたり1および10mg/kg 投与ではB_1-HCl投与時と同じ傾度をしめしたが50mg/kg 投与では結合型 LiA 合成能は低下した．

3) B_2大量投与は蛋白結合型 LiA 合成能になんら

*7) TAD = Thiamine allyldisulfide

の影響をおよぼさなかつた.

4) B_6の大量投与は一時的に蛋白結合型 LiA 合成能が低下した.

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1. INTRODUCTION

With regard to the relationship between the vitamin B_2 complex and the metabolism of carbohydrates, Schröder and Stepp maintained that vitamin B_2 depressed the blood sugar level in diabetic patients and normal rabbits, and vitamin B_6 and nicotinic acid exhibited a similar effect. In their experiment using white rats, Suzuki et al. observed that vitamin B_2 and B_6 exerted preventive and therapeutic effects on the intoxication due to excessive intake of sugar. Oshima stated that vitamin B_2, nicotinic acid, and vitamin B_6 all depressed the blood sugar level in diabetic patients and, in rabbits, B_6 even brought about rapid recovery from hyperglycemia following an overloading of sugar.

The present experiment was designed to study the effects of individual vitamin B_2 complex factors, particularly vitamin B_2, nicotinic acid, and vitamin B_6 on the metabolism of carbohydrates by observing their individual and combined actions on the blood sugar level of rabbits.

2. EXPERIMENTAL PROCEDURE

Male rabbits weighing approximately 2 kg were used. They were raised on a mixed feed comprising bean curd refuse, vegetables and grass. Those with vigorous appetite and no sign of diarrhea were chosen for the experiment.

The vitamin B_2 complex factors used were all products of Sankyo Pharmaceutical Co. The sample of vitamin B_2 contained 0.5 mg of bioflavin (translator's note: should be riboflavin?) per 2 cc, the samples of nicotinamide, 20 and 50 mg per cc, the sample of sodium nicotinate, 50 mg per cc, and the sample of B_6, 20 mg per cc.

Experiments were carried out in the morning at approximately the same time. The animals were starved for about 20 hours before the experiment in order to avoid a change in blood sugar due to food intake. During the experiment, the rabbits were kept in a box-type fixing device in the natural position with their head fixed. Blood samples were drawn from one of the auricular veins, and the drug was injected into the auricular vein on the other side. Blood samples were drawn immediately before drug injection and during a period of 3 hours thereafter, more specifically, at 15 minutes intervals during the first 1 hour and at 30 minutes intervals during the subsequent 2 hours, a total of 9 times. No anticoagulant was added to the blood samples. The blood sugar level was determined by Hagedorn-Jensen's method.

3. RESULTS

A. CONTROL

The control animals were given (1) 0.5, (2) 1, (3) 2, (4) 3, and (5) 4 cc per kg of body weight of physiological solution. All experiments followed the conditions of the control experiment.

The results are shown in Table 1. The average values of these groups are compared in Table 2 and Figure 1. When the amount of physiological saline solution was small, the blood sugar level was elevated, and the level was depressed with larger amount of injection. When the amount of injection was below a specific level, the blood sugar indicated a downward trend immediately after injection, and the subsequent increase was also moderate. The blood sugar value in this paper is expressed in mg%, and the time, in minutes.

B. EXPERIMENTAL RESULTS

a. Injection of Individual Vitamin B_2 Complex Factors

(1) Injection of Vitamin B_2

The effect of B_2 was studied in (i) a group given 0.25 mg/kg of body weight (1 cc) and (2) a group given 0.5 mg per kg of b.w. (2 cc). The entire first group (5) showed a depression of blood sugar level after injection for approximately 3 hours. The latter consisted of 7 cases. In terms of the average value, the value somewhat rose for the first 45 minutes and dropped subsequently. The results are summarized in Table 3. In comparison of the curves, a notable rise was shown by the 1 cc control group, while the corresponding B_2 group indicated a downward trend. At 2 cc, the difference was small, but the pattern was similar (Table 4 and Figure 2).

(2) Injection of Nicotinamide and Sodium Nicotinate

Nicotinamide was injected to (i) a group of 3 rabbits at 20 mg per kg of body weight (1 cc) and (2) a group of 4 at 50 mg per kg of b.w. (1 cc). The first group showed fluctuations within 6% of the experimental value. The curve remained low. The second group showed greater fluctuations, with a slightly upward trend. Sodium nicotinate was injected to 7 rabbits at 50 mg per kg of body weight (1 cc). Following the injection, a notable increase was present (Table 5).

In comparison with the control group, the nicotinamide groups exhibited the inhibitory effect of the compound on the elevation of blood sugar level, the 0.25 mg group showing a stronger effect of the drug than the 0.5 mg group. The inhibitory effect of sodium nicotinate was weakest (Table 6, Figure 3).

(3) Injection of Vitamin B_6

The effect of B_6 was studied in (1) a group of 2 receiving B_6 at 10 mg (0.5 cc) per kg. b.w. and (2) a group of 4 receiving at 20 mg (1 cc) per kg.b.w. After injection, the first group showed a slight

drop and a subsequent rise, but from approximately 1 hour after injection, the value began to drop again. This pattern of change was shown clearly by one of the 2 rabbits and less notably by the other. The second group showed a notable rise immediately after injection, and a gradual decrease thereafter (Table 7)

In the comparison of the curves between the control and the experimental groups, the B_6 groups indicated an inhibitory effect of the agent on the elevation of blood sugar level in the latter part of the 3-hour period, and the 10 mg group indicated a stronger effect than the 20 mg group (Table 8, Figure 4).

B. Injection of Combinations of vitamin B_2 Complex Factors

B_2, nicotinamide, and B_6 were combined and simultaneously injected at different rates in order to investigate the interaction between the factors on the blood sugar level.

(1) B_2 + Nicotinamide

The effect of a combined injection at 0.25 mg/cc/kg b.w. of B_2 and 50 mg/cc of nicotinamide was studied in 4 rabbits. Following the injection, 2 out of 4 indicated a notable rise, the other 2 showed a moderate increase. The curve of average values shows peaks at 46 and 60 minutes after injection, and maintains the same level thereafter (Tables 9, 10, 11, 14, and Figures 5, 6, and 9).

(2) B_2 + B_6

Five rabbits were given 0.25 cc of B_2 and 20 mg/cc of B_6 at the same time. Following the injection, the blood sugar level indicated a slight depression for the first 45 minutes, and a gradual rise thereafter (Tables 9, 10, 12, 14; Figures 5, 7, 9).

(3) Nicotinamide + Vitamin B_6

Three rabbits received a combined injection of nicotinamide at 50 mg/cc and B_6 at 20 mg/cc. After injection, the blood sugar level rose to a peak 60 minutes later and dropped subsequently to almost the preexperimental value (Tables 9, 10, 13, 14; Figures 5, 8, 9).

(4) B_2 + Nicotinamide + B_6

Three rabbits received a combined injection of B_2 at 0.25 mg/cc, nicotinamide at 50 mg/cc, and B_6 at 20 mg/cc per kg of body weight. Following the injection, 1 out of the 3 showed a marked rise, and the remaining 2, a gradual rise. All three indicated a notable elevation in value as of 3 hours after the injection (Table 9). In order to compare with the control group, graphs were prepared showing the average values. As noted in Table 15 and Figure 10, the 2 groups indicated entirely different patterns. The curve of the 3-factor group is compared with the curves of the 2-factor, combined injection groups in Figure 5. As noted in Table 10 and Figure 5, the former showed a similar pattern of change with 2 of the 2-factor groups, excluding the B_2 + B_6 group during the 1st half of the 3-hour period, but it showed a significant rise during the latter half.

4. SUMMARY

The effects of B_2, nicotinamide, sodium nicotinate, and B_6, injected intravenously to normal rabbits at starvation, on the blood sugar level were studied for a period of 3 hours.

When each factor was individually injected, it appeared that no significant effect in absolute value was produced, but in comparison with the effect of the physiological saline solution given to the control, all factors depressed the blood sugar level, exhibiting an inhibitory effect on the elevation of blood sugar. The effect of B_2 was most notable, followed in rank by nicotinamide, sodium nicotinate, and B_6 in that order. Tn terms of the amount of injection, the inhibitory effect was stronger at 0.25 mg/2 cc (per kg of body weight) rather than at 0.5 mg/cc of B_2, 20 mg/cc rather than 50 mg/cc of nicotinamide, and 10 mg rather than 20 mg of B_6. Thus, within this range of dose, the small dose administration produced more notable effect. A similar pattern of action was described by Yatani and Okyo with regard to the effect of B_2 on the blood sugar level in healthy rabbits. Bicknell and Prescott quoted the reports of various investigators to the effect that neither nicotinic acid nor nicotinamide exerts any significant effect on the blood sugar leve of healthy individuals or diabetic patients, but it seems that the effect of these compounds depends upon the amount administered.

When these compounds were combined, the blood sugar level was elevated, as is noted in the results of the B_2 + nicotinamide, B_2 + B_6, nicotinamide + B_6, and B_2 + B_6 + nicotinamide injections. The combination of the 3 B_2 complex factors exerted the strongest effect among the four combinations, followed in rank by the nicotinamide + B_6, B_2 + nicotinamide, and B_2 + B_6 combinations in that order.

InIn other words, the vitamin B_2 complex factors lower the blood sugar level when administered individually, but in combination, they elevated the value to a significant extent. The 3 factors indicated no potentiating action in reducing the blood sugar, but potentiated one another in their blood sugar increasing action.

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TABLES AND FIGURES

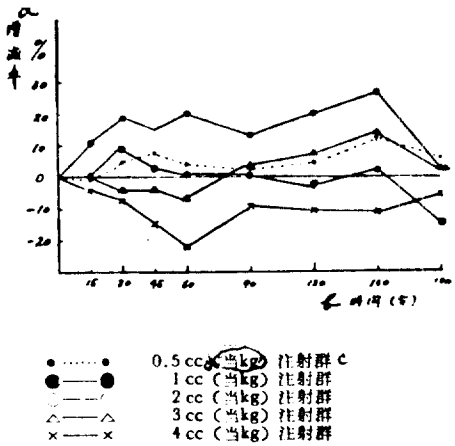


Figure 1. Graphic Presentation of the data in Table 2
KEY: a, increase/decrease ratio; b, time (min); c, group; d, per kg of body weight.

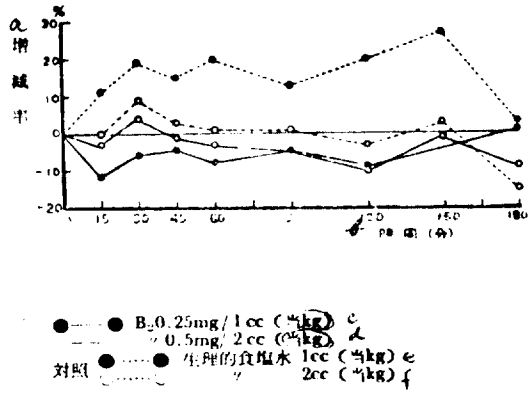


Figure 2. Graphic Presentation of the data in Table 4.
KEY: a, increase/decrease ratio; b, time (min); c,d, kg.b.w.; e, physiological saline 1 cc per kg of b.w.; f, control; physiological saline, 2 cc per kg of b.w.

TABLE 1. BLOOD SUGAR LEVELS (mg%) OF THE CONTROL GROUPS GIVEN PHYSIOLOGICAL SALINE SOLUTION.

a 番号	b 体重 kg	c 血糖値 d 注射前	e 注射後 (分) 15	30	45	60	90	120	150	180
(1) 0.5cc (〃kg) 注射群 0.5 cc per kg of b.w. group										
1	2.300	117	117	117	131	124	119	124	124	132
2	〃	119	104	124	124	124	124	124	146	119
3	2.500	113	125	125	119	115	113	115	122	120
f 平均値		116	115	122	125	121	119	121	131	124
g 増減率%			-0.9	4.6	7.1	4.0	2.0	4.0	12.3	6.4
(2) 1cc (〃kg) 注射群 1 cc group										
1	1.950	102	110	95	111	90	31	108	102	90
2	3.000	95	106	113	84	84	110	115	115	117
3	2.650	83	113	113	111	117	108	110	113	122
4	2.200	93	93	111	93	93	93	97	104	104
5	2.000	77	75	113	119	125	129	119	119	150
6	2.050	113	163	159	182	181	177	182	195	193
7	2.900	99	117	97	104	99	81	124	110	115
8	2.000	108	117	111	108	97	95	106	90	88
9	2.300	97	99	119	79	119	122	119	122	99
10	2.400	110	79	99	120	101	83	63	135	77
11	1.800	117	129	129	125	170	122	131	129	132
12	1.850	127	70	120	97	92	124	131	168	111
13	2.200	163	183	172	172	179	184	157	146	141
f 平均値		100	111	119	115	120	113	120	127	103
g 増減率%			11	19	15	20	13	20	27	3
(3) 2cc (〃kg) 注射群 2 cc group										
1	2,000	83	92	92	93	93	96	66	--	57
2	1,850	97	79	75	83	79	65	57	--	57
3	2,100	138	125	136	134	132	129	122	115	86
4	1,860	101	92	124	117	122	127	122	129	122
5	1,950	115	129	131	81	74	120	131	132	138
6	2,000	113	125	127	122	127	120	120	86	86
7	2,000	61	72	77	90	83	54	57	57	39
8	2,050	113	113	131	129	125	125	124	124	122
f 平均値		103	103	112	106	104	104	100	107	88
g 増減率%			0	8.7	2.9	1	1	-2.9	3.9	-14.6
(4) 3cc (〃kg) 注射群 3 cc group										
1	2,000	106	106	97	95	97	111	122	122	113
2	1,950	119	117	117	117	113	125	136	136	120
3	2,200	101	101	97	101	92	104	92	117	104
f 平均値		109	108	104	104	101	113	117	125	112
g 増減率%			-0.9	-4.6	-4.6	-7.3	3.7	7.3	14.7	2.8
(5) 4cc (〃kg) 注射群 4 cc group										
1	1,700	79	70	75	70	65	75	65	75	63
2	1,700	108	106	106	102	95	95	102	95	102
3	2,100	102	102	86	74	66	92	92	86	108
f 平均値		96	92	89	82	75	87	86	85	91
g 増減率%			4.2	-7.3	-14.6	-21.9	-9.5	-10.4	-11.4	-5.5

KEY: a, case No.; b, body weight (kg); c, blood sugar value; d, before injection; e, after injection (min); f, mean value; g, fluctuation rate (%)

TABLE 2. FLUCTUATION OF BLOOD SUGAR LEVEL DUE TO THE INJECTION OF PHYSIOLOGICAL SALINE SOLUTION (CONTROL GROUPS)

a 経過時間 (分) / b 各群増減率(%)	15	30	45	60	90	120	150	180
0.5cc(〃kg)注射群	− 0.9	4.6	7.1	4.0	2.0	4.0	12.3	6.4
1.0 〃 〃	11.0	19.0	15.0	20.0	13.0	20.0	27.0	3.0
2.0 〃 〃	0.	8.7	2.9	1.0	1.0	- 2.9	3.9	−14.6
3.0 〃 〃	− 0.9	- 4.6	− 4.6	− 7.3	3.7	7.3	14.7	2.8
4.0 〃 〃	− 4.2	− 7.2	−14.6	−21.9	− 9.5	−10.4	−11.4	− 5.5

KEY: a, increas

KEY: a, elapsed time (min); b, increase/decrease ratio (%); c, 0.5 cc (per kg of body weight) group

TABLE 3. BLOOD SUGAR LEVEL OF B_2 INJECTION GROUPS (mg%)

i) B_2 0.25 mg/cc (〃kg) 注射群 B_2 0.25 mg/cc(per kg) group

a 番号	b 体重 kg	c 血糖値 d 注射前	e 注射後 (分) 15	30	45	60	90	120	150	180
1	1,800	97	90	95	95	90	86	86	−	92
2	1,900	119	119	113	106	119	129	131	−	132
3	1,800	127	99	129	124	124	120	119	−	129
4	1,900	115	102	102	101	95	111	83	−	108
5	1,900	113	93	95	119	95	101	99	−	115
f 平均値		114	101	107	109	105	109	104	−	115
g 増減率%			−11.4	−6.1	−4.4	−7.9	−4.4	−8.8	−	0.9

ii) B_2 0.5 mg/2cc (〃kg) 注射群 B_2 0.5 mg/2 cc (per kg) group

1	2,000	120	120	125	122	124	132	136	129	125
2	2,000	108	108	110	108	92	75	86	86	70
3	2,000	113	113	124	113	113	115	95	175	134
4	2,000	125	125	127	124	115	104	93	122	84
5	2,150	83	70	86	75	90	83	75	95	81
6	2,200	68	57	66	66	66	65	52	47	47
7	1,800	95	102	101	101	90	104	108	102	108
f 平均値		102	99	106	101	99	97	92	101	93
g 増減率%			−2.9	3.9	−1.0	−2.9	- 4.9	−9.8	- 1.0	−8.8

KEY: a, case No.; b, body weight; c, blood sugar level; d, before injection; e, after injection; f, mean value; g, increase/decrease ratio (%).

TABLE 4. FLUCTUATIONS OF THE BLOOD SUGAR LEVEL DUE TO B_2 INJECTION

c 各群 / a 経過時間(分) 増減率(%)	15	30	45	60	90	120	150	180
B_2 0.25mg/cc(〃kg)注射群	−11.4	− 6.1	− 4.4	− 7.9	− 4.4	− 8.8	−	0.9
〃 0.5mg/2cc(〃kg) 〃	− 2.9	3.9	− 1.0	− 2.9	− 4.9	− 9.8	− 1.0	− 8.8

KEY: a, elapsed time (min); b, fluctuation (%); c, (per kg) group

TABLE 5. BLOOD SUGAR LEVELS OF THE GROUPS GIVEN NICOTINAMIDE AND SODIUM NICOTINATE INDIVIDUALLY (mg%)

a 番号	b 体重 kg	c 血糖値 d 注射前	e 注射後 (分) 15	30	45	60	90	120	150	180
i) ニコチン酸アミド 20mg/cc (当kg) Nicotinamide 20 mg/cc (kg)										
1	1,900	119	124	131	111	119	115	110	110	111
2	2,200	104	83	104	110	110	93	110	110	110
3	2.450	129	148	124	145	143	129	136	138	132
f 平均値		117	118	119	122	124	112	119	119	118
g 増減率%			0.9	1.7	4.3	6.0	−4.3	1.7	1.7	0.9
ii) ニコチン酸アミド 50mg/cc (当kg) Nicotinamide 50 mg/cc/kg										
1	2,100	106	93	111	106	99	99	102	111	106
2	1,700	110	113	122	119	95	72	129	129	132
3	2,100	74	113	110	110	139	134	143	152	154
4	1,850	115	95	119	110	101	125	117	122	120
f 平均値		112	104	116	111	109	108	123	120	128
g 増減率%			7.1	3.6	−0.9	−2.7	−3.6	10.7	7.1	14.2
iii) ニコチン酸ナトリウム 50mg/cc (当kg) Sodium nicotinate 50 mg/cc										
1	2,200	93	104	139	143	155	166	143	129	132
2	1,650	106	120	122	129	125	122	134	139	139
3	1,900	113	106	104	84	108	113	115	154	168
4	1,650	106	95	104	111	111	113	113	111	110
5	1,950	125	143	146	145	148	143	143	146	124
6	1,650	131	138	113	111	108	111	115	102	131
7	1,950	106	92	111	131	117	111	102	106	117
f 平均値		111	114	120	122	125	127	123	127	132
g 増減率%			2.7	8.2	10.0	12.6	14.4	10.8	14.4	18.9

KEY: a, case No.; b, body weight; c, blood sugar level; d, before injection; f, mean value; g, increase/decrease ratio (%)

TABLE 6. FLUCTUATIONS OF BLOOD SUGAR LEVEL OF THE GROUPS GIVEN NICOTINAMIDE AND SODIUM NICOTINATE

a 増減率(%) \ b 経過時間(分)	15	30	45	60	90	120	150	180
c ニコチン酸アミド 20mg/cc	0.9	1.7	4.3	6.0	−4.3	1.7	1.7	0.9
d 〃 50mg/cc	−7.1	3.6	−0.9	−2.7	−3.6	10.7	7.1	14.2
e ニコチン酸ナトリウム (〃)	2.7	8.2	10.0	12.6	14.4	10.8	14.4	18.9

KEY: a, increase/decrease ratio (%); elapsed time (min); c, nicotinamide - 20 mg/cc; d, nicotinamide - 50 mg/cc; e, sodium nicotinate-50 mg/cc

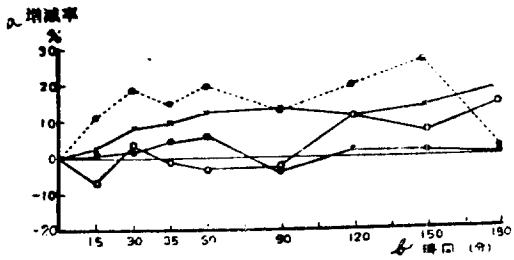
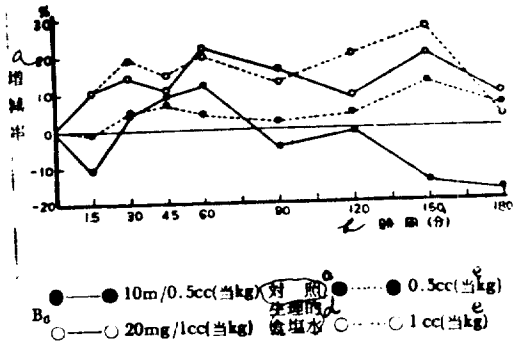


Figure 3. Graphic Presentation of the data in Table 6.

第3図 第6表の曲線とせるもの

●——● ニコチン酸アミド 20mg/cc (当kg) c
△——△ 〃 50mg/cc (当kg) d
×——× ニコチン酸ナトリウム 50mg/cc (当kg) e
●……● 対照 生理的食塩水 1 cc (当kg) f

KEY: a, increase/decrease ratio (%); b, time (min); c, nicotinamide-20 mg/cc(per kg); d, nicotinamide-50 mg/cc (per kg); e, sodium nicotinate 50 mg/cc (per kg); f, control, physiological solution, 1 cc per kg.



KEY: a, increase/decrease ratio; b, time (min); b, control; d, physiological saline; e, (per kg).

Figure 4. GRAPHIC PRESENTATION OF THE DATA IN TABLE 8.

TABLE 7. BLOOD SUGAR LEVEL (mg%) OF THE B_6 - 10 mg/0.5 cc/kg GROUP

i) B_6 10mg/0.5cc (当kg)

a 番号	b 体重 kg	c 血糖値 d 注射前	e 注射後 (分) 15	30	45	60	90	120	150	180
1	1,900	110	102	111	108	117	90	101	63	34
2	1,700	117	102	127	139	138	125	125	131	106
f 平均値		114	102	119	124	128	108	113	97	95
g 増減率%			−10.5	4.3	8.8	12.3	−5.3	−0.9	−14.9	−16.7

ii) B_6 20mg/cc (当kg)

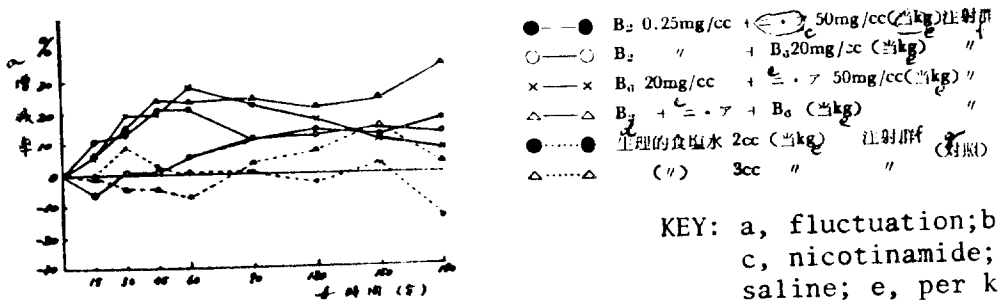
1	2,900	102	101	127	120	125	115	117	145	132
2	2,500	101	117	110	108	129	117	113	117	113
3	1,800	77	81	88	75	81	74	77	72	57
4	1,900	106	129	120	129	138	141	129	129	122
f 平均値		97	107	111	108	118	112	106	116	106
g 増減率%			10.3	14.4	11.3	21.6	15.5	9.3	20.0	9.3

KEY: a, case No.; b, body weight; c, blood sugar value; d, before injection (min); f, mean value; g, increase/decrease ratio (%)

TABLE 8. FLUCTUATIONS OF BLOOD SUGAR LEVEL OF THE B_6 GROUPS

a 各群増減率(%) \ b 経過時間(分)	15	30	45	60	90	120	150	180
B_6 0.5cc 10mg(c当kg)注射時 d	−10.5	4.3	8.8	12.3	−5.3	−0.9	−14.9	−16.7
B_6 1cc 20mg(c当kg) 〃 d	10.3	14.4	11.3	21.6	15.5	9.3	20.0	9.3

KEY: a, fluctuation (%); b, elapsed time (min); c, (per kg); d, group.



KEY: a, fluctuation;b, time (min); c, nicotinamide; d, phys. saline; e, per kg; f, group; g, control

Figure 5. Graphic Presentation of Table 10.

TABLE 9. BLOOD SUGAR LEVELS (mg%) OF THE GROUPS GIVEN COMBINED INJECTIONS OF VITAMIN B_2 COMPLEX FACTORS

(1) B_2 0.25mg/cc, ニコチン酸アミド 50mg/cc (当kg) nicotinamide per kg

a 番号	b 体重 kg	c 血糖値 d 注射前	e 注射後 (分) 15	30	45	60	90	120	150	180
1	1,750	122	138	122	122	117	101	122	120	122
2	1,700	113	131	138	136	134	155	152	154	159
3	2,200	125	150	161	197	195	184	154	148	148
4	1,750	111	106	110	111	122	83	113	106	120
f 平均値		118	131	133	142	142	131	135	132	137
g 増減率%			11.0	12.7	20.3	20.3	11.0	14.4	11.9	17.0

(2) B_2 0.25mg/cc, B_6 20mg/cc (当kg) per

1	2,300	120	101	99	99	122	136	136	138	131
2	1,900	96	104	111	108	111	120	104	113	108
3	2,000	117	106	117	117	120	124	127	134	134
4	1,800	99	82	119	119	104	111	104	101	101
5	1,850	110	111	99	104	113	110	127	132	138
f 平均値		108	101	109	109	114	120	121	124	122
g 増減率%			−6.5	0.9	0.9	5.9	11.1	12.0	14.8	13.0

(3) B_6 20mg/cc, ニコチン酸アミド 50mg/cc (当kg) nicotinamide per

1	1,900	95	92	104	101	104	115	115	122	120
2	1,750	99	102	104	93	115	106	102	97	93
3	1,900	104	117	146	159	159	141	132	111	104
f 平均値		99	104	118	118	126	121	116	110	106
g 増減率%			5.1	19.3	19.3	27.3	22.2	17.2	11.1	7.8

(4) B_2 0.25mg/cc, ニコチン酸アミド 50mg/cc, B_6 20mg/cc (当kg) nicotinamide per

1	1,950	111	127	152	157	164	143	154	146	170
2	1,750	119	111	119	129	119	124	125	141	146
3	1,770	97	110	104	119	119	111	117	117	131
f 平均値		109	116	125	135	134	136	132	135	147
g 増減率%			6.4	14.7	23.8	22.9	24.7	21.1	23.8	34.9

KEY: a, case No.; b, body weight; c, blood sugar level; d, before injection; e, after injection (min); f, mean value; g, fluctuation (%)

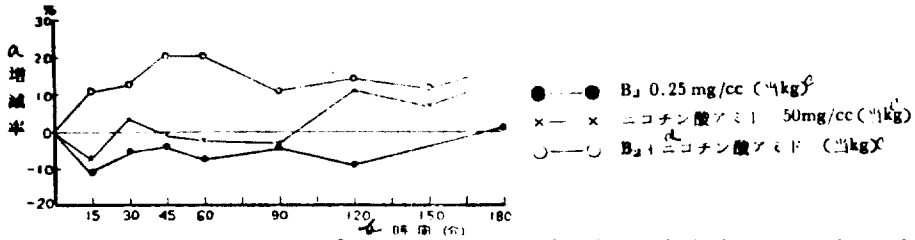
TABLE 10. FLUCTUATIONS OF BLOOD SUGAR LEVEL OF THE GROUPS GIVEN COMBINED INJECTION OF VITAMIN B_2 COMPLEX FACTORS

a 各群増減率(%) \ b 経過時間(分)	15	30	45	60	90	120	150	180
B_2 + ニコチン酸アミド c	11.0	12.7	20.3	20.3	11.0	14.4	11.9	17.0
B_2 + B_6	−6.5	0.9	0.9	5.9	11.1	12.0	14.8	13.0
+ ニコチン酸アミド B_6 d	5.1	19.3	19.3	27.3	22.2	17.2	11.1	7.8
B_2 + ニコチン酸アミド + B_6 e	6.4	14.7	23.8	22.9	24.7	21.1	23.8	34.9

KEY: a, fluctuation (%); b, elapsed time (min); c, B_2 + nicotinamide; d, + nicotinamide B_6; e, B_2 + nicotinamide + B_6.

TABLE 11. COMPARISON BETWEEN THE INJECTION OF B_2 + NICOTINAMIDE AND SEPARATE INJECTIONS OF B_2 AND NICOTINAMIDE

a 各群増減率(%) / b 経過時間(分)	15	30	45	60	90	120	150	180
B_2	−11.4	− 6.1	− 4.4	− 7.9	− 4.4	− 8.8	−	0.9
ニコチン酸アミド c	− 7.1	3.6	− 0.9	− 2.7	− 3.6	10.7	7.1	14.2
B_2 + ニコチン酸アミド d	11.0	12.7	20.3	20.3	11.0	14.4	11.9	17.0



KEY: a, fluctuation (%); b, elapsed time (min); c, nicotinamide; d, B_2 + nicotinamide

Figure 6. GRAPHIC PRESENTATION OF TABLE 11.
KEY: a, fluctuation rate; b, time (min); c, per kg); d, nicotinamide

TABLE 12. COMPARISON OF THE COMBINED INJECTION OF B_2 AND B_6, AND INDIVIDUAL INJECTIONS OF B_2 AND B_6.

a 各群増減率(%) / b 経過時間(分)	15	30	45	60	90	120	150	180
B_2	−11.4	− 6.1	− 4.4	− 7.9	− 4.4	− 8.8	−	0.9
B_6	10.3	14.4	11.3	21.6	15.5	9.3	20.0	9.3
B_2 + B_6	− 6.5	0.9	0.9	5.9	11.1	12.0	14.8	13.0

KEY: a, fluctuation rate (%); b, elapsed time (min)

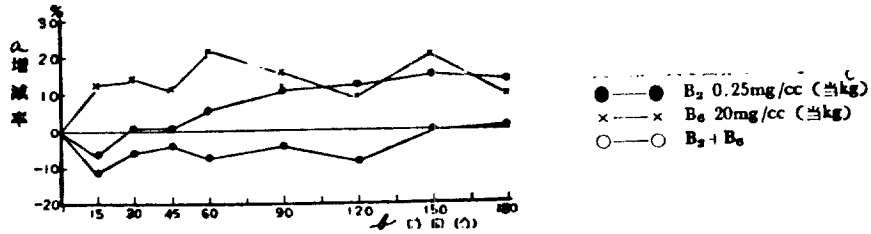


Figure 7. GRAPHIC PRESENTATION OF TABLE 12.
KEY: a, fluctuation rate; b, time (min); c,(per kg).

TABLE 13. COMPARISON BETWEN THE COMBINED INJECTION OF NICOTINAMIDE AND B_6, AND SEPARATE INJECTIONS OF B_6 AND NICOTINAMIDE.

a 各群増減率(%) / b 経過時間(分)	15	30	45	60	90	120	150	180
ニコチン酸アミド c	− 7.1	3.6	− 0.9	− 2.7	− 3.6	10.7	7.1	14.2
B_6	10.3	14.4	11.3	21.6	25.5	9.3	20.0	9.3
ニコチン酸アミド + B_6 d	5.1	19.3	19.3	27.3	22.2	17.2	11.1	7.8

KEY: a, fluctuation rate (%); elapsed time (min); c, nicotinamide; d, nicotinamide + B_6

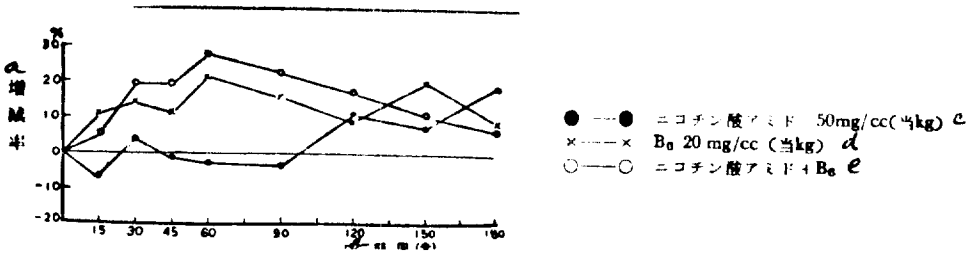


Figure 8. GRAPHIC PRESENTATION OF TABLE 13
(KEY: a, fluctuation rate; b, time in min; c, nicotinamide-50 mg/cc/kg; d, B_6-20 mg/cc/kg; e, nicotinamide + B_6.)

TABLE 14. COMPARISON OF COMBINED INJECTIONS OF MORE THAN 2 FACTORS

a 各群増減率(%) / b 経過時間(分)	15	30	45	60	90	120	150	180
B_2+ニコチン酸アミド c	11.0	12.7	20.3	20.3	11.0	14.4	11.9	17.0
B_2+B_6	− 6.5	0.9	0.9	5.9	11.1	12.0	14.8	13.0
ニコチン酸アミド+B_6 d	5.1	19.3	19.3	27.3	22.2	17.2	11.1	7.8

KEY: a, fluctuation; b, elapsed time (min); c, B_2 + nicotinamide; d, nicotinamide + B_6.

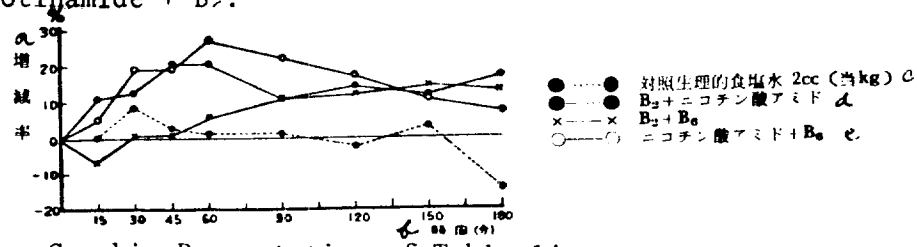


Figure 9. Graphic Presentation of Table 14.
(KEY: a, fluctuation; b, time in min; c, physiological saline at 2 cc per kg; d, B_2 + nicotinamide; e, nicotinamide + B_6)

TABLE 15. B_2 + NICOTINAMIDE + B_6 GROUP (FLUCTUATION RATE)

a 各群増減率(%) / b 経過時間(分)	15	30	45	60	90	120	150	180
c B_2+ニコチン酸アミド+B_6	6.4	14.7	23.8	22.9	24.7	21.1	23.8	34.9
d 対照 3cc (当kg)	− 0.9	− 4.6	− 4.6	− 7.3	3.7	7.3	14.7	2.8

KEY: a, fluctuation (%); b, elapsed time (min); c, B_2 + nicotinamide + B_6; d, control (3 cc per kg)

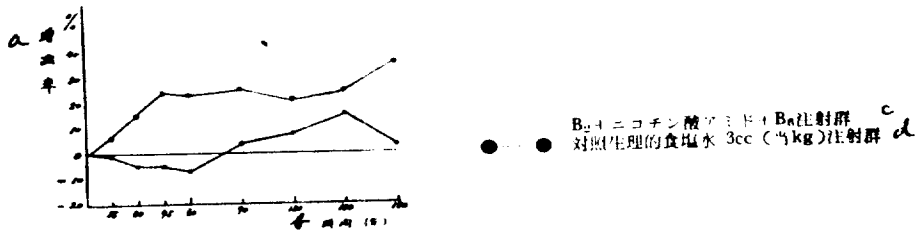


Figure 10. Graphic Presentation of Table 15.
(KEY: a, fluctuation rate; b, time in min; c, B_2 + nicotinamide + B_6; d, control - physiological saline at 3 cc per kg)

第1表 生理的食塩水注射群（対照群）血糖（mg%）

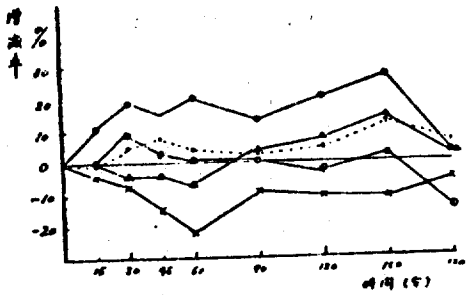
(1) 0.5cc（当kg）注射群

番号	体重 kg	血糖値 注射前	注射後（分） 15	30	45	60	90	120	150	180
1	2.300	117	117	117	131	124	119	124	124	132
2	〃	119	104	124	124	124	124	124	146	119
3	2.500	113	125	125	119	115	113	115	122	120
平均値		116	115	122	125	121	119	121	131	124
増減率%			−0.9	4.6	7.1	4.0	2.0	4.0	12.3	6.4
(2) 1cc（当kg）注射群										
1	1.950	102	110	95	111	90	31	108	102	90
2	3.000	95	106	113	84	84	110	115	115	117
3	2.650	83	113	113	111	117	108	110	113	122
4	2.200	93	93	111	93	93	93	97	104	104
5	2.000	77	75	113	119	125	129	119	119	150
6	2.050	113	163	159	182	181	177	182	135	193
7	2.900	99	117	97	104	99	81	124	110	115
8	2.000	108	117	111	108	97	95	106	90	88
9	2.300	97	99	119	79	119	122	119	122	99
10	2.400	110	79	99	120	101	83	63	135	77
11	1.800	117	129	129	125	170	122	131	129	132
12	1.850	127	70	120	97	92	124	131	168	111
13	2.200	163	183	172	172	179	184	157	146	141
平均値		100	111	119	115	120	113	120	127	103
増減率%			11	19	15	20	13	20	27	3
(3) 2cc（当kg）注射群										
1	2,000	83	92	92	93	93	96	66	—	57
2	1,850	97	79	75	83	79	65	57	—	57
3	2,100	138	125	136	134	132	129	122	115	86
4	1,860	101	92	124	117	122	127	122	129	122
5	1,950	115	129	131	81	74	120	131	132	138
6	2,000	113	125	127	122	127	120	120	86	86
7	2,000	61	72	77	90	83	54	57	57	39
8	2,050	113	113	131	129	125	125	124	124	122
平均値		103	103	112	106	104	104	100	107	88
増減率%			0	8.7	2.9	1	1	−2.9	3.9	−14.6
(4) 3cc（当kg）注射群										
1	2,000	106	106	97	95	97	111	122	122	113
2	1,950	119	117	117	117	113	125	136	136	120
3	2,200	101	101	97	101	92	104	92	117	104
平均値		109	108	104	104	101	113	117	125	112

増減率%			−0.9	−4.6	−4.6	−7.3	3.7	7.3	14.7	2.8
(5) 4cc（当kg）注射群										
1	1,700	79	70	75	70	65	75	65	75	63
2	1,700	108	106	106	102	95	95	102	95	102
3	2,100	102	102	86	74	66	92	92	86	108
平均値		96	92	89	82	75	87	86	85	91
増減率%			−4.2	−7.3	−14.6	−21.9	−9.5	−10.4	−11.4	−5.5

第2表　生理的食塩水注射による血糖増減表（対照群）

経過時間（分）／各群増減率(%)	15	30	45	60	90	120	150	180
0.5cc(当kg)注射群	− 0.9	4.6	7.1	4.0	2.0	4.0	12.3	6.4
1.0 〃 〃	11.0	19.0	15.0	20.0	13.0	20.0	27.0	3.0
2.0 〃 〃	0.	8.7	2.9	1 0	1.0	− 2.9	3.9	−14.6
3.0 〃 〃	− 0.9	− 4.6	− 4.6	− 7.3	3.7	7.3	14.7	2.8
4.0 〃 〃	− 4.2	− 7.2	−14.6	−21.9	− 9.5	−10.4	−11.4	− 5.5



第1図　第2表を曲線にて示せるもの

●……●　0.5cc（当kg）注射群
●——●　1cc（当kg）注射群
○——○　2cc（当kg）注射群
△——△　3cc（当kg）注射群
×——×　4cc（当kg）注射群

固定した。採血は，耳翼静脈により行い，薬液注射は，他側同名静脈へ行つた。採血は，薬液注射直前及び其後3時間に亘り，最初の1時間は，15分毎に，其後の2時間は，30分毎に施行，即，前後9回採血した。血液には，凝血阻止剤を混じなかつた。血糖測定法は，Hagedorn-Jensen 氏法に準拠した。

3. 結　　果

イ. 対　照

生理的食塩水を用い，注射量は，(1) 体重当 kg 0.5cc，(2) 1cc，(3) 2cc，(4) 3cc及び(5) 4cc宛の5群に分け，実験上の諸条件は，凡て本実験と同様にした。

各群の成績は第1表の如くである。平均値を以て，各群を比較すれば，第2表及び第1図の如くである。即，生理的食塩水注射容量が，少量の場合は血糖上昇し，注射液量増加に従つて，血糖低下の傾向を見た。而して，その少量注射も一定限度を超えれば，注射直後は，むしろ，低下の傾向を，その後の上昇程度も，著明に低く経過することを見た。本論文中の血糖値は mg % を以て表し，経過時間は分を以て表わす。

ロ. 実験成績

甲，V.B_2 複合体各因子単独注射の場合

(1) B_2 注射の場合

B_2 は，i) 家兎体重当量 0.25 mg（1cc）注射群及び ii) 0.5 mg（2cc）注射群の2群に就いて，観察した。前者にて，全例（5例），注射後，血糖の降下を見，これはほぼ3時間に亘る。後者は7例に就いて見た。平均値を以て見れば，注射後45分迄は，軽度の上昇を，その後は低下を見た。第3表の如くである。対照曲線と比較すれば，1cc注射群に於て，対照の著明な上昇曲線に対し B_2 群による曲線は，明かな下降曲線を示し，2cc注

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Kansai Ika-Daigaku Zasshi 8:7-17. 1956.

原　　著

V. B_2 複合体各因子の含水炭素代謝に及ぼす影響に就いて

第1編　正常家兎血糖に対する影響

関西医科大学　第二内科教室（指導　前田東作教授）

川那部喜美子

Studies on Influence of Agents, Contained in V.B_2-Complex, upon the Metabolism of Carbohydrate

Report 1. Influences upon the Blood Sugar Content of Normalrabbit in Fasting

Kimiko Kawanabe

Department of II. Internal Medicine, Kansai Medical School (Supervised by T. Maeda MD)

Observation is made on influences of Vitamine B_2, Nicotinamide, Sodium nicotinate and Vitamine B_6 injected intravenous upon the blood sugar content of fasting rabbits. The results obtained as follows:

(1) The content decreased or at least had an increasing trend inhibited when each agent was injected alone.

(2) The content increased, when two or three of these agents were injected together.

1. 緒　　言

V.B_2 複合体と含水炭素代謝の関係に就いては，Schröder, Stepp は V.B_2 が糖尿病患者及び正常家兎の血糖を降下せしめ，V.B_6，ニコチン酸等に就いても，ほぼ同様の作用のあることを暗示し，鈴木等は，白鼠を用いて，V.B_2，V.B_6 等が砂糖過剰投与による中毒の予防並びに治療に有効なるを報告し，大島は，V.B_2，ニコチン酸及び V.B_6 等，いづれも糖尿病患者の血糖を降下せしめ，一方，V.B_6 は，家兎の糖負荷後の過血糖をも速に恢復せしめ得ると報告している。

余は，V.B_2 複合体因子，就中，V.B_2，ニコチン酸及び V.B_6 等，各因子個々の，家兎血糖に対する作用を観察し，更に，その作用に関して，各因子相互の，相関々係を検索し，以て，これら因子の含水炭素代謝に及ぼす影響の一端を知らんとする目的を以て，本研究に着手した。

2. 実 験 方 法

実験動物，体重は 2kg 前後の雄性家兎。一定の場所にて，玉蜀黍，野菜及び青草等の混合飼料を以て，数日間飼養し，食慾旺盛，且つ下痢のないものを用いた。

V.B_2 複合体因子に関しては，いづれも三共製薬会社製品を使用し，V.B_2 はビオフラビン（以下 B_2 と記載）の 2cc 中 0.5 mg 含有のものを，ニコチン酸アミド（以下ニ・アと記載）は 1cc 中 20mg 及び 50mg 含有の 2 種を，ニコチン酸ナトリウム（以下ニ・ナと記載）は 1cc 中 50mg 含有のものを，V.B_6（以下 B_6 と記載）は 1cc 中 20mg 含有のものを使用した。

実験は，午前中，ほぼ一定の時刻に行い，動物は，実験前，約20時間の絶食により，食物摂食による血糖変化を，可及的に避けた。実験時には，家兎は，箱型固定器中に，自然位に置き，頭部を

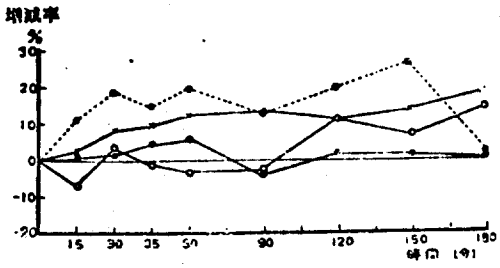
対照に比較し，観察すれば，ニ・ア注射群は，いづれも，血糖上昇に対し，抑制作用を示し，その程度は，0.25 mg 群に於て，0.5 mg 群より明かである。ニ・ナ注射群に於ては，この抑制作用は最も弱い（第6表，第3図）。

第5表　ニコチン酸アミド及びナトリウム単独注射群血糖（mg%）

番号	体重 kg	血糖値 注射前	注射後（分） 15	30	45	60	90	120	150	180
i) ニコチン酸アミド 20mg/cc（当kg）										
1	1,900	119	124	131	111	119	115	110	110	111
2	2,200	104	83	104	110	110	93	110	110	110
3	2.450	129	148	124	145	143	129	136	138	132
平均値		117	118	119	122	124	112	119	119	118
増減率%			0.9	1.7	4.3	6.0	−4.3	1.7	1.7	0.9
ii) ニコチン酸アミド 50mg/cc（当kg）										
1	2,100	106	93	111	106	99	99	102	111	106
2	1,700	110	113	122	119	95	72	129	129	132
3	2,100	74	113	110	110	139	134	143	152	154
4	1,850	115	95	119	110	101	125	117	122	120
平均値		112	104	116	111	109	108	123	120	128
増減率%			−7.1	3.6	−0.9	−2.7	−3.6	10.7	7.1	14.2
iii) ニコチン酸ナトリウム 50mg/cc（当kg）										
1	2,200	93	104	139	143	155	166	143	129	132
2	1,650	106	120	122	129	125	122	134	139	139
3	1,900	113	106	104	84	108	113	115	154	168
4	1,650	106	95	104	111	111	113	113	111	110
5	1,950	125	143	146	145	148	143	143	146	124
6	1,650	131	138	113	111	108	111	115	102	131
7	1,950	106	92	111	131	117	111	102	106	117
平均値		111	114	120	122	125	127	123	127	132
増減率%			2.7	8.2	10.0	12.6	14.4	10.8	14.4	18.9

第6表　ニコチン酸アミド及びナトリウム注射群増減率

増減率(%) ＼ 経過時間(分)	15	30	45	60	90	120	150	180
ニコチン酸アミド 20mg/cc	0.9	1.7	4.3	6.0	−4.3	1.7	1.7	0.9
〃 50mg/cc	−7.1	3.6	−0.9	−2.7	−3.6	10.7	7.1	14.2
ニコチン酸ナトリウム（〃）	2.7	8.2	10.0	12.6	14.4	10.8	14.4	18.9



第3図　第6表を曲線とせるもの

●——● ニコチン酸アミド　20mg/cc（当kg）
○——○ 〃　50mg/cc（当kg）
×——× ニコチン酸ナトリウム　50mg/cc（当kg）
●……● 対照　生理的食塩水　1cc　（当kg）

(3) B_n 注射の場合

B_n は，i) 家兎体重当 kg 10 mg (0.5cc) 注射群(2例)及び，ii) 20 mg (1cc) 注射群(4例)の2群に就いて観察した。前者に於て，注射後，初め下降，後上昇を示し，後1時間よりは，漸次低下を示し，2例中1例は著明に，他の1例に軽度にこれを表している。後者に於ては，注射後，著明に上昇を示し，その後稍下降的である（第7表）。

対照曲線に，各群のそれを比較すれば，注射後3時間の経過中，後者に於て，いづれも，B_n 注射群は，血糖上昇に対し抑制を示し，その度は，10 mg 群は 20 mg 群に優る（第8表，第4図）。

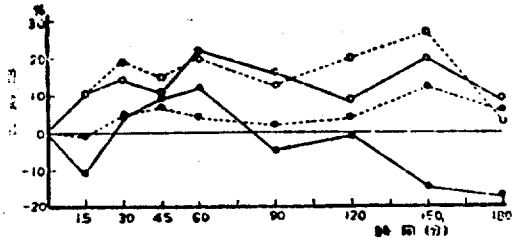
第7表　B_n 単独注射群血糖 (mg%)

i) B_n 10mg/0.5cc（当kg）

番号	体重 kg	血糖値 注射前	注射後（分） 15	30	45	60	90	120	150	180
1	1,900	110	102	111	108	117	90	101	63	34
2	1,700	117	102	127	139	138	125	125	131	106
平均値		114	102	119	124	128	108	113	97	95
増減率%			−10.5	4.3	8.8	12.3	−5.3	−0.9	−14.9	−16.7
ii) B_n 20mg/cc（当kg）										
1	2,900	102	101	127	120	125	115	117	145	132
2	2,500	101	117	110	108	129	117	113	117	113
3	1,800	77	81	88	75	81	74	77	72	57
4	1,900	106	129	120	129	138	141	129	129	122
平均値		97	107	111	108	118	112	106	116	106
増減率%			10.3	14.4	11.3	21.6	15.5	9.3	20.0	9.3

第8表　B_n 単独注射各群増減率

経過時間(分) / 各群増減率(%)	15	30	45	60	90	120	150	180
B_n 0.5cc 10mg(当kg)注射時	−10.5	4.3	8.8	12.3	−5.3	−0.9	−14.9	−16.7
B_n 1cc 20mg(〃kg) 〃	10.3	14.4	11.3	21.6	15.5	9.3	20.0	9.3



第4図　第8表とせるもの

●——● 10m/0.5cc(当kg)　対照 生理的食塩水 ●……● 0.5cc(当kg)
B_6
○——○ 20mg/1cc(当kg)　○……○ 1cc(当kg)

乙 V.B_2複合体各因子同時複合注射の場合

B_2，ニ・ア及びB_6の各因子の，種々な組合せによる，同時複合注射を行い，血糖作用に関して各因子相互の相関々係を観察した。

(1) B_2とニ・ア注射の場合

家兎体重当 kg，B_2 0.25 mg/cc 加，ニ・ア50 mg/cc 宛の注射群（4例）に就いて，観察した。注射後，4例中2例に於て，著明な血糖上昇を，他の2例に於ては，極軽微の上昇を認めた。平均値による曲線は，注射後45分，60分に最高を示し後はほぼ同じ高さを以て経過している（第9，10，11，14表，第5，6，及び9図）。

第9表　B_2複合体各因子同時注射群血糖（mg%）

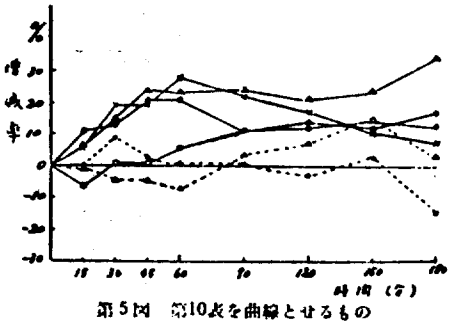
番号	体重 kg	血糖値 注射前	注射後(分) 15	30	45	60	90	120	150	180
(1) B_2 0.25mg/cc, ニコチン酸アミド 50mg/cc（当kg）										
1	1,750	122	138	122	122	117	101	122	120	122
2	1,700	113	131	138	136	134	155	152	154	159
3	2,200	125	150	161	197	195	184	154	148	148
4	1,750	111	106	110	111	122	83	113	106	120
平均値		118	131	133	142	142	131	135	132	137
増減率%			11.0	12.7	20.3	20.3	11.0	14.4	11.9	17.0
(2) B_2 0.25mg/cc, B_6 20mg/cc（当kg）										
1	2,300	120	101	99	99	122	136	136	138	131
2	1,900	96	104	111	108	111	120	104	113	108
3	2,000	117	106	117	117	120	124	127	134	134
4	1,800	99	82	119	119	104	111	104	101	101
5	1,850	110	111	99	104	113	110	127	132	138
平均値		108	101	109	109	114	120	121	124	122
増減率%			-6.5	0.9	0.9	5.9	11.1	12.0	14.8	13.0
(3) B_6 20mg/cc, ニコチン酸アミド 50mg/cc（当kg）										
1	1,900	95	92	104	101	104	115	115	122	120
2	1,750	99	102	104	93	115	106	102	97	93
3	1,900	104	117	146	159	159	141	132	111	104
平均値		99	104	118	118	126	121	116	110	106
増減率%			5.1	19.3	19.3	27.3	22.2	17.2	11.1	7.0

(4) B_2 0.25mg/cc, ニコチン酸アミド 50mg/cc, B_6 20mg/cc (当kg)

1	1,950	111	127	152	157	164	143	154	146	170
2	1,750	119	111	119	129	119	124	125	141	146
3	1,770	97	110	104	119	119	111	117	117	131
平均値		109	116	125	135	134	136	132	135	147
増減率%			6.4	14.7	23.8	22.9	24.7	21.1	23.8	34.9

第10表 B_2 複合体各因子同時注射各群増減率

経過時間(分) / 各群増減率(%)	15	30	45	60	90	120	150	180
B_2+ニコチン酸アミド	11.0	12.7	20.3	20.3	11.0	14.4	11.9	17.0
B_2+B_6	−6.5	0.9	0.9	5.9	11.1	12.0	14.8	13.0
+ニコチン酸アミドB_6	5.1	19.3	19.3	27.3	22.2	17.2	11.1	7.8
B_2+ニコチン酸アミド+B_6	6.4	14.7	23.8	22.9	24.7	21.1	23.8	34.9

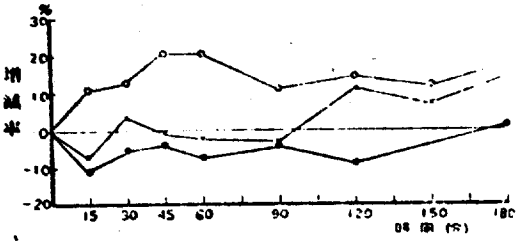


●——● B_2 0.25mg/cc + ニ・ア 50mg/cc(当kg)注射
○——○ B_2 〃 + $B_6$20mg/cc (当kg) 〃
×——× B_6 20mg/cc + ニ・ア 50mg/cc(当kg) 〃
△——△ B_2 + ニ・ア + B_6 (当kg) 〃
●……● 生理的食塩水 2cc (当kg) 注射群 (対照)
△……△ (〃) 3cc 〃 〃

第5図 第10表を曲線とせるもの

第11表 B_2 加ニコチン酸アミド注射時と B_2 及びニコチン酸アミド単独注射時比較

経過時間(分) / 各群増減率(%)	15	30	45	60	90	120	150	180
B_2	−11.4	− 6.1	− 4.4	− 7.9	− 4.4	− 8.8	−	0.9
ニコチン酸アミド	− 7.1	3.6	− 0.9	− 2.7	− 3.6	10.7	7.1	14.2
B_2+ニコチン酸アミド	11.0	12.7	20.3	20.3	11.0	14.4	11.9	17.0



第6図 第11表を曲線せるもの

●——● B_2 0.25 mg/cc (当kg)
×——× ニコチン酸アミド 50mg/cc(当kg)
○——○ B_2+ニコチン酸アミド (当kg)

(2) B_2 と B_6 注射の場合

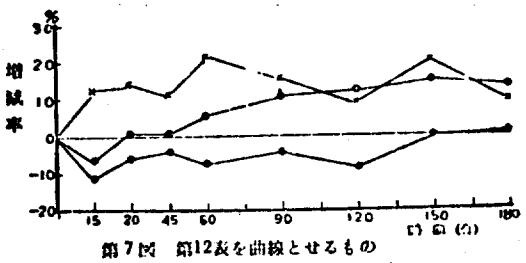
家兎体重当低, B_2 0.25/cc 加, B_6 20mg/cc 宛同時注射群 (5例) に就いて, 観察した。血糖は注射後, 45分迄は若干の低下を, その後, 漸次上昇を示した (第 9, 10, 12, 14表, 並びに第5, 7, 9図)。

(3) ニ・アと B_6 注射の場合

家兎体重当低, ニ・ア 50mg/cc 加, B_6 20mg/cc 宛同時注射群 (3例) に就いて, 観察した。注射後, 血糖は上昇し, 60分に於てほぼ最高値, 後漸次下降し, 注射前の血糖値に近くなる (第9, 10, 13, 14表並びに第5, 8, 9図)。

第12表 B_2 加 B_6 注射時と B_2 及び B_6 単独注射時の比較

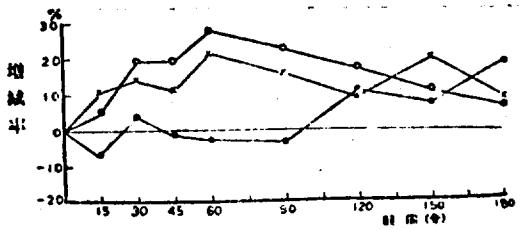
各群増減率(%) ＼ 経過時間(分)	15	30	45	60	90	120	150	180
B_2	−11.4	− 6.1	− 4.4	− 7.9	− 4.4	− 8.8	−	0.9
B_6	10.3	14.4	11.3	21.6	15.5	9.3	20.0	9.3
B_2+B_6	− 6.5	0.9	0.9	5.9	11.1	12.0	14.8	13.0



第7図 第12表を曲線とせるもの
●——● B_2 0.25mg/cc (当kg)
×——× B_6 20mg/cc (当kg)
○——○ B_2+B_6

第13表 ニコチン酸アミド加 B_6 注射時とニコチン酸アミド及び B_6 単独注射時の比較

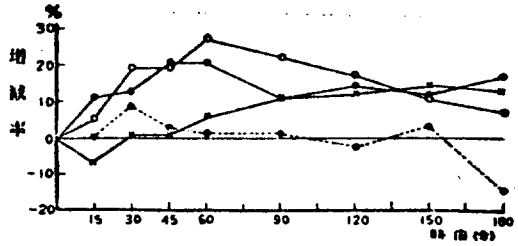
各群増減率(%) ＼ 経過時間(分)	15	30	45	60	90	120	150	180
ニコチン酸アミド	− 7.1	3.6	− 0.9	− 2.7	− 3.6	10.7	7.1	14.2
B_6	10.3	14.4	11.3	21.6	25.5	9.3	20.0	9.3
ニコチン酸アミド+B_6	5.1	19.3	19.3	27.3	22.2	17.2	11.1	7.8



第8図 第13表を曲線とせるもの
●——● ニコチン酸アミド 50mg/cc(当kg)
×——× B_6 20 mg/cc (当kg)
○——○ ニコチン酸アミド+B_6

第14表 2種複合注射時の比較

各群増減率(%) ＼ 経過時間(分)	15	30	45	60	90	120	150	180
B_2+ニコチン酸アミド	11.0	12.7	20.3	20.3	11.0	14.4	11.9	17.0
B_2+B_6	− 6.5	0.9	0.9	5.9	11.1	12.0	14.8	13.0
ニコチン酸アミド+B_6	5.1	19.3	19.3	27.3	22.2	17.2	11.1	7.8



第9図　第14表を曲線とせるもの

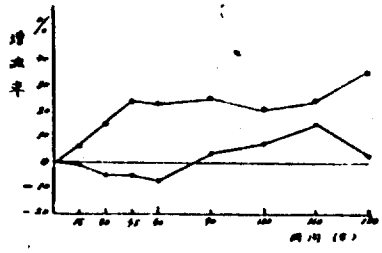
●……● 対照生理的食塩水 2cc（当kg）
●——● B_2＋ニコチン酸アミド
×——× B_2＋B_6
○——○ ニコチン酸アミド＋B_6

(4)　B_2，ニ・ア及びB_6注射の場合

家兎体重当近，B_2 0.25 mg/cc 加，ニ・ア 50 mg/cc 加，B_6 20 mg/cc 宛同時注射群（3例）に就いて，観察した。注射後，3例中1例は著明に，他の2例は漸次に，血糖の上昇を示し，注射後3時間値に於ては，3例共，更に著明な上昇を示している（第9表）。平均値による増減率を以て曲線を作り，対照群のそれと比較すれば，その推移は第15表及び第10図に見られる如く，逆の傾向を表す。他の2因子複合注射群の増減率曲線との比較は，第10表及び第5図の如く，注射後3時間の経過中，前半にては，B_2加B_6群を除く他の2群と，ほぼ同様の経過を表し，後半に於て，3因子群は，著明な上昇を示している。

第15表　B_2加ニコチン酸アミド加B_6複合注射群（増減率）

経過時間(分) ／ 各群増減率(%)	15	30	45	60	90	120	150	180
B_2＋ニコチン酸アミド＋B_6	6.4	14.7	23.8	22.9	24.7	21.1	23.8	34.9
対　照 3cc（当kg）	− 0.9	− 4.6	− 4.6	− 7.3	3.7	7.3	14.7	2.8



第10図　第15表を曲線とせるもの

〜〜〜〜 B_2＋ニコチン酸アミド＋B_6注射群
●——● 対照生理的食塩水 3cc（当kg）注射群

4. 結　　論

正常家兎，空腹時血糖に対して，静脈内注射により投与した，B_2，ニ・ア，ニ・ナ及びB_6等の因子が惹起する影響を，注射後3時間に亘り，観察した。

単独注射時には，各因子は，血糖に対し，絶対値に於ては，著明な影響を及ぼさないかの如く見えるが，対照実験の，生理的食塩水の同容量注射時の成績と，比較検討すれば，いづれも降下的に少くとも，血糖上昇に抑制的に作用していることが認められた。その中，B_2，最も著明，次にニア，ニ・ナ及びB_6の順位である。その注射量と作用の強弱に就いてみれば，B_2は，0.5 mg/2 cc（当kg）

注射群より0.25mg/cc，ニ・アは，50mg/cc(当kg)より20mg cc/(当kg)，B_6に於ても，20mg群より10mg群の如く，この範囲に於ては，少量注射群に於て，却てその作用の著明なることを見た。同様の関係を，矢谷・大髮も，健康家兎負荷過血糖に及ぼすビタミンB_2の影響に就いての報告中に，認めている。Bicknell and Prescott は，ニコチン酸及びニコチン酸アミドが，健康人並びに，糖尿病患者の血糖に対し，何等の作用も及ぼさぬとする，諸家の報告を紹介，引用しているが，これも，使用量による差違ではないかと考える。

複合注射時，即，B_2加ニ・ア，B_2加B_6，ニ・ア加B_6及び3因子同時注射等，4群のいづれの場合に於いても，明かに，血糖上昇を示し，その度は，3因子複合注射時に，最も著明，ニ・ア加B_6注射時，これに次ぎ，B_2加ニ・ア注射時は更に低く，B_2加B_6注射時は最低であつた。

即，V.B_2複合体各因子は，単独にては，血糖に対し，多少の，低下乃至，血糖上昇に抑制的作用を，有するを認めたが，それらの同時複合注射時には，かえつて，著明な，血糖上昇作用を呈するを認めた。換言すれば，3因子相互間に，血糖降下的な相乗作用は認めず，むしろ，上昇的相乗作用を認めた。

擱筆に当り，御指導を戴いた大島，遠藤前教授並びに御指導御校閲を戴いた恩師前田教授に深甚の謝意を表します。

本研究の，要旨は昭和16年3月，第15回消化器病研究会総会に於て発表した。

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Compt. Rend. 224:1123-1125. 1947.

Lecoq-'47
Bib No. 1495

PHYSIOLOGY. - SYNERGISTIC AND ANTAGONISTIC PHENOMENA BETWEEN ADRENALINE AND CERTAIN VITAMINS.

Authors (1): Messrs. Raoul Lecoq, Paul Chauchard & Mrs. Henriette Mazoué.
Presented by Mr. Emmanuel Leclainche.

When a rat is injected with adrenaline (1 to 5 drops of officinal solution at .001) repeated daily, from the 4th or 5th day on, an increase in nerve chronaxia is obtained which is permanently maintained. These disturbances, an index of a depressive condition of the encephalic nerve centers, are probably related to the hyperglycemiant action of the hormone, as the antagonism, previously noted by two of us, between this adrenaline effect and the stimulating action of caffeine proves. We thought that we had there a method which could be generalized and which could provide interesting information, especially with regard to the influence of various vitamins on the effects of adrenaline. The chronaximetric method has already revealed itself as valuable in the study of the antagonisms of certain vitamins to another hormone, thyroxin.

Either for preventative or curative purposes, we thus injected rats receiving as above adrenaline injections (all subcutaneously and in separate injections) with vitamin A (axerophtol (12γ.5), vitamin K (2-methyl-1.4 naphtoquinone 100γ), vitamin C (ascorbic acid 250γ), vitamine P (epicatechin 500γ) or vitamin B_6 or G (pyridoxin 50γ).

The only vitamins which were revealed to be antagonistic to adrenaline, i.e., likely to suppress or prevent adrenalinic variations of the nerve chronaxia, were: vitamin A; vitamin K; vitamin B_6(or G). On the contrary, vitamin P and vitamin C accentuated chronaxic disturbances, making them appear earlier and increasing their amplitude.

In this way we came upon again, with the aid of the chronaximetric test, and vis-a-vis the nerve action of adrenaline, the protective power of vitamin P, already pointed out by Parrot and Lavollay, and that of vitamin C, previously observed by Kreitmair and G. & A. Ungar as concerns the vascular activity of adrenaline. This power would seem to have a relationship with the reducing properties of these vitamins; since the time of Bacq's experiments, the exaggeration of the effects of adrenaline by antioxygens and especially by phenols is, in fact, well known.

As for the antagonism which we noted between adrenaline and vitamins A and K, all the evidence would seem to point here to inverse phenomena which cancel each other out. In addition, our observations are found to be in agreement with the hypotensive action of vitamin A and vitamin K, brought out by Bruel and Lecoq, in the treatment of high blood pressure, quantitatively higher in the laboratory for the former. Without forgetting the possible intervention of other processes, it is most likely that the impedance to the action of adrenaline brought by these vitamins plays a role in their use in vascular therapy. It is interesting to recall that the introduction of phenolic antiseptics in certain

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commercial preparations of vitamin A is enough to prevent its hypotensive activity, the action of these derivatives being inverse to that of the vitamin.

The similar effects which were observed with vitamin B_6 (or G) call for clarification. The presence of this vitamin (pyridoxine or adermine) is shown to be vital for preventing or curing chronaxic disturbances due to an excess of tyrosine in the diet or to repeated injections of this amino acid. Given the close chemical relationships which exist between tyrosine and adrenaline, it is not surprising to find that vitamin B_6 is also active in both cases.

CONCLUSIONS. - Carried out on the white rat, the chronaximetric analysis of the neural effects of adrenaline confirms the synergic action of ascorbic acid and epicatechin (vitamins C and P).

On the other hand, the analysis shows the antagonistic action excercized by vitamins A, K and B_6 (or G) on adrenaline.

The experimental results concerning vitamins A and K explain, at least partially, the therapeutic effectiveness of these vitamins recommended by Lecoa and Bruel, through intramuscular injections, in the treatment of high blood pressure.

Lecoq-'47
Bib No. 1495

Compt. Rend. 224:1123-1125. 1947.

PHYSIOLOGIE. — *Phénomènes de synergie et d'antagonisme entre l'adrénaline et certaines vitamines.* Note [1] de MM. **Raoul Lecoq**, **Paul Chauchard** et Mme **Henriette Mazoué**, présentée par M. Emmanuel Leclainche.

Quand on soumet un rat à des injections d'adrénaline (1 à 5 gouttes du soluté officinal au millième) répétées chaque jour, on obtient dès le 4e jour ou le 5e jour, une augmentation des chronaxies nerveuses qui se maintient en permanence. Indice d'un état dépressif des centres nerveux encéphaliques, ces perturbations sont en rapport probable avec l'action hyperglycémiante de l'hormone, ainsi que le prouve l'antagonisme précédemment noté, par deux d'entre nous, entre cet effet de l'adrénaline et l'action excitante de la caféine [2]. Nous avons pensé qu'il y avait là une méthode susceptible d'être généralisée et de fournir des renseignements intéressants, en ce qui regarde notamment l'influence de diverses vitamines sur les effets de l'adrénaline. Déjà, la méthode chronaximétrique est apparue précieuse dans l'étude des antagonismes de quelques vitamines vis-à-vis d'une autre hormone, la thyroxine [3].

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(1) *Comptes rendus*, 222, 1946, p. 1408.

(1) Séance du 9 avril 1947.

(2) P. Chauchard et H. Mazoué, *C. R. Soc. Biol.*, 139, 1945, p. 594.

(3) G. Mouriquand, J. Coisnard et V. Eddi, *C. R. Soc. Biol.*, 138, 1944, p. 552; P. Chauchard et H. Mazoué, *C. R. Soc. Biol.*, 139, 1945, p. 640; R. Lecoq, P. Chauchard et H. Mazoué, *Rev. Pathol. comp. et Hyg. gén.*, 46, 1946, p. 554.

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Soit à titre préventif, soit à titre curatif, nous avons donc injecté à des rats recevant comme ci-dessus des injections d'adrénaline (le tout par voie sous-cutanée et en injections séparées) de la vitamine A (axérophtol 12γ,5), de la vitamine K (2-méthyl-1,4 naphtoquinone 100γ), de la vitamine C (acide ascorbique 250γ), de la vitamine P (épicatéchine 500γ) ou de la vitamine B_6 ou G (pyridoxine 50γ).

Seules se sont révélées antagonistes de l'adrénaline, c'est-à-dire susceptibles de supprimer ou d'empêcher les variations adrénaliniques des chronaxies nerveuses : la vitamine A, la vitamine K et la vitamine B_6 (ou G). Au contraire, la vitamine P et la vitamine C ont accentué les troubles chronaxiques, les rendant un peu plus précoces et accroissant leur amplitude.

Nous retrouvons ainsi, à l'aide du test chronaximétrique, et vis-à-vis de l'action nerveuse de l'adrénaline, le pouvoir protecteur de la vitamine P, signalé déjà par Parrot et Lavollay (1), et celui de la vitamine C, antérieurement observé par Kreitmair et G. et A. Ungar (2) en ce qui concerne l'activité vasculaire de l'adrénaline. Ce pouvoir paraît être en rapport avec les propriétés réductrices desdites vitamines; depuis les expériences de Bacq, l'exagération des effets de l'adrénaline par les antioxygènes et en particulier par les phénols est en effet bien connue (3).

En ce qui concerne l'antagonisme que nous avons constaté entre l'adrénaline et les vitamines A et K, tout porte à croire qu'il s'agit ici de phénomènes inverses et qui s'annulent. Nos observations se trouvent par ailleurs en accord avec l'action hypotensive de la vitamine A et de la vitamine K, mise en évidence par Bruel et Lecoq (4), dans le traitement de l'hypertension artérielle; l'importance de la première l'emportant toutefois en clinique sur celle de la seconde. Sans préjudice de l'intervention possible d'autres processus, il est vraisemblable que l'entrave à l'action de l'adrénaline apportée par ces vitamines joue un rôle dans leur utilisation en thérapeutique vasculaire. Il est intéressant de souligner que l'introduction d'antiseptiques phénoliques dans certaines préparations commerciales de vitamine A suffit à empêcher l'activité hypotensive de celle-ci, ces dérivés ayant une action inverse de la vitamine.

Les effets analogues qui ont été observés avec la vitamine B_6 (ou G) appellent quelques explications. La présence de cette vitamine (pyridoxine ou adermine) se montre indispensable pour prévenir ou guérir les troubles chronaxiques dus

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(1) *C. R. Soc. Biol.*, 138, 1944, p. 82.

(2) H. Kreitmair, *Arch. exp. Pathol. Pharm.*, 176, 1934, p. 326; G. et A. Ungar, *C. R. VII Réun. Ass. Physiol.*, Louvain, 1938, p. 344.

(3) *Arch. intern. Physiol.*, 42, 1936, p. 340.

(4) *Presse méd.*, 54, 1946, p. 740; *Gaz. méd. France*, 53, 1946, p. 467; *Concours méd.*, 69, 1947, p. 546; *Gaz. méd. France*, 54, 1947, p. 42.

à un excès de tyrosine dans le régime ou à des injections répétées de cet amino acide (*). Étant donnés les rapports chimiques étroits qui existent entre la tyrosine et l'adrénaline, il n'est pas surprenant que la vitamine B_6 se révèle également active dans les deux cas.

Conclusions. — Poursuivie sur le Rat blanc, l'analyse chronaximétrique des effets nerveux de l'adrénaline confirme l'action synergique de l'acide ascorbique et de l'épicatéchine (vitamines C et P).

Elle montre, par contre, l'action antagoniste qu'exercent les vitamines A, K et B6 (ou G) vis-à-vis de l'adrénaline.

Les résultats expérimentaux concernant les vitamines A et K expliquent, au moins pour une part, l'efficacité thérapeutique de ces vitamines préconisées par Lecoq et Bruel, en injections intramusculaires, dans le traitement de l'hypertension artérielle.

J. Am. Med. Assoc. 218(27):1924-1927, 1971.

Leon-'71
Bib No. 1514

Symposium on Levodopa

Pyridoxine Antagonism of Levodopa in Parkinsonism

Arthur S. Leon, MS, MD; Herbert E. Spiegel, PhD; Gordon Thomas, MS; and William B. Abrams, MD

Since the decarboxylation of dopa to form dopamine is dependent upon pyridoxal phosphate, Duvoison and co-workers[1] hypothesized that pyridoxine might enhance the therapeutic effects of levodopa. Accordingly, they administered pyridoxine hydrochloride to patients with parkinsonism who were receiving maintenance doses of levodopa. They found that large doses of pyridoxine completely eliminated the clinical effects of levodopa, while smaller doses reduced or abolished its therapeutic activity or only dyskinetic side effects. This was associated with a reduced rise in plasma dopa levels following levodopa administration. Other workers have also reported reduced clinical activity of levodopa following pyridoxine.[2-5] The purpose of the present study was to correlate the effects of pyridoxine on the therapeutic activity of levodopa with its effects on absorption and metabolism.

Materials and Methods

The study population consisted of three men and one woman with parkinsonism who had achieved stable improvement (Table 1). Maintenance levels of levodopa ranged from 3.5 to 6.0 gm daily in divided doses. Three of the subjects were hospitalized for the study in the Special Treatment Unit, Newark (NJ) Beth Israel Medical Center. The fourth (subject 1) was followed up as an outpatient. Informed consent was obtained.

Patients were maintained on their previous dosage schedules of levodopa during a control period of one to two weeks before pyridoxine administration, and during an eight- to ten-day period while receiving this vitamin. Clinical observations were made daily on the hospitalized patients and twice a week on the outpatient. In addition, a parkinsonism rating sheet (Parkinson's Disease Report Form, Division of Medical Affairs, Hoffman-La Roche, Inc, Nutley, NJ) listing 36 symptoms and signs of the disease was completed once a week for each subject by a neurological consultant. Plasma levels of dopa and urinary levels of dopa, dopamine, dihydroxyphenylacetic acid (dopac), and homovanillic acid (HVA) were determined several times during the control period and during the period the subject was receiving pyridoxine, and in one subject (subject 1) five days after pyridoxine was discontinued. Blood was collected with the subject in the fasting state before and 1½ and 2½ hours after the initial morning doses of levodopa. Plasma dopa levels are ordinarily at their peak one to two hours after dosing.[1] (See also page 1912). The test dose of levodopa was 1.0 gm for three subjects (subjects 1, 3, and 4) and 0.5 gm for the fourth (subject 2). Subjects resumed their usual levodopa dose schedule following collection of the 2½-hour blood specimen. Urine specimens were collected for eight hours following the initial dose of levodopa. During the period the patient was receiving pyridoxine hydrochloride, 50 mg of the vitamin was administered orally along with the initial dose of levodopa on the days of specimen collections. On other days, 25 mg was given twice daily.

Handling of Specimens

Blood specimens of 10 ml were collected in heparinized tubes and immediately centrifuged for ten minutes. Four milliliters of plasma were added to 20 ml of chilled 0.4N perchloric acid to precipitate protein. Urine specimens were acidified with 6N hydrochloric acid (1% volume in volume). Plasma and urine specimens were then frozen and stored until analyzed.

Separation of dopa from the plasma was accomplished by adsorption of alumina and fractionation.[6] Dopa levels were then determined spectrofluorimetrically following oxidation by the method of Sourkes and Murphy.[7]

Urine specimens were also subjected to alumina adsorption.[8] With this technique, dopa, dopamine, and dopac are adsorbed on the alumina while HVA remains in the supernatant. Dopac was eluted from the alumina with 0.1N hydrochloric acid and separated on cationic resin (Dowex 50 W × 4). Dopa and dopamine were fractionated by the method of Sedvall and co-workers, and levels were determined in a similar manner as for the plasma. The

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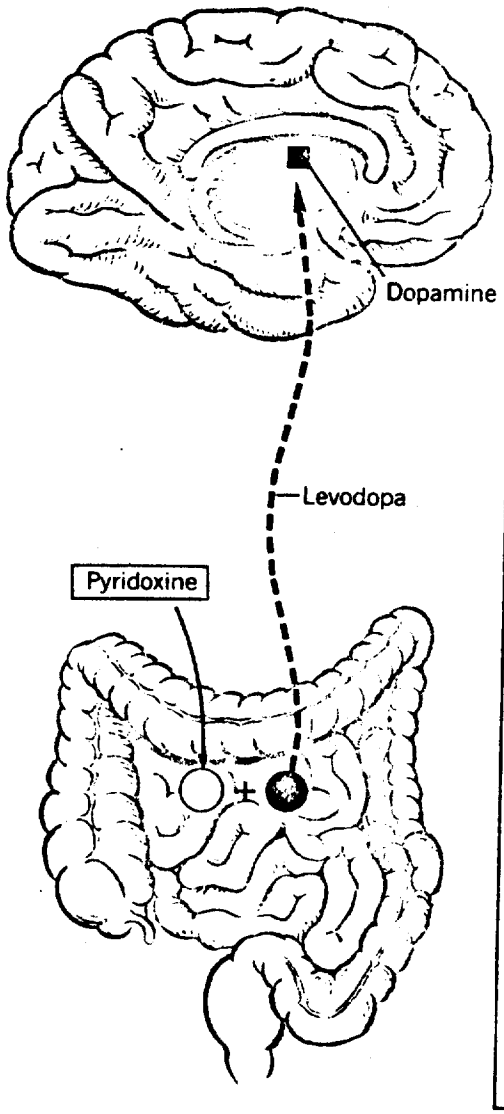

Levodopa enters the brain and is then presumably decarboxylated to dopamine

(Dopamine is depleted in the basal ganglia and substantia nigra of patients with parkinsonism)

(Dopamine does not cross the blood brain barrier)

EFFECTS OF PYRIDOXINE:

Reduces amount of intact levodopa available to enter the brain

Does not interfere with absorption of levodopa

May accelerate peripheral decarboxylation trans-amination, and/or o-methylation of dopa

Accelerates metabolism of levodopa to dopac and HVA proximal to central circulation in the gastrointestinal tract during absorption and/or during initial passage through the liver via the portal circulation

Hypothesis of possible mechanisms by which pyridoxine interferes with effectiveness of levodopa in treatment of parkinsonism.

Table 1.—Patient Population and Therapeutic Response

Patient	Age	Sex	Maintenance Dose Levodopa (gm/Day)	Stage of Parkinsonism (I-V) Before Treatment	Levodopa Alone	Levodopa + Pyridoxine	After Pyridoxine Stopped
1	61	M	6.0	V	II	IV	I
2	47	F	3.5	V	IV	V	IV
3	78	M	5.5	IV	II	III	0
4	64	M	4.0	IV	I	I	I

HVA was assayed spectrofluorimetrically as described by Sato.[7] Dopac was coupled to nitrate and measured by an automated colorimetric procedure (H.E. Spiegel and R.P. Christian, unpublished data). This procedure measures dopac and dihydroxymandelic acid (DOMA). However, the presence of DOMA was investigated by thin-layer chromatography using authentic DOMA as a standard and none was detectable.

Results

Clinical Changes.—Three of the four patients experienced an increase in symptoms and signs of parkinsonism ranging from 1 to 3 stages of severity when pyridoxine was given concomitantly with levodopa (Table 1). In subject 1, a marked exacerbation of symptoms and signs of parkinsonism began on the third day of pyridoxine administration and persisted for five days after the vitamin was discontinued. It was subsequently necessary to increase this patient's daily dose of levodopa from 6.0 to 8.0 gm to obtain the previous level of clinical improvement. Subjects 2 and 3 showed a less-marked increase in symptoms and signs of parkinsonism while receiving pyridoxine. Subject 3 had a rapid remission of disease following withdrawal of the vitamin and subject 2 gradually reverted to the previous level of improvement. The fourth patient, subject 4, did not show any reversal of the clinical improvement resulting from levodopa therapy when pyridoxine was added. However, he experienced a clearing of nausea and involuntary movements attributed to levodopa. Following this study his levodopa dose was reduced and he remained free of these side effects.

Laboratory Findings.—A rapid rise in plasma dopa levels was noted in all subjects 1½ and 2½ hours after levodopa administration (Table 2). Following concomitant administration of pyridoxine, a much smaller rise occurred. Mean plasma dopa levels at 1½ and 2½ hours during the period the subject was receiving pyridoxine were 60.0% to 73.1% lower than the levels before pyridoxine (mean, 65.9% lower). Significant decreases in levels were still evident five days after the vitamin was discontinued in the one subject tested (subject 1). The effects of concomitant administration of pyridoxine on eight-hour urinary excretion of dopa, dopamine, and HVA following levodopa are seen in Table 3. The eight-hour urinary excretion levels of dopa and dopamine decreased over 60% ($P < 0.01$) during pyridoxine administration. Significant reductions in excretion levels were evident as early as the first day the patient was given pyridoxine. Urinary excretion of dopac and HVA was not significantly altered by pyridoxine ($P < 0.05$). Dopa and dopamine excretion decreased proportionately as evidenced by the lack of

significant change in the dopamine to dopa excretion ratio (Table 4). The ratio of dopa to HVA was markedly reduced, however.

Comment

The current hypothesis of the mechanism of action of levodopa in the treatment of parkinsonism is based on studies which showed that dopamine is depleted in the basal ganglia and substantia nigra of patients with parkinsonism.[illegible] Since dopamine does not cross the blood-brain barrier, it cannot be used for the treatment of this disorder. However, levodopa does enter the brain and is then presumably decarboxylated to dopamine. Levodopa must be administered in large amounts to obtain therapeutic benefits because rapid peripheral metabolism to dopamine and the phenolcarboxylic acids, dopac and HVA, reduces the amount of levodopa available for transport into the brain.

This study confirms the previous reports that pyridoxine interferes with the clinical effects of levodopa in parkinsonism (Figure).[illegible] The findings of reduced plasma and urinary levels of dopa when pyridoxine is administered along with levodopa strongly suggests that this interference results from a further reduction in the amount of levodopa available to enter the brain. Since the quantity of HVA and dopac excreted in the urine have been shown to reflect the dosage of levodopa (see also page 1912),[illegible] the absence of a significant reduction in the excretion of these metabolites during pyridoxine administration leads one to conclude that pyridoxine does not interfere with the absorption of levodopa. Duvoison and co-workers[1] came to a similar conclusion after finding reduced plasma levels of dopa associated with unchanged plasma levels of HVA following levodopa administration to patients pretreated with pyridoxine. Accelerated absorption of levodopa with peak plasma levels occurring earlier than the usual one to two hours after dosing (shift of plasma dopa curve to the left) can also be excluded by the demonstration of reduced urinary excretion of dopa and dopamine.

Since pyridoxal phosphate is a cofactor of the enzyme dopa decarboxylase, it has been postulated that pyridoxine accelerates the peripheral decarboxylation of dopa to dopamine.[illegible] (See also page 1912.) Evidence in favor of this hypothesis is the recent demonstration that peripheral decarboxylase inhibitors block the negative effects of pyridoxine on patients with parkinsonism receiving levodopa.[illegible] If such acceleration occurred after the absorption process, an increase in urinary excretion of dopamine would be expected in addition to the demonstrated reductions in plasma and urinary dopa levels. The excretion of dopamine was found instead to be decreased proportionately to the decrease in dopa excretion. It thus appears from our findings that the reduced dopa levels

Table 2.—Plasma Dopa Levels (μg/ml ± SE) After Initial Morning Dose of Levodopa

Patient	Pyridoxine, Study Period*	Time, hr: 0	1½	2½	Mean†	Percent Decrease‡
1	Before (N = 3)	0.04 ± 0.04	0.91 ± 0.63	0.50 ± 0.18	0.67 ± 0.22	
	During (N = 3)	0.06 ± 0.03	0.23 ± 0.10	0.14 ± 0.05	0.18 ± 0.06	73.1
	After (5 days) (N = 1)	0.03	0.18	0.26	0.22	
2	Before (N = 4)	0.20 ± 0.01	0.73 ± 0.12	0.37 ± 0.06	0.55 ± 0.10	
	During (N = 4)	0.15 ± 0.09	0.32 ± 0.07	0.12 ± 0.05	0.22 ± 0.02	60.0
3	Before (N = 5)	0.27 ± 0.01	2.13 ± 0.66	2.58 ± 0.28	2.38 ± 0.32	
	During (N = 4)	0.02 ± 0.01	0.98 ± 0.27	0.60 ± 0.13	0.79 ± 0.15	66.8
4	Before (N = 2)	0.07 ± 0.06	1.65 ± 0.03	0.70 ± 0.04	1.08 ± 0.15	
	During	0.06 ± 0.05	0.56 ± 0.12	0.27 ± 0.07	0.39 ± 0.04	63.7
Mean	Before (N = 14)	0.15 ± 0.03	1.36 ± 0.36	1.04 ± 0.14	1.17 ± 0.20	
	During (N = 14)	0.07 ± 0.05	0.52 ± 0.12	0.28 ± 0.08	0.37 ± 0.08	65.9

*N signifies the number of determinations made.
†Mean of the plasma dopa values after 1½ and 2½ hours.
‡Mean plasma dopa value at 1½ and 2½ hours before pyridoxine administration, minus the mean value during pyridoxine administration, divided by the mean value pyridoxine administration.

Table 3.—Eight-Hour Urine Excretion of Dopa, Dopamine, Dopac, and HVA* (mg/8 hr ± SE) After Levodopa

Patient	Pyridoxine, Study Period†	Dopa	Dopamine	Dopac	HVA
1	Before (N = 3)	5.75 ± 1.20	66.52 ± 10.80	340.9 ± 28.6	471.3 ± 46.5
	During (N = 3)	1.49 ± 0.60	18.37 ± 6.45	274.5 ± 90.2	500.4 ± 189.4
2	Before (N = 3)	9.49 ± 0.62	38.77 ± 14.20	353.9 ± 41.2	410.3 ± 24.0
	During (N = 4)	3.02 ± 0.72	16.93 ± 5.46	300.0 ± 78.8	347.9 ± 130.0
3	Before (N = 4)	14.40 ± 2.82	121.83 ± 17.35	246.9 ± 49.0	510.1 ± 160.1
	During (N = 3)	6.03 ± 1.70	50.69 ± 12.40	350.6 ± 82.9	383.8 ± 115.3
4	Before (N = 2)	4.50 ± 0.83	30.95 ± 1.99	144.6 ± 39.2	226.0 ± 31.2
	During (N = 3)	1.34 ± 0.22	8.59 ± 1.04	176.1 ± 38.1	195.9 ± 64.1
Mean	Before (N = 12)	8.54 ± 1.37	64.52 ± 11.09	267.1 ± 39.5	404.43 ± 65.5
	After (N = 13)	2.97‡ ± 0.81	23.65§ ± 6.34	275.3 ± 72.5	357.0 ± 124.7

*HVA signifies homovanillic acid.
†N signifies the number of determinations made.
‡65.22% decrease from control levels with $P<0.01$ by analysis of variance.
§63.34% decrease from control levels with $P<0.01$ by analysis of variance.

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A STUDY OF THE TOXICITY OF CERTAIN VITAMIN PREPARATIONS IN AN EXPERIMENT

G. N. Lipkan, Kiev

Vitamins are a group of compounds which have been widely studied. However in the literature available to us we encountered only isolated works touching on the toxicity of these biologically active substances (A. M. Timofeyeva and G. A. Tsofina, 1943; Ya. B. Maksimovich, 1963; Yu. M. Ostrovskiy et al., 1968; Winter et al., 1967).

We studied the toxicity, in acute experiments, of several water-soluble vitamins of the B group, ascorbic acid, a preparation of vitamin P - rutin, and galascorbine, which possesses C and P vitamin activity. The experiments were conducted on white rats which had been placed on the usual diet. Toxicity was determined using a probe-analysis method proposed by Litchfield and Wilcoxson (M. L. Belen'kiy, 1959). In all the experiments, the vitamin dosage was determined which caused the death of all the experimental animals, as well as the size of the dose which caused no deaths. Five animals were used for testing each dose. Solutions of the preparations were made immediately before administration in distilled water. The incompletely soluble substances were administered in the form of a suspension. Death of the animals was calculated over 2-3 days. The data obtained are reflected in Tables 1 and 2.

Of all the preparations we studied, vitamin B_1 and folic acid had a relatively high toxicity with intraperitoneal administration. The phosphorylated form of thiamine, co-carboxylase, under the experimental conditions, was approximately 5 times less toxic than vitamin B_1 itself.

In clinical practice, the pharmacodynamic effect of vitamin preparations is even more widely utilized. However increased doses of vitamins are often used without sufficient experimental grounds resulting from empirical observations and conclusions, while only isolated attempts in some way or other occur on which to base the possible application of increased doses of the citamins with the aim of therapeutics. Z. T. Malkin (1952) concluded that in order to

Table 1. Acute Toxicity of Vitamin Preparations with a Single Intraperitoneal Administration to Rats (mg/kg)

Preparation	Max. Tolerab. Dose (LD_0)	LD_{16}	LD_{50} & its Reliab. Lim. for p = 0.05	LD_{84}	Abs. Leth. Dose (LD_{100})
Vitamin B_1 (thiamine)	300	335	390 (342-445)	475	500
Co-carboxylase	1600	1655	1955 (1746-2190)	2300	2300
Vitamin B_2 (riboflavin)	3000	-	4000	-	-
Nicotinic acid	700	820	1080 (900-1296)	1400	1500
Vitamin B_6 (pyridoxine)	1600	1675	1925 (1750-2118)	2195	2200
Folic acid	500	610	720 (600-864)	850	900
Orotic acid	1500	1875	2200 (1880-2574)	2810	3000

Table 4.—Ratios of Excretion of Dopa Homovanillic Acid (HVA) and Dopamine to Dopa After Levodopa Administration

Patient	Dopa/HVA (x 10^{-2})		Dopamine/Dopa	
	Before Pyridoxine	While Receiving Pyridoxine*	Before Pyridoxine	While Receiving Pyridoxine†
1	1.22	0.30	11.57	12.32
2	2.31	0.87	4.09	5.61
3	2.82	1.57	8.46	8.41
4	1.99	0.68	6.88	6.41
Mean	2.09	0.86	7.75	8.19

* $P<0.01$ when mean ratios before pyridoxine administration were compared by analysis of variance with those during pyridoxine administration.
† $P>0.05$ when mean ratios before pyridoxine administration were compared by analysis of variance with those during pyridoxine administration.

result from an acceleration by pyridoxine of the metabolism of levodopa to dopac and HVA, proximal to the central circulation, ie, in the gastrointestinal tract or during the absorption process (including the initial passage through the liver via the portal circulation).

An alternate explanation for the findings presented here is that pyridoxine might cause a shift of the peripheral metabolism of levodopa to pathways usually subordinate to decarboxylation with the formation of dopa derivatives not analyzed in this study. For example, Sourkes and Missala[13] have demonstrated in rats that the activity of transaminase is very susceptible to dietary pyridoxine. If this also holds true for man, pyridoxine might cause an increased rate of transamination of levodopa relative to decarboxylation, which would lead to increased formation of the corresponding keto acid, dihydroxyphenylpyruvic acid (DOPP), and decreased formation of dopamine. The DOPP might then be decarboxylated to dopac and O-methylated to HVA. The urinary excretion levels of these phenolcarboxylic acids would thus remain unchanged for a given dose of levodopa. Since pyridoxine is involved in methionine intestinal transport regulation,[14] it could also conceivably make more methyl groups available for O-methylation of dopa. Dopa and dopamine would then be diverted to O-methyl derivative not measured by us. Further studies are required to fully explain the alterations in dopa metabolism induced by pyridoxine.

Summary

Pyridoxine was administered to four patients with parkinsonism who received maintenance doses of levodopa. Three reverted to more severe stages of parkinsonism, while the fourth had clearing of levodopa-induced side effects. There was an associated decrease of 60% or more in plasma dopa levels and in urinary excretion of dopa and dopamine in all subjects, while the excretion of dopac and HVA remained essentially unchanged. Pyridoxine thus appears to interfere with the therapeutic effect of levodopa in parkinsonism by increasing its rate of metabolism in the gastrointestinal tract or during absorption, making less available for transport to the brain.

Ira Ross, MD, served as neurological consultant.

Nonproprietary and Trade Names of Drugs

Levodopa—*Bendopa, Dopar, Larodopa.*
Pyridoxine hydrochloride—*Beadox, Beesix, Hexa-Betalin, Hexavibex, Hydoxin.*

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Table 1 concluded

Ascorbic acid	2100	2200	2499 (2251-2774)	2810	3000
Rutin	15,000	-	20,000	-	-
Galascorbine	2800	2850	3120 (2943-3707)	3450	3500

obtain the pharmacodynamic effect of the vitamin preparations, it is necessary to use doses 10-20 times greater than the physiological doses, which reflect man's requirements for these vitamins. If you start with the physiological norms of man's daily requirement of these vitamins, established in 1960 by the Ministry of Health Protection in the USSR (N. S. Yarusov, 1961) and with the calculation principle presented above for doses of vitamin preparations having a pharmacodynamic effect, then for a man weighing 70 kg on the average, the curative doses are within the limits of 20-60 mg for vitamin B_1, 25-70 mg for vitamin B_2, 1000-2400 mg for vitamin C, 150-500 mg for nicotinic acid, and 20-40 mg for vitamin B_6. According to P. I.

Table 2. Acute Toxicity of Vitamin Preparations with a Single Subcutaneous Administration to Rats (mg/kg)

Preparation	Max. Tolerab. Dose (LD_0)	LD_{16}	LD_{50} & its Reliab. Lim. for p = 0.05	LD_{84}	Abs. Leth. Dose (LD_{100})
Vitamin B_1 (thiamine)	700	760	840 (785-899)	930	1000
Co-carboxylase	4000	4250	4650 (4346-4976)	5170	5200
Vitamin B_2 (riboflavin)	5000	-	8000	-	-
Nicotinic acid	2000	2050	2550 (2179-2984)	3125	3200
Vitamin B_6 (pyridoxine)	2600	2750	3130 (2653-3693)	3600	3800
Folic acid	3200	3250	3550 (3318-3799)	3950	4000
Orotic acid	3000	3300	3990 (3500-4519)	4750	5000
Ascorbic acid	3500	3850	4100 (3832-4387)	4400	4500
Rutin	25,0000	-	30,000	-	-
Galascorbine	3800	4050	4320 (4037-4620)	4650	4800

Shilov and T. I. Yakovlev (1964), in proposing a requirement for folic acid of 1-2 mg and for vitamin P of 50 mg, the therapeutic doses of these vitamins are, consequently, 10-40 mg and 500-1000 mg, respectively. Comparing the results obtained with those presented above, it must be noted that the therapeutic vitamin dosages are significantly less than their toxic doses. Thus, for vitamin B_1, the maximum therapeutic dose (1 mg/kg weight) is altogether 0.3% of the LD_{50} obtained in the experiment, for vitamin B_2 0.025%, nicotinic acid 0.65%, vitamin C 1.37%, vitamin B_6 0.029%, folic acid 0.079%, galascorbine 0.45%, and rutin 0.07%. In calculating these figures we used the LD_{50} of the vitamins for their intraperitoneal administration. Even if you take the maximum amount of the vitamins which shows a pharmacodynamic effect, for an average effective dose,

the therapeutic coefficient (ratio of the average lethal dose to the average effective dose) will in all cases exceed 100 (with the exception of ascorbic acid), which confirms the wide range of the therapeutic effect of vitamins. In addition, these data indicate the possibility of using vitamins for therapeutics in large doses.

In order to establish the optimal amount of vitamins for therapeutic application, the biological study of the effect of increased doses of these preparations is necessary above all. It is expedient to investigate the effect of significant doses of vitamins on the course of various pathological processes in the organism which are caused by extreme reactions. In this relation, the work of the Italian scientists is representative. In experiments on dogs, Barusco et al. (1968) formed conclusions about the usefulness for neuroleptanalgesia of thiamine and its monophosphoric ester, which have a narcotic effect in high dosages. Manami et al. (1968) administered thiamine internally in doses up to 250 mg/kg to patients at the time of narcosis and to healthy subjects - up to 350 mg/kg. Sauly et al. (1968) administered thiamine internally, on the average of 20 g to a man, in a 10% solution with the addition of 0.6 g of Pentothal. According to the data of Violani et al. (1968), 20 hours after administering 12-13 g of thiamine, it was not found in the blood.

According to our data, the doses of thiamine used by the Italian investigators are within the zone of toxic effect and could cause significant secondary effects.

The use, in anesthesiology, of thiamine, which has significant toxicity, inidcates the possibility of increasing the therapeutic doses of other less toxic vitamin preparations within the zone of their nontoxic pharmacodynamic effect. However the use of increased doses of vitamin preparations must be substantiated on all sides.

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ИЗУЧЕНИЕ ТОКСИЧНОСТИ НЕКОТОРЫХ ВИТАМИННЫХ ПРЕПАРАТОВ В ЭКСПЕРИМЕНТЕ

Г. Н. Липкан. Киев

Витамины относятся к всесторонне изученным соединениям, однако в доступной нам литературе мы встретили лишь единичные работы, касающиеся изучения токсичности этих биологически активных веществ (А. М. Тимофеева, Г. А. Цофина, 1963; Я. Б. Максимович, 1963; Ю. М. Островский и соавт., 1963; Winter и соавт., 1967).

Нами изучена токсичность в острых опытах некоторых водорастворимых витаминов группы В, аскорбиновой кислоты, препарата витамина Р — рутина и галаскорбина, обладающего С- и Р-витаминной активностью. Опыты проведены на белых крысах, находившихся на обычном рационе вивария. Токсичность определяли при помощи метода пробит-анализа, предложенного Литчфильдом и Вилкоксоном (М. Л. Беленький, 1959). Во всех опытах определяли дозу витаминов, вызывающую гибель всех подопытных животных, и величину дозы, не вызывающей гибели. Для испытания каждой дозы брали 5 животных. Растворы препаратов готовили непосредственно перед введением на дистиллированной воде. Плохо растворимые вещества вводили в виде взвеси. Гибель животных учитывали в течение 2—3 дней. Полученные данные отражены в табл. 1 и 2.

Из всех изученных нами препаратов относительно высокой токсичностью обладает витамин B_1 и фолиевая кислота при внутрибрюшинном введении. Фосфорилированная форма тиамина — кокарбоксилаза в условиях опыта примерно в 5 раз менее токсична, чем сам витамин B_1.

В клинической практике все более широко используется фармакодинамическое действие витаминных препаратов. Однако повышенные дозы витаминов часто применяются без достаточных экспериментальных обоснований, исходя из эмпирических наблюдений и выводов, причем встречаются лишь единичные попытки каким-либо образом обосновать возможность применения повышенных доз витаминов с терапевтической целью. З. Н. Малкин

Таблица 1. Острая токсичность витаминных препаратов при однократном внутрибрюшинном введении крысам (*мг/кг*)

Препарат	Максимально переносимая доза ($ЛД_0$)	$ЛД_{16}$	$ЛД_{50}$ и ее доверительные границы при $p = 0,05$	$ЛД_{84}$	Абсолютно смертельная доза ($ЛД_{100}$)
Витамин B_1 (тиамин)	300	335	390 (342 ÷ 445)	475	500
Кокарбоксилаза	1600	1655	1955 (1746 ÷ 2190)	2300	2300
Витамин B_2 (рибофлавин)	3000	—	>4000	—	—
Никотиновая кислота	700	820	1080 (960 ÷ 1206)	1400	1500
Витамин B_6 (пиридоксин)	1600	1675	1925 (1750 ÷ 2118)	2195	2200
Фолиевая кислота	500	610	720 (600 ÷ 864)	850	900
Оротовая кислота	1500	1875	2200 (1880 ÷ 2574)	2675	3000
Аскорбиновая кислота	2100	2200	2499 (2251 ÷ 2774)	2810	3000
Рутин	15 000	—	>20 000	—	—
Галаскорбин	2800	2850	31200 (2843 ÷ 3307)	3450	3500

(1952) считает, что для получения фармакодинамического эффекта витаминных препаратов необходимо применять дозы, в 10—20 раз больше физиологических, отражающих потребность человека в этих витаминах. Если исходить из физиологических норм суточной потребности человека в витаминах, утвержденных в 1960 г. Министерством здравоохранения СССР (Н. С. Ярусова, 1961), и вышеприведенного принципа расчета доз витаминных препаратов, обладающих фармакодинамическим эффектом, то для человека со средним весом 70 *кг* лечебные дозы находятся в пределах 20—60 *мг* для витамина B_1, 25—70 *мг* — для витамина B_2, 1000—2400 *мг* — для витамина С, 150—500 *мг* — для никотиновой кислоты, 20—40 *мг* — для витамина B_6. По П. П. Шилову и

Таблица 2. Острая токсичность витаминных препаратов при однократном подкожном введении крысам (*мг/кг*)

Препарат	Максимально переносимая доза ($ЛД_0$)	$ЛД_{16}$	$ЛД_{50}$ и ее доверительные границы при $p = 0,05$	$ЛД_{84}$	Абсолютно смертельная доза ($ЛД_{100}$)
Витамин B_1 (тиамин)	700	760	840 (785 ÷ 899)	930	1000
Кокарбоксилаза	4000	4250	4650 (4346 ÷ 4976)	5170	5200
Витамин B_2 (рибофлавин)	5000	—	8000	—	—
Никотиновая кислота	2000	2050	2550 (2179 ÷ 2984)	3125	3200
Витамин B_6 (пиридоксин)	2600	2750	3130 (2653 ÷ 3693)	3600	3800
Фолиевая кислота	3200	3250	3550 (3318 ÷ 3799)	3950	4000
Оротовая кислота	3000	3300	3900 (3560 ÷ 4539)	4750	5000
Аскорбиновая кислота	3500	3850	4100 (3832 ÷ 4387)	4400	4500
Рутин	25000	—	30000	—	—
Галаскорбин	3800	4050	4320 (4037 ÷ 4622)	4650	4800

Т. И. Яковлеву (1964), предположительная потребность в фолиевой кислоте — 1—2 *мг* и в витамине Р — 50 *мг*, следовательно, терапевтические дозы этих витаминов составляют соответственно 10—40 и 500—1000 *мг*. Сравнивая полученные данные с приведенными выше, следует отметить, что терапевтические дозы витаминов значительно меньше их токсических доз. Так, для витамина B_1 максимальная терапевтическая доза (1 *мг/кг* веса) составляет всего около 0,3% от $ЛД_{50}$, полученной в эксперименте, для витамина B_2 — 0,025%, никотиновой кислоты — 0,65%, витамина С — 1,37%, витамина B_6 — 0,029%, фолиевой кислоты — 0,079%, галаскорбина — 0,45%, рутина — 0,07%. При расчетах этих цифр мы пользовались $ЛД_{50}$ витаминов при их внутрибрюшинном введении. Если принять даже максимальные количества витаминов, оказывающие фармакодинамическое действие, за среднеэффективную дозу, терапевтический коэффициент (отношение среднесмертельной дозы к среднеэффективной) будет во всех случаях превышать 100 (за исключением аскорбиновой кислоты), что свидетельствует о большой широте терапевтического действия витаминов. Кроме того, эти данные указывают на возможность применения витаминов с терапевтической целью в больших дозах.

Для того, чтобы установить оптимальные количества витаминов для применения их с терапевтической целью, прежде всего необходимо биологическое изучение действия повышенных доз этих препаратов. Целесообразно исследовать влияние значительных доз витаминов на течение различных патологических процессов в организме, вызываемых экстремальными воздействиями. В этом отношении показательны работы итальянских ученых. В опытах на собаках Baruscо и соавт. (1968) сделали вывод о пригодности тиамина и его монофосфорного эфира, обладающих в высоких дозировках наркотическим действием, для нейролептанальгезии. Manani и сотр. (1968) вводили тиамин внутривенно в дозе до 250 *мг/кг* больным во время наркоза внутривенно и здоровым испытуемым — до 350 *мг/кг*. Sauly и сотр. (1968) вводили внутривенно тиамин в среднем 20 *г* на человека в 10% растворе с добавлением 0,6 *г* тиопентала. По данным Violani и соавт. (1968), через 20 часов после введения 12—13 *г* тиамина последний в крови не обнаруживается.

По нашим данным, используемые итальянскими исследователями дозы тиамина находятся в зоне токсического действия и могут вызывать значительные побочные эффекты.

Применение в анестезиологии тиамина, обладающего значительной токсичностью, указывает на возможность использования терапевтических дозировок других, менее токсичных витаминных

препаратов в зоне их нетоксического фармакодинамического действия. Однако использование повышенных доз витаминных препаратов должно быть всесторонне обосновано.

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SOME BIOCHEMICAL ASPECTS OF VITAMIN B_6 ADMINISTRATION TO RABBITS FED DIETARY CHOLESTEROL

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SUMMARY

The effect of long-term administration of vitamin B_6 to rabbits fed dietary cholesterol was studied.

The data obtained show that the dose of pyridoxine administered did not affect significantly serum, liver or heart cholesterol levels of normal animals, nor did it affect the synthesis of cholesterol in serum or liver.

When the vitamin was administered to rabbits receiving an atherogenic diet, serum, liver and heart cholesterol levels were significantly increased, relative to the cholesterol-fed animals. Pyridoxine deficiency increased serum and liver cholesterol levels with concomitant decreases in cholesterol synthesis. When vitamin B_6 was administered to pyridoxine deficient rabbits receiving the dietary cholesterol supplement, serum, liver and heart cholesterol levels were further increased.

The significance of these findings and the possible relationship between these factors are discussed.

INTRODUCTION

There are indications in the literature that vitamin B_6 affects animal and human lipid metabolism. Vascular changes and fatty infiltration of the liver, identical to atherosclerosis in man, have been noted in various species[1-3]. The administration of vitamin B_6 to patients with atherosclerosis and to rabbits with alimentary hypercholesteremia cause a reduction in blood cholesterol[4,5].

The investigations cited above dealt mainly with the effect of pyridoxine on blood cholesterol.

The purpose of the present investigation was to determine the effect of vitamin B_6 on blood, liver and heart cholesterol levels as well as on cholesterol biosynthesis in the blood and liver of rabbits fed dietary cholesterol.

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MATERIALS AND METHODS

One hundred rabbits of approximately the same age and weight (2 kg) were fed a control diet for 1 week and were divided into 6 groups:

Group N. Twenty animals received the control pelleted diet*.

Group N + B_6. Sixteen animals received the control diet and vitamin B_6 subcutaneously (6 mg/kg body weight per day).

Group N + 1 %. Sixteen animals received the control diet supplemented with 1 % cholesterol.

Group N + 1 % + B_6. Sixteen rabbits received the cholesterol diet and vitamin B_6 subcutaneously (6 mg/kg body weight per day).

Group D. Sixteen animals received a pyridoxine deficient diet**.

Group D + 1 %. Sixteen animals of this last group received the pyridoxine deficient diet with the 1 % dietary cholesterol supplement.

All rabbits were placed in individual cages, watered and fed *ad libitum*. The animals were weighed once weekly. The rabbits were killed at various periods of time in the following way: 6 rabbits of the N group at the beginning of the experiment and thereafter 6 rabbits (when available) of each group at 3, 5 and 8 weeks.

Three h 30 min before they were killed, the rabbits were injected intraperitoneally with [^{14}C]acetate solution, 0.4 ml/kg body weight (5.7 μmoles, 6.2 $\times$ 10^7 counts/min). Ten ml of blood was removed by heart puncture and the animals were euthanized. Hearts and livers were quickly removed, weighed and frozen.

Plasma and liver cholesterol determinations were made according to the method of FERRO AND HAM[8]. For radioactivity measurements the cholesterol was extracted with petroleum ether in the conventional way and precipitated with digitonine. The digitonide pads were dissolved in 1 ml hyamine–NaOH (10 $\times$) in counting vials and 14 ml of scintillation liquid (redistilled toluene–PPO–POPOP) was added. The samples were counted for 30 min on a Nuclear Chicago, Model 720, scintillation counter.

RESULTS AND DISCUSSION

In Table 1 are presented mean serum, liver and heart cholesterol levels, for all experimental groups. Table 2 shows serum and liver cholesterol specific activities for the same groups of rabbits.

Serum, liver and heart cholesterol levels of the N group were relatively constant throughout the experimental period (Table 1). Although slightly decreased, [^{14}C]-acetate incorporation rates in both serum and liver cholesterol were not statistically affected throughout the experimental (Table 2).

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* The control diet had the following percent composition: vitamin free casein 18 %, sucrose 68 %, vegetable oil 10 %, U.S.P. Salt mixture 2.4 % and was supplemented with all the necessary vitamin. The diet was offered to the animals in pelleted form.

** The pyridoxine deficient diet was the same as the control diet except that it did not contain any pyridoxine.

TABLE 2

SERUM AND LIVER CHOLESTEROL SPECIFIC ACTIVITIES (COUNTS/MIN PER MG CHOLESTEROL) FOR ALL EXPERIMENTAL GROUPS

Number of animals in each group given in parentheses.

Groups	*0 Weeks*		*3 Weeks*		*5 Weeks*		*8 Weeks*	
	serum (S.A.)[a]	*liver (S.A.)*	*serum (S.A.)*	*liver (S.A.)*	*serum (S.A.)*	*liver (S.A.)*	*serum (S.A.)*	*liver (S.A.)*
N	1100 ± 200 (6)	1775 ± 219	1084 ± 214 (5)	2372 ± 422	907 ± 180 (6)	1569 ± 188	805 ± 161 (6)	1221 ± 143
N + 1%			21 ± 6 (5)	36 ± 10	25 ± 4 (5)	65 ± 10	—	—
N + B_6			260 ± 80 (6)	1349 ± 118	1034 ± 258 (5)	1721 ± 283	687 ± 105 (6)	984 ± 35
N + B_6 + 1%			43 ± 14 (6)	63 ± 18	13 ± 4 (5)	34 ± 8	—	—
D			61 ± 22 (6)	954 ± 116	65 ± 45 (4)	474 ± 45	45 ± 13 (4)	102 ± 38
D + 1%			42 ± 20 (5)	66 ± 16	58 ± 16 (4)	80 ± 22	—	—

[a] S.A. = specific activity (counts/min per mg cholesterol).

TABLE 1

MEAN SERUM, LIVER AND HEART CHOLESTEROL LEVELS FOR ALL EXPERIMENTAL GROUPS

Number of animals in each group given in parentheses.

Groups	0 Weeks			3 Weeks			5 Weeks			8 Weeks		
	serum (mg/100 ml)	*liver (mg/g)*	*heart (mg/g)*	*serum (mg/100 ml)*	*liver (mg/g)*	*heart (mg/g)*	*serum (mg/100 ml)*	*liver (mg/g)*	*heart (mg/g)*	*serum (mg/100 ml)*	*liver (mg/g)*	*heart (mg/g)*
N	42±4.7	1.8±0.30 (6)	0.54±0.06	38± 5.4	2.2±0.18 (5)	0.54±0.05	37± 3.9	1.8± 0.15 (6)	0.61±0.05	52±10	2.2±0.11 (6)	0.56±0.06
N + 1%				546±188	7.2±1.0 (5)	1.0 ±0.28	906±40	10 ± 3.5 (5)	1.1 ±0.06	—	—	—
N + B_6				87± 10	2.2±0.21 (6)	0.63±0.04	36± 5	1.8± 0.09 (6)	0.67±0.04	41± 6	1.9±1.0 (6)	0.42±0.03
N + B_6 + 1%				560±146	8 ±2.0 (6)	1.2 ±0.07	1195±84	18 ± 2.3 (5)	1.6 ±0.02	—	—	—
D				327± 69	3.7±0.87 (6)	0.85±0.09	498±97	4.2±0.48 (4)	0.71±0.07	383±82	5.9±0.88 (4)	0.65±0.06
D + 1%				617±138	7.9±1.9 (5)	0.82±0.16	693±50	7.1±1.5 (4)	1.2 ±0.5	—	—	—

daily subcutaneous injections of vitamin B_6 to normal rabbits (N + B_6) (6 mg/kg b.w.) did not produce any sustained significant changes in the serum, liver or heart cholesterol levels. A very marked hypercholesteremic effect ($P < 0.01$) was observed however, at 3 weeks concomitantly with a sharp decrease ($P < 0.01$) in serum cholesterol specific activities (Tables 1 and 2). Liver levels of the same group at this time were not significantly disturbed although [^{14}C]acetate incorporation rates in this tissue differed significantly ($P < 0.05$) from the normals. The transient effect could possibly be explained by an increased absorption rate or decreased catabolic rate, or both.

The pyridoxine treated cholesterol-fed rabbits (N + B_6 + 1 %) showed significantly increased ($P < 0.01$) serum, liver and heart cholesterol levels at 5 weeks relative to the cholesterol-fed group (N + 1 %) (Table 1). Although significant ($P < 0.05$), differences between serum and liver cholesterol specific activities suggest that the cholesterol supplement was perhaps too high overshadowing possibly the occurrence of greater differences between the two groups (Table 2).

Pyridoxine deficiency significantly increased ($P < 0.01$) serum and liver cholesterol levels of the animals (D group) compared to the N group throughout the experiment while specific activities for serum and liver cholesterol decreased concomitantly (Tables 1 and 2). The results indicate that unlike the rat[6] the rabbit behaves somewhat similarly to the chick in this respect[7].

The dietary cholesterol supplement given the pyridoxine deficient rabbits (D + 1 %) further increased serum and liver cholesterol levels, relative to the D group. These findings suggest an additive effect of the dietary cholesterol supplement to the hypercholesteremic effect of the pyridoxine deficiency itself.

Plasma and liver cholesterol levels observed for the groups of rabbits receiving dietary cholesterol decreased in the following order: N + 1 % + B_6 > N + 1 % > D + 1 %, while synthesis of cholesterol in the same groups inversely increased.

Under the experimental conditions enumerated above, and unlike the observations of other workers[4,5] the data justifies the conclusion that the administration of vitamin B_6 to normal rabbits does not cause a sustained significant effect on serum, liver or heart cholesterol levels. If however, pyridoxine is administered against a background of alimentary hypercholesteremia, it causes a significant rise in cholesterol levels. Cholesterol synthesis in the plasma and liver is greatly diminished. The exact mechanism(s) of action of pyridoxine under these conditions has not been determined.

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Interconversions of Vitamin B_6 in Mammalian Tissue

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A review of interconversions of vitamin B_6 in mammalian tissues is presented. Orally administered (PN) pyridoxine is rapidly absorbed from the intestinal tract. Intravenously administered PN is rapidly transformed to the several forms of B_6 in liver, brain, and carcass. The amount retained and pattern of interconversion is characteristic for each tissue. The enzymes involved in the interconversions have been studied but little data are available on factors which control the rates of interconversions. Chronic administration of marijuana to mice alters the distribution of retained phosphorylated forms of vitamin B_6 in brain.

Vitamin B_6 is the generic term used for the three phosphorylated and three nonphosphorylated forms of this vitamin. Pyridoxal phosphate and pyridoxamine phosphate are essential for the metabolism of amino acids in transamination, decarboxylation, elimination, and replacement and dehydratase reactions (Braunstein, 1960). The functions of vitamin B_6 can be assessed either by *in vitro* studies of enzyme reactions (Snell and Ayling, 1968) or by observation of animal or human species where vitamin B_6 is deprived (Greenberg, 1964) or a dependency state exists.

A number of vitamin B_6 functions can be demonstrated in the vitamin B_6 dependency states. Vitamin B_6-dependent seizures (Scriver, 1960) occur shortly after birth and are resistant to usual anticonvulsant medications but are controlled by pyridoxine given daily at 10 to 20 times the usual daily requirement. The manifestations of the disease are thought to be due to decreased synthesis of γ amino butyric acid which is catalyzed by the PLP dependent enzyme glutamic decarboxylase. A decreased binding of PLP to this enzyme is thought to be the basic defect in this disease. Another example of vitamin B_6 function is illustrated by the disease pyridoxine responsive anemia. Individuals with this condition have a microcytic, hypochromic anemia on a normal diet (Harris and Horrigan, 1964), are unresponsive to other forms of therapy, but respond to 10 to 20 mg of pyridoxine per day with return to normal hemoglobin values. Pyridoxal phosphate catalyzes the formation of δ-aminolevulinic acid from glycine and succinyl CoA as a step in heme synthesis. It is thought that defective binding of PLP to δ-aminolevulinic acid synthetase is the defect in this condition. Other examples of vitamin B_6 dependency states are cystathioninuria and xanthurenic aciduria.

Although an extensive literature exists on the enzymatic reactions catalyzed by pyridoxal phosphate and pyridoxamine phosphate, little work has been carried out on the enzymes involved in the metabolism of vitamin B_6 and even less on the rates of interconversions of vitamin B_6 and factors which influence the metabolism and distribution of vitamin B_6. A number of drugs such as amphetamine (Bilodeau, 1965), chlorpromazine (Ebadi, 1970), reserpine, isoniazid (Vilter, 1964), birth control pills (Brown *et al.*, 1969) and marijuana, have been shown to affect either the concentration of vitamers or the enzymes of vitamin B_6 metabolism. The present paper reviews data on the absorption of pyridoxine (PN) and the enzymatic reactions involved in vitamin B_6 interconversions. In addition, detailed studies are reported on the interconversion of ^{14}C-PN to pyridoxine phosphate (PNP), pyridoxal phosphate (PLP), pyridoxamine phosphate (PMP), pyridoxal (PL), and pyridoxamine (PM) in liver, brain, and carcass of normal male Swiss-Webster mice and the effect of marijuana or B_6 depletion on the process.

ABSORPTION AND EXCRETION OF PYRIDOXINE

The absorption of PN has been studied (Booth and Brain, 1962). These authors administered ^{3}H-PN orally in doses varying from 0.05 to 5.0 mg, alone or accompanied by intraperitoneal nonradioactive PN. The amount absorbed was determined either by the percent of administered ^{3}H-PN excreted or the percent of ^{3}H-PN not absorbed at varying levels of the gut. When 0.05 mg of ^{3}H-PN containing 1 μCi of radioactivity was administered, an average of 34% of administered ^{3}H-PN was excreted, primarily in that form.

When animals were killed at intervals between 7.5 and 60 min after the administration of 0.05 mg ^{3}H-PN and the percent of ^{3}H-PN remaining in the intestinal tract was calculated, it was found that the absorption of pyridoxine was remarkably rapid. Within 15 min 74.7% and by 60 min greater than 90% had been absorbed. From a study of the amount of radioactivity remaining in the gut at varying levels and times, it was concluded that: virtually no absorption occurred in the stomach; the greater part of PN was absorbed in the jejunum; a smaller amount of PN was absorbed in the ileum than jejunum but the ileum had the capacity to absorb PN; virtually no PN was absorbed in the colon but this portion of the gut was capable of absorbing some PN. Their data favor the concept that absorption of PN occurs by passive diffusion rather than active transport.

When 1.0 mg of ^{3}H-PN containing 20 μCi was administered to human volunteers (Brain and Booth, 1964), it was found that approximately 17% was excreted. When large amounts of cold PN were administered simultaneously the amount of ^{3}H-PN excreted rose to an average of 50%. Absorption appeared to be maximal between 60 and 90 min after administration and was virtually complete by 12 hr. It was found that conditions which contribute to malabsorption, such as idiopathic steatorrhea, as well as resection of major sections of the small intestine had no effect on the amount of PN absorbed.

It has been shown (Contractor and Shane, 1968) that after oral administration of 100 mg of PN to humans, the highest concentration in blood appeared 2 hr later. Large increases in the concentration of pyridoxal phosphate, pyridoxal, and 4-pyridoxic acid occurred but no significant increase in concentration of PMP or PM was noted.

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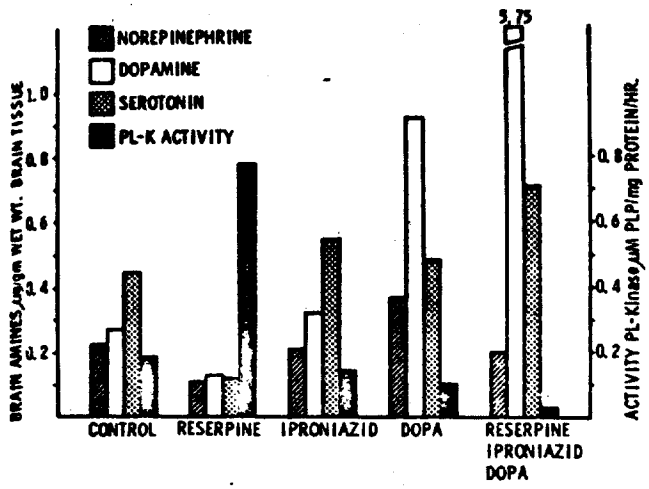

Figure 1. Relationship of brain amine concentration to PL kinase activity. New Zealand white rabbits were administered reserpine 5 mg/kg I.V. or iproniazid 100 mg/kg I.V. or DOPA 150 mg/kg I.V. at varying times prior to decapitation. Brain amines extracted and measured fluorometrically; brain PL kinase activity measured by method of McCormick and Snell

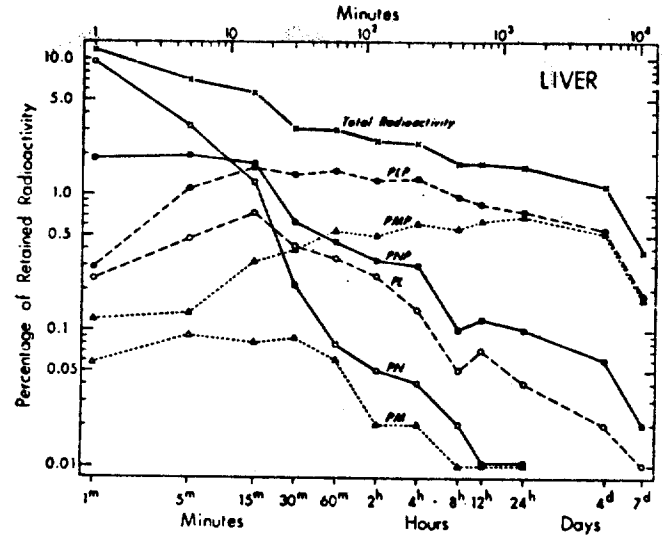

Figure 2. Retention and distribution of ^{14}C-pyridoxine in liver. Swiss-Webster mice were injected I.V. with ^{14}C-PN and then sacrificed at times as shown. Liver was homogenized, B_6 vitamers extracted, separated and radioactivity determined as described. Results expressed as individual vitamer percent retained of total injected radioactive ^{14}C-PN at times indicated

Johansson and coworkers (Johansson *et al.*, 1966) administered ^{3}H-PN to human volunteers to study the turnover of vitamin B_6 in man. From the radioactive substances excreted in urine, data on B_6 turnover were calculated. Isotope retention was characterized by an initial rapid fall in the first day and then a slow decline with a half-life of 13 to 38 days of the administered radioactive ^{3}H-PN.

ENZYMES WHICH CATALYZE VITAMIN B_6 TRANSFORMATION

Pyridoxal Phosphokinase. Ingested pyridoxine may be either phosphorylated to PNP or oxidized to PL. Pyridoxal is phosporylated directly by the same kinase, pyridoxal phosphokinase, that phosphorylates PN. McCormick, Gregory and Snell (McCormick *et al.*, 1961) were the first to report detailed studies of pyridoxal phosphokinase. Their work showed that in mammalian tissue PL was the preferred substrate but PN was also readily phosphorylated. The enzyme occurred in all tissues with the highest activity in the supernatant of the particle-free cell fraction.

The author's studies of the properties of PL kinase stemmed from the observation that when an animal was depleted of the amines norepinephrine, dopamine, and serotonin by reserpine, the activity of brain PL kinase increased but when amine levels were increased by administration of monamine oxidase inhibitors such as iproniazid, a decrease in enzyme activity occurred (Ebadi *et al.*, 1968). These results are shown in Figure 1. This inverse relation between the concentration of brain amines and PL kinase activity suggested that amine concentration may control the rate of PLP synthesis in brain. Pyridoxal phosphokinase from rabbit brain has been highly purified and the activity of the enzyme in the presence of added amines studied (Henn and McCoy, 1971). When amines such as dopamine, norepinephrine, or serotonin were added at 1 m*M* concentration to the incubation mixture of the purified enzyme in the presence of excess PL, inhibition of 68–75% of normal activity was observed. These results showed that *in vitro* as well as *in vivo* increased concentration of amines inhibited pyridoxal kinase.

Pyridoxine Oxidase. Pyridoxine may be oxidized to pyridoxal or phosphorylated to pyridoxine phosphate which, in turn, is oxidized to pyridoxal phosphate. Present data suggest that two distinct enzymes catalyze these two oxidation reactions. Marino and coworkers (Marino *et al.*, 1960) purified pyridoxine oxidase from rabbit liver and studied the properties of the enzyme. The purified enzyme did not oxidize PNP, the pH optimum was 5.7 to 5.9, and was not activated by addition of FMN or metals. There was no report of inhibition of enzyme activity by adding pyridoxal or pyridoxamine. Wada and Snell (Wada and Snell, 1961) purified pyridoxine phosphate oxidase which catalyzes the oxidation of both PNP to PLP and PMP to PLP. These authors noted that the oxidation of PMP was another pathway in addition to amine oxidase for the disposition of the amine group of amino acids and that the oxidation of PMP was a mechanism for the net conversion of PMP to PLP. Their data showed that purified PNP oxidase did not convert PN to PL; the enzyme was FMN dependent, was inhibited by its product PLP, and used either PNP or PMP as substrate at pH 8.0. At this pH, PNP was bound preferentially to PMP. In crude tissue extracts of liver, PNP was oxidized much more rapidly than PN.

INTERCONVERSIONS OF ^{14}C-PYRIDOXINE

Although data were available on the individual steps of B_6 transformation, little information was available on the rate of transformation and distribution of PN was determined simultaneously in brain, liver, and carcass. For these reasons studies were initiated which used ^{14}C-PN rather than ^{3}H-PN which, depending on the method of preparation, has a 10 to 60% loss of the tritium label during transformation. In the present studies, male Swiss-Webster mice were given intravenously 0.5 ml of a solution containing 5.3 μCi and 115 μg of ^{14}C-PN. They were sacrificed at time intervals of 1 min to 7 days by immersion in liquid nitrogen, dissected while frozen, and B_6 vitamers were extracted in 7% TCA. The concentrated extracts were applied to a thin layer cellulose plate and B_6 vitamers separated by electrophoresis (Colombini and McCoy, 1970a). Using this technique it was possible to separate all six forms of the vitamer found in brain, liver, and carcass (Colombini and McCoy, 1970b).

Table I. Percentages of the Recovered Radioactivity and Distribution of the B_6 Vitamers in the Mouse Brain

Tissue	Experiment	Total dpm	% of injected radio-activity	% PN	% PM	% PL	% PNP	% PMP	% PLP
Brain	Controls	9,571	0.16	3.17	2.17	17.98	13.03	14.17	49.57
	Red Oil (Acute)	5,825	0.09	9.18	7.35	10.36	12.75	15.47	44.89
	Red Oil (8 days)	13,795	0.22	4.14	0.70	8.73	39.73	21.39	25.32

Total radioactivity injected as ^{14}C-pyridoxine = 6,093,000 dpm.

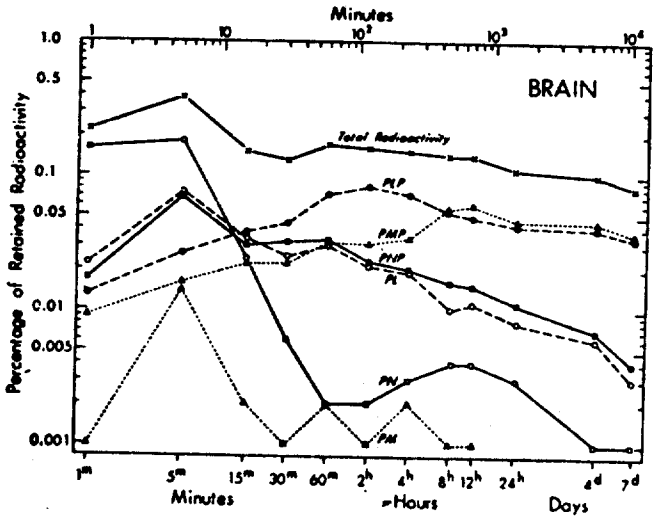

Figure 3. Retention and distribution of ^{14}C-pyridoxine in brain. Conditions of administration of ^{14}C-PN, extraction of B_6 vitamers and measurement of retained radioactivity as described in Figure 2

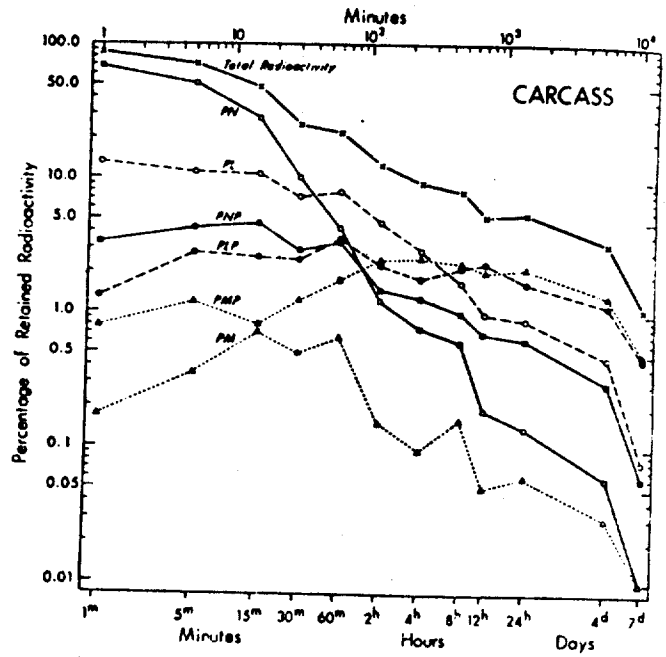

Figure 4. Retention and distribution of ^{14}C-pyridoxine in carcass. Conditions of administration of ^{14}C-PN, extraction of B_6 vitamers and measurement of retained radioactivity as described in Figure 2

RETENTION AND DISTRIBUTION OF VITAMIN B_6 IN LIVER

The shortest time interval between ^{14}C-PN injection and sacrifice was 1 min. The total retained radioactivity was 12.2% distributed as 9.6% PN, 1.8% PNP, 0.3% PLP, and 0.25% PL (Figure 2). These results indicated that phosphorylation proceeded more rapidly than oxidation and that PLP was formed within the first minute after PN administration. By 5 min after injection the total retained radioactivity had decreased to 7.5%, of which 3.4% was PN, 2.0% PNP, 1.2% PLP, and 0.5% PL. These data again indicated that phosphorylation proceeds more rapidly than oxidation as the amount of PNP was greater than PLP which is derived from both PL and PNP. By 60 min the total radioactivity retained was 3.4%, of which 1.7% was PLP, 0.5% PMP, 0.4% PNP, 0.3% PL, and 0.07% PN. These results illustrated that the principal reactions occurring after PN administration were a conversion to PLP and a continued interconversion of PLP to PMP. Of the 2% of total radioactivity retained at 24 hr, 1.5% was almost equally divided as PLP and PMP.

RETENTION AND DISTRIBUTION OF VITAMIN B_6 IN BRAIN

The brain, unlike liver, did not reach peak retention of radioactivity until 5 min after injection of ^{14}C-PN. Initial conversion of PN differed in brain compared to liver. Whereas phosphorylation was the dominant initial reaction in liver, oxidation appeared equally active in brain as indicated by the equal percent of retained radioactivity in PL and PNP at 1 and 5 min (Figure 3). The maximum total radioactivity retained was much less in brain (0.4%) compared to liver (12.2%) but that radioactivity which was retained by brain was lost more slowly than that of the liver. At 60 min the distribution of retained radioactivity was 0.08% PLP and almost equal amounts (0.03%) of PMP, PNP and PL while at 24 hr there were almost equal amounts of PLP and PMP (0.05%) and approximately 0.01% of both PNP and PL. The distribution of retained radioactivity predominantly as PLP and PMP reflected the distribution of actual amount of vitamin B_6 in brain which is predominantly PMP and PLP (Bain and Williams, 1960).

RETENTION AND DISTRIBUTION OF VITAMIN B_6 IN CARCASS

It was an unexpected finding that 1 min after ^{14}C-PN injection 86.7% of the total radioactivity injected was found in carcass (Figure 4). The radioactivity was distributed as 68.3% PN, 12.9% PL, 3.3% PNP, and 1.3% PLP. Fifteen minutes after injection, there was still retention of ^{14}C-PN distributed as 30% PN, 11% PL, 4.8% PNP, and 2.6% PLP. These results indicated that in contrast to liver, the predominant initial pathway of PN metabolism in carcass is oxidation to PL and that PL kinase activity is decreased compared to liver. At 24 hr the total radioactivity was 7% of the injected dose compared to 1.6% in liver and 0.1% in brain. These data further support the proposal that the carcass is a storage site for vitamin B_6 in the body. Although like liver, the predominant forms at this time were PMP and

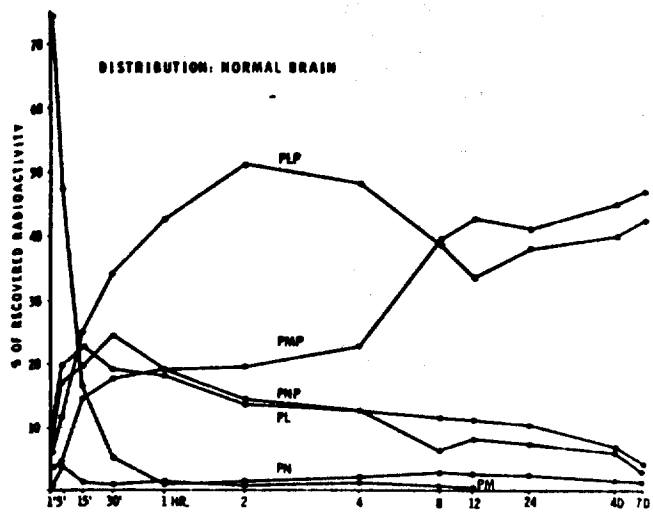

Figure 5. Distribution of retained radioactivity in normal brain. Swiss-Webster mice were injected with ^{14}C-PN and treated as described in Figure 2. Results are expressed as distribution among B_6 vitamers of radioactivity retained by normal brain

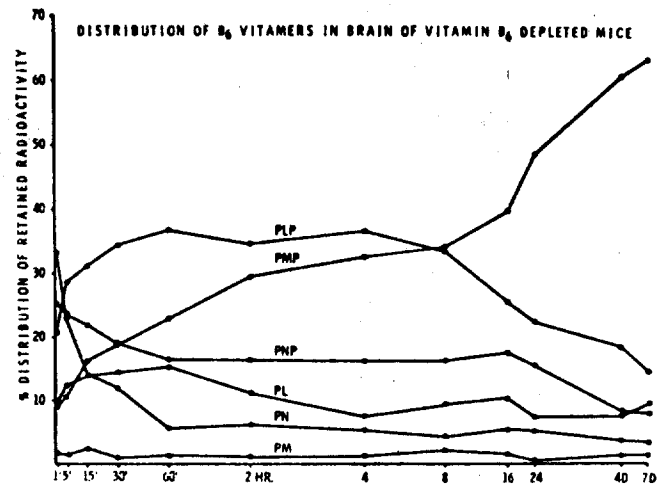

Figure 6. Distribution of retained radioactivity in brain of vitamin B_6 deficient mice. Conditions of administration and results expressed as in Figure 5

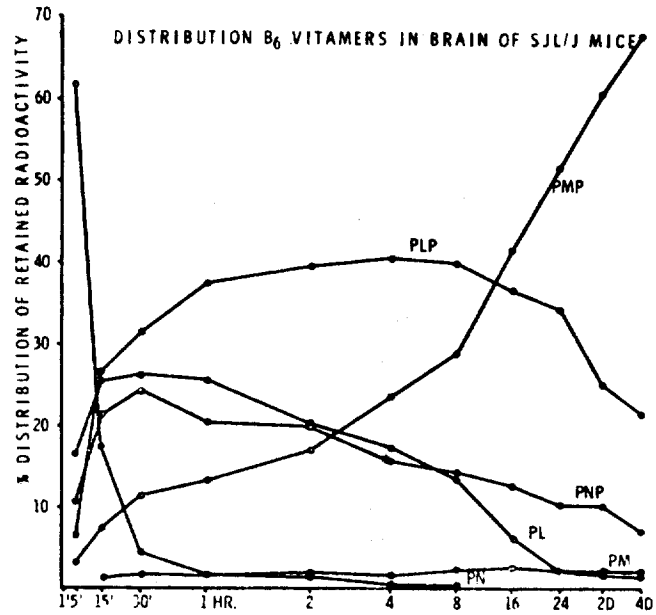

Figure 7. Distribution of retained radioactivity in brain of convulsogenic mice. Conditions of administration and results expressed as in Figure 5

PLP, a larger proportion was present as PL, PNP, and PN than in liver.

EFFECTS OF MARIJUANA ON PN TRANSFORMATION

The data on transformation of vitamin B_6 in liver, brain, and carcass of normal mouse indicated that PN was rapidly transformed to PLP and PMP and retained in these forms. The described technique was used to study the effects of various drugs on vitamin B_6 metabolism in brain. The red oil of marijuana was administered as a single dose of 500 μg/kg or daily for 8 days at a dose of 300 μg/kg intraperitoneally to Swiss-Webster mice and contained 20% Δ^9-tetrahydrocannabinol. Continued red oil administration resulted in increased retention of radioactivity when the animals were sacrificed 30 min after ^{14}C-pyridoxine administration but the distribution of retained radioactivity was markedly altered (Table I). The percent of radioactivity retained as PLP and PL was decreased while that of PNP and PMP was increased. This suggested that prolonged administration of the red oil of marijuana inhibited the activity of pyridoxine oxidase in brain.

PN TRANSFORMATION IN BRAIN OF B_6 DEPLETED AND CONVULSOGENIC MICE

The distribution of retained radioactivity was studied in young adult male Swiss-Webster mice after receiving for 40 days a vitamin B_6 deficient diet (Nutritional Biochemicals Corporation). The metabolism of vitamin B_6 in mice that had received the deficient diet was compared to normal and audiogenic seizure-prone mice strain SJL/J obtained from Jackson Laboratories, Bar Harbor, Maine. Normal, B_6 depleted and seizure-prone mice all received 0.5 ml of ^{14}C-PN containing 5.3 μCi and 115 μg of PN. The results are expressed as the percent distribution of total retained radioactivity at times indicated, among all B_6 vitamers. In normal mice the highest percent of retained PLP occurred 2 hr after injection of ^{14}C-PN, whereas in depleted and convulsogenic mice the maximum percent retention occurred at 1 hr (Figure 5). This finding suggests a more rapid conversion of retained radioactivity to PLP in these mice compared to normal. The percent distribution of retained radioactivity in brain for the first 8 hr (Figures 5, 6, and 7) after administration of radioactive ^{14}C-PN was similar for normal, B_6 depleted, and convulsogenic mice. By 24 hr, however, the percent of radioactivity retained as PMP was greater in both SJL/J and B_6 depleted mice compared to control animals (Figures 6 and 7) while the percent retained as PLP was lower. These studies together with a study in progress of effects of amphetamine on B_6 metabolism suggest that where the metabolism of vitamin B_6 is increased, the percent retention of PMP is increased. The mechanism of this observation is not clear at this time.

CONCLUSION

The pathways for the normal metabolism of vitamin B_6 are known and some data exist on the rate of transformation of PN to the phosphorylated forms. Although considerable information is available on properties of the enzymes which catalyze phosphorylation (White and Dempsey, 1970) and oxidation (Wada and Snell, 1961) of the vitamers, there is limited data on control mechanisms for pyridoxal kinase and very little data on control mechanisms operating on pyridoxine

phosphate oxidase and aldehyde oxidase. Although the total body half-life of administered ^{3}H-PN has been calculated (Contractor and Shane, 1968), there is no data on turnover of vitamin B_6 in brain or liver. However, techniques are now available for this work to be done. The work on interconversion and distribution of vitamin B_6 in liver, brain, and carcass of normal mice (Colombini and McCoy, 1970b) has laid the foundation for further work of the overall effects on B_6 metabolism of drugs such as amphetamines or reserpine known to alter single steps in the metabolism of this vitamin. It is hoped that soon data will be available on the turnover of each of the B_6 vitamers and of the factors which control B_6 turnover in brain, liver, and carcass.

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Certain Effects of Dietary Pyridoxine and Casein on the Carcinogenicity of *p*-Dimethylaminoazobenzene*

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Miner and his associates (11) studied the incidence of liver tumors in rats fed *p*-dimethylaminoazobenzene in synthetic diets containing 12 per cent of purified casein and various levels of the B vitamins. They observed that lowering the pyridoxine content of the diet from 2.5 to 0.2 mgm. per kgm. of diet decreased the incidence of hepatomas at 6 months from 67 per cent to 7 per cent. For a further test of this dietary effect Kline and his co-workers (4) inoculated rats and mice receiving high or low levels of pyridoxine with malignant tumors. In both species the number of takes, the percentage of regressions, and the size of the surviving neoplasms indicated that the diets low in pyridoxine retarded the growth of these tumors. Similar experiments by Bischoff, Ingraham, and Rupp (1) are in agreement with these observations. Kline and his group (4) further showed that the tumor incidence of mice painted with methylcholanthrene was 36 per cent on a low pyridoxine diet and 62 per cent when a high level of this vitamin was fed.

However, subsequent experiments in this laboratory have indicated that the effect of low levels of pyridoxine on the carcinogenicity of *p*-dimethylaminoazobenzene is more variable than the protection given by hydrogenated coconut oil (9, 10) or by high levels of riboflavin (3, 11). Furthermore, Miller and Baumann (6) showed that adult rats on diets free of pyridoxine survived much longer if they had received abundant supplies of pyridoxine during the period of growth than if only minimal amounts had been fed during this period. The present study was therefore designed to determine if the amount of pyridoxine fed to young rats would subsequently influence the formation of hepatic tumors due to *p*-dimethylaminoazobenzene on diets low in pyridoxine. Since pyridoxine or its derivatives appear to be important in protein metabolism (2, 5, 7), both high and low levels of casein were fed with two levels of pyridoxine.

METHODS

Equal numbers of male and female albino rats of the Sprague Dawley strain were raised from weaning on synthetic diets (Table I) containing either 1.5 or 6.0 mgm. of pyridoxine per kgm. The rats fed the high pyridoxine diet reached an average weight of 175 gm. in 8 weeks, while those receiving the lower level of this vitamin required 9 weeks to attain the same weight. At these times they were divided into groups of 15 to 17 members of comparable weight and sex, and fed several different diets (Table I) containing 0.06 per cent of *p*-dimethylaminoazobenzene. The quantities of vitamins in the latter diets were those previously found to permit the induction of liver tumors under our conditions (11), although higher levels of some of the vitamins were fed for the growth of the young rats. Of those raised on either the high or low pyridoxine diets, one group was given 0.2 mgm. and another 2.5 mgm. of pyridoxine per kgm. of a 12 per cent casein diet. In addition,

TABLE I: COMPOSITION OF DIETS

	For growth	For tumor induction
	gm. per kgm.	gm. per kgm.
Purified casein (9)	180	120 or 480
Corn oil	50	50
Salts	40	40
Glucose monohydrate	730	790 or 430
p-Dimethylaminoazobenzene		0.6
	mgm. per kgm.	mgm. per kgm.
Pyridoxine hydrochloride	1.5 or 6.0	0.2 or 2.5
Thiamine hydrochloride	3.0	3.0
Nicotinic acid	3.0	—
Calcium pantothenate	13.0	7.0
Riboflavin	2.0	2.0
Choline chloride	1000.0	300.0

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two groups of the rats raised on the low pyridoxine diet were fed 48 per cent casein diets containing 0.2 or 2.5 mgm. of pyridoxine per kgm. The rats received 1 drop of halibut liver oil weekly during the growth period, and 1 drop monthly for the rest of the experiment. They were kept in groups of 5 to 8 in screen-bottomed cages, and food and water were given *ad libitum*. At 3 week intervals the amount of diet consumed by each group of rats was determined over a 3 day period.

The azo dye was incorporated in the diets by dissolving it in the fat with heat. The rations were mixed every 2 to 4 weeks and stored at 0° C. After the rats had been on the diets containing the azo dye for 120 days, their livers were examined by laparotomy and the animals were continued on the same diet as before, but minus the carcinogen, for an additional 60 days. The rats were then killed and a final examination of the livers was made.

RESULTS

The level of pyridoxine fed during the period of tumor induction was reflected by the health of the animals. The rats on the high pyridoxine diets (Table II, Groups 1, 3, and 5) ate about 10 gm. of food daily and gained 20 to 40 gm. during the dye feeding period, whereas those receiving the low level of this vitamin (Table II, Groups 2, 4, and 6) ate only 8 gm. daily and lost 20 to 40 gm. in the first 4 months. It was necessary to supply each of these rats with 2 mgm. of pyridoxine every day by dropper during the ninth and 12th weeks, and to raise the pyridoxine content of the diets from 0.2 to 0.3 mgm. per kgm. during the 13th to 16th weeks. After feeding of the dye was stopped at 4 months it was possible to maintain the rats for the rest of the experiment with 0.2 mgm. per kgm. of diet.

Effect of Pyridoxine Fed during Growth

In each case the rats receiving the low pyridoxine diets with the carcinogen had fewer hepatomas than their controls, but the protection afforded by the low pyridoxine diets was much greater for rats that had received only minimal amounts of this vitamin prior to the dye. Thus those raised on the low level of pyridoxine and then fed the 12 per cent casein diets containing *p* dimethylaminoazobenzene and 0.2 or 2.5 mgm. of pyridoxine per kgm. had tumor incidences of 19 and 60 per cent respectively at 4 months, and 50 and 87 per cent at 6 months (Groups 3 and 4). Of the rats raised on the high level of pyridoxine and subsequently fed the above diets, 19 and 41 per cent of those receiving 0.2 or 2.5 mgm. of pyridoxine respectively had neoplasms at 4 months, and 81 and 100 per cent developed tumors by 6 months (Groups 1 and 2). Of these animals (Groups 1 to 4) the lowest tumor incidence was observed in Group 4; this group also consumed the least food. Similarly, the extent of cirrhosis in all the groups paralleled the number of neoplasms; it was mild in the groups fed the low level of pyridoxine and moderate in those given the higher level.

Table II: The Incidence of Hepatic Tumors in Rats Raised and Maintained on Synthetic Diets High and Low in Pyridoxine and Casein

Group no.	Growth diet. Pyridoxine per kgm., mgm.	Maintenance diet * Pyridoxine per kgm. mgm.	Maintenance diet * Casein, per cent	Average weight increment at 4 mo., gm.	Average daily food intake,† gm. per rat	Survival ‡ at 4 mo.	Incidence § of tumors 4 mo.	Incidence § of tumors 6 mo.	Negative survivors at 6 mo.	Cirrhosis
1	6.0	2.5	12	56	10.1	15/15	7/15	15/15	0	Moderate
2	6.0	0.2	12	19	8.3	16/17	3/16	13/16	3	Mild
3	1.5	2.5	12	20	9.7	15/15	9/15	13/15	1	Moderate
4	1.5	0.2	12	-29	7.7	16/17	3/16	8/16	8	Mild
5	1.5	2.5	48	41	11.3	12/15	9/12	11/12	1	Mild
6	1.5	0.2	48	-43	7.6	11/17	0/11	1/14	10	Mild

* Contained 0.06 per cent *p* dimethylaminoazobenzene for first 4 months.
† The average of 5 determinations at 3 week intervals of the food consumed over a 3 day period.
‡ Survival = number alive at end of 4 months over number at start.
§ Incidence = number with tumors over number of survivors at 4 months.

Effect of Level of Protein

A second factor that affected the carcinogenicity of *p* dimethylaminoazobenzene in diets low in pyridoxine was the level of casein. This is seen by comparing the four groups of rats raised on the low level of pyridoxine. The rats fed 48 per cent of casein and the low level of pyridoxine (Group 6) had no tumors at 4 months and only 1 (7 per cent) was noted at 6 months, while the rats that received 12 per cent of casein and the same level of pyridoxine (Group 4) had an incidence of 19 and 50 per cent at 4 and 6 months respectively. Each of these groups consumed

approximately the same amount of food, and the livers were mildly cirrhotic. Most of the rats receiving 12 or 48 per cent of casein and the high level of pyridoxine (Groups 3 and 5) had hepatomas by 4 months; these incidences were comparable despite the higher food consumption and milder cirrhosis of Group 5.

DISCUSSION

These experiments demonstrate several facts that have both specific and general implications concerning the control of the formation of experimental tumors by dietary means. Specifically they illustrate the necessity of adequate amounts of pyridoxine for the genesis of tumors induced by *p*-dimethylaminoazobenzene. Of greater importance, perhaps, is the demonstration that the nutritional history of an animal can have a definite influence on the subsequent formation of hepatic neoplasms. It is possible that the level of other dietary ingredients fed during the growth period may have analogous effects, and variations in the results of similar experiments could be explained by the differences in the nutritional background of the animals employed. For instance, since large amounts of dietary riboflavin protect rats from liver tumors due to the azo dye (3, 11), animals with large stores of this vitamin might be more resistant to the carcinogen than those with minimal stores. It is apparent, therefore, that in any comparison of results among groups of rats obtained from different sources, it is important to consider the dietary history of the animals. The data also demonstrate that efficient tumor induction by *p*-dimethylaminoazobenzene is feasible in rats both raised and maintained on highly purified diets.

In an earlier study (6) adult rats that had been raised from weaning on diets containing high levels of pyridoxine were depleted of the vitamin less readily than those given less during the growth period. Similarly, in this series the animals fed the low pyridoxine growth diet prior to the low-pyridoxine:low-casein diet containing *p*-dimethylaminoazobenzene appeared to be more deficient than those fed the same azo dye diet but given more pyridoxine during the period of growth. This was evident from the smaller amounts of food consumed and the greater loss of weight of the rats raised on the low level of the vitamin. Accordingly, it is probable that the lower tumor incidence of the rats raised on the low-pyridoxine diet was due to their greater average degree of deficiency during the period of tumor induction and growth.

Several studies have indicated that pyridoxine is involved in protein metabolism. For example, the requirement of mice for pyridoxine was found to be 3 to 4 times greater on 60 per cent of casein than when only 20 per cent was fed (7). Rats (5, 8) or mice (7) deficient in pyridoxine excreted 10 to 20 per cent of ingested *l* tryptophane as xanthurenic acid, while practically none of this compound was excreted by control animals. Furthermore, pyridoxal stimulated greatly the decarboxylation of tyrosine by cell suspensions of *Streptococcus faecalis R* (2). The results of the present experiment also suggest a relationship between protein metabolism and pyridoxine. With adequate pyridoxine an increase in the level of casein in the diet from 12 to 48 per cent did not affect the tumor incidence; but when a low level of pyridoxine was fed, the rats receiving 48 per cent of casein developed fewer hepatomas than those on 12 per cent of casein. After 6 months only 1 of 14 rats on the high protein diet had developed a tumor, as compared to a 50 per cent incidence in the group on the lower level of casein. Since the metabolism of large amounts of casein appears to cause a more rapid depletion of the pyridoxine stores of rats, those fed the high casein: low-pyridoxine diet were probably more deficient than those on the low casein:low-pyridoxine diet. This would explain also the greater weight loss and poorer physical condition of the deficient rats on the high protein diet.

Although the amount of food eaten by the rats in each of the groups varied from 7.6 to 11.3 gm. per day, these differences apparently were not responsible for the effects observed. Of the rats receiving the azo dye in the low pyridoxine diets, those on the high and low levels of casein (Groups 4 and 6) ate approximately the same amount of food; yet the tumor incidences were 7 and 50 per cent respectively at 6 months. Further, while the daily food intakes of the groups fed the high level of pyridoxine differed by as much as 1.6 gm. the percentage of neoplasms in the three groups was very similar. These results are comparable to those observed in earlier studies (9, 10, 11), in which rats with the highest incidences of tumors have frequently consumed less diet and carcinogen than the protected animals.

The mechanism by which a lack of pyridoxine inhibits tumor formation has not been studied, but it seems possible that after the animals reach a certain state of deficiency they may be unable to utilize a sufficient amount of one or more amino acids for the synthesis of protein. In such a situation the synthesis of protein in both normal and neoplastic tissue could not proceed, which would account for the loss in weight and for the failure of tumor induction and growth.

SUMMARY

1. Weanling albino rats were raised for 8 to 9 weeks on synthetic diets containing 1.5 or 6.0 mgm. of pyridoxine per kgm. Groups of 15 to 17 were then fed 0.06 per cent of *p*-dimethylaminoazobenzene in synthetic diets containing 12 or 48 per cent of casein and 0.2 or 2.5 mgm. of pyridoxine per kgm. After 4 months the livers were examined by laparotomy; the rats were continued on the same diet without the dye for another 2 months, and were then killed for a final tumor count.

2. In each case the rats on the low pyridoxine diets containing the azo dye had fewer tumors than their controls receiving more of this vitamin. However, of the rats fed the low-pyridoxine:azo-dye diets those raised on the low pyridoxine diet had fewer hepatomas than the animals that received a higher level during the period of growth.

3. Substitution of 48 per cent of casein for the usual 12 per cent level in the low pyridoxine diets decreased the incidence of liver tumors from 50 to 7 per cent. When the rats received adequate pyridoxine the tumor incidence on 48 per cent of casein was equal to that on the 12 per cent casein diet.

4. The results demonstrated that the number of hepatomas induced on a given diet might be influenced by the nutritional history of the animals. Since high incidences of tumors were observed in rats raised and maintained on highly purified diets, such diets can be used in experiments where it is desirable to control the nutritional background.

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VITAMINS AS PHARMACOLOGICAL AGENTS

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A discussion of vitamins as pharmacological agents appears at first to be a rather uninteresting undertaking, since in comparison with other potent remedies these substances fail to produce conspicuous pharmacodynamic effects. Indeed, the *range* between the therapeutic and the lethal dose is so wide, that one might feel justified to place vitamins in the same category as salt, sugar, or other basic food elements which are essential from the nutritional viewpoint, but practically inert in the pharmacological sense. However, such an opinion would be erroneous since vitamins are pharmacologically quite active, provided they are investigated in animals suffering from a specific vitamin deficiency.

The fact that a drug, in order to exhibit its pharmacodynamic effect, has to be tested under certain pathological conditions, is not unusual. Antipyretics, spasmolytics and analgesics may be cited as other examples. However, while the pathological condition necessary for demonstrating the effect of these drugs may be produced in a variety of ways, a vitamin affects only those conditions which result from a deficiency of that particular agent; in this respect, vitamins resemble in their therapeutic specificity the immune sera

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or antitoxins. Failure to recognize this accounts not only for a large number of ill-founded therapeutic claims, but also for many of the disappointments of vitamin therapy and the skepticism originating therefrom.

It can readily be seen, that a discussion of vitamins as pharmacological agents must distinguish between their effect in the normal and in the vitamin deficient animal. In normal animals their action is rather unspecific and pharmacodynamic effects are produced only by doses several thousand times larger than those needed for a therapeutic effect (table 1).

The only vitamin which produces a distinct pharmacologic effect even in therapeutic doses, is nicotinic acid. Its administration is followed in humans by a transitory peripheral vasodilatation. However, this effect cannot be ascribed to its nature as a vitamin, since it fails to occur on the administration of nicotinamide, which is equally effective as a vitamin.

TABLE 1

Lethal doses of B vitamins as compared with their daily requirement

	SPECIES	DAILY REQUIREMENT	L.D. 50 ORAL	RATIO
Thiamin	mouse	3γ	100 mgm.	30,000
Riboflavin	mouse	3γ	>7 mgm.	>2,000
Nicotinic acid	dog	0.25 mgm.[1]	>2 grams[1]	>8,000
Pyridoxine	rat	10γ	1 gram[2]	100,000
Pantothenic acid	rat	100γ	>2 gram[2]	>20,000

[1] Per kilogram body weight.
[2] Per rat (200 grams body weight).

With therapeutic margins of from 1:1000 upward, the danger of *acute* toxicity is obviously of very minor concern to practical vitamin therapy. The question of *chronic* toxicity deserves more attention since vitamins, due to their nature as nutritional factors, are likely to be taken over a prolonged period of time and without supervision by a physician. Fortunately, all information presently available indicates that the danger of chronic hypervitaminosis is practically nonexistent. Daily feeding of up to several hundred times the maintenance doses of thiamin, riboflavin, pyridoxine, nicotinic acid and pantothenic acid over the entire life span of rats fails to produce toxic effects. However, since pathologic changes resulting from prolonged administration of large doses of vitamins may become manifest only in the second generation, toxicity experiments should be extended beyond the first generation. Thus, Perla (1) has shown that in rats prolonged administration of thiamin causes in the third generation loss of maternal instinct and interferes with lactation; Sure (2) has made similar observations and we obtained such findings in an experiment extended over 4 generations.

According to Perla (3), this toxicity is due to a relative lack of manganese in the diet and can be prevented by addition of traces of this element. Thus, ordinarily innocuous doses of vitamins may become harmful under certain conditions. Unfortunately, not much experimental data is as yet available to elaborate on this point. V. A. Drill (4) reported, that hyperthyroid female rats, which had lost weight and were still receiving a thyroid preparation, regained their weight when both thiamin and vitamin B complex were administered; and Sure and Smith (5) found that in such animals the weight loss could be retarded by administration of a potent vitamin B concentrate. In a later paper, Sure and Buchanan (6) state that pure thiamin in daily doses of 30 to 100 micrograms counteracts the toxic effects of 0.2 mgm. thyroxine daily. In our laboratory we have

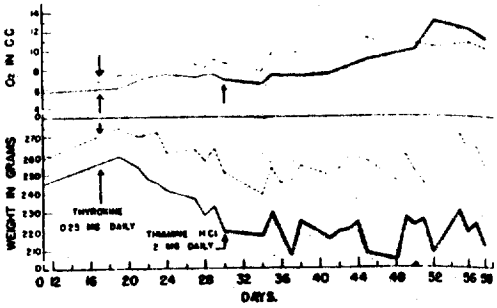

Fig. 1. Effect of thiamine on weight and O_2 consumption of hyperthyroid rats. ..... indicates the average weight. Curves represent the averages of a group of 4 rats. All animals received 0.25 mgm. thyroxine and those represented in the lower curve received 2 mgm. thiamin hydrochloride at the point indicated.

investigated the influence of hyperthyroidism on the toxicity of very large doses of thiamin. Rats were given daily doses of thyroxine and simultaneously, or after establishment of a hyperthyroid state, large amounts of thiamin were administered. In addition to daily determination of the oxygen consumption and body weight frequent records were taken of the heart and respiratory rate and the body temperature. Although more than 200 rats were used in these experiments, the normal and hyperthyreotic rats did not differ significantly in their sensitivity to thiamin (fig. 1).

A question of practical importance is whether an imbalance in a combination of vitamins may lead to toxic effects which are not observed if the vitamins are given in their naturally occurring mutual relationship. This problem has attained particular prominence since the enrichment of foods with selected members of the vitamin B

complex has become an established practice, although even those investigators who believe in the possibility of an increased vitamin toxicity due to imbalance have always emphasized, that this danger does not exist for the relatively minute quantities of vitamins incorporated into flour, bread or other foodstuffs. The only published experimental data indicating an adverse effect of vitamin imbalance are those of A. F. Morgan (7), who showed that dogs suffering from a combined nicotinic acid and pantothenic acid deficiency lived longer than those on nicotinic acid or pantothenic acid deficiency alone.

In our laboratory the effect of pantothenic acid or riboflavin was studied in adult dogs during a combined pantothenic acid-riboflavin deficiency (table 2). No increase in toxicity of either vitamin was found in these conditions. Whether the dogs were on the combined deficiency or only on riboflavin deficiency, the same length of time

TABLE 2

Progress of depletion symptoms in riboflavin and riboflavin-pantothenic acid deficient dogs

DOG NO.	DEFICIENCY	COLLAPSE AFTER	RIBOFLAVIN EXCRETION IN γ/24 HOUR				
			1st week	15th week	22nd week	28th week	29th week
		weeks					
108	Riboflavin	23	77	15	*6*		
115	Riboflavin	29	30	15	17	*8*	
103	Riboflavin and Pantothenic Acid	30	175	28	27	36	*11*
113	Riboflavin and Pantothenic Acid	22	37	12	*8*		

was required to produce collapse and the precollapse level of riboflavin in the urine was essentially the same in both groups.

It has been reported that during a multiple deficiency correction of only one component tends to aggravate the symptoms of the other deficiencies. Sydenstricker (8), Spies and others described the development of nicotinic acid and riboflavin deficiency symptoms in sub-acute cases of pellagra treated only with thiamin. György (9), Chick (10) and Harris (11) have shown that in rats maintained on diets deficient in both riboflavin and vitamin B_6, the dermatitis characteristic of the lack of the latter does not become manifest until riboflavin is supplied. Daft and Sebrell (12), and Lepkovsky, Jukes and Krause (13) have made similar observations on the appearance of pantothenic acid deficiency lesions in rats deficient both in pantothenic acid and pyridoxine. However, such observations must not necessarily be regarded as evidence for an adverse effect of a vitamin imbalance because they may merely indicate that some deficiencies require more time for development than others, time which is provided in these experiments through the cure of that deficiency which occurred first.

In a series of experiments undertaken in our laboratory, the effect of prolonged administration of large doses of single vitamins of the B group on the course of multiple deficiencies was investigated. There was no indication of an increased toxicity (14). Similarly, we have been unable to demonstrate a greater toxicity of thiamin in animals suffering from riboflavin deficiency (table 3).

As with other medicinal agents, the question of optimal dosage, most effective spacing and preferred way of administration deserves careful consideration.

It appears advisable to distinguish between a *maintenance* dose, sufficient to provide an adequate supply of the vitamin in a non-depleted organism and a *curative* dose, necessary to restore

TABLE 3

Acute toxicity of thiamine in normal and riboflavin deficient rats

MODE OF ADMINISTRATION	NORMAL RATS		RIBOFLAVIN DEFICIENT RATS	
	L.D. 0	L.D. 50	L.D. 0	L.D. 50
	mgm./kgm.	*mgm./kgm.*	*mgm./kgm.*	*mgm./kgm.*
S.C.	500	1400	>750	1500
P.O.	6000	9500	6000	9000

to normal a vitamin depleted animal. Obviously, the curative dose is usually much larger than the maintenance dose, making it advisable to administer the vitamin in its pure form and, quite frequently, by parenteral injection.

Contrasted to this strictly medicinal use is the equally essential use of vitamins for the maintenance of dietary balance. The doses required for this purpose are much smaller than those needed for therapy and can be supplied through a carefully selected diet or specially prepared and enriched foodstuffs.

Since there is practically no danger of overdosing with any of the vitamins, the principal points of interest are those related to absorption, storage and excretion as they have a direct bearing on the mode of administration. The various groups of vitamins differ greatly in these properties. Generally speaking, the fat soluble vitamins A and D are stored to a greater extent than the water soluble vitamins B and C. This influences not only the rapidity with which deficiencies may develop, but also the efficiency of different modes of administration.

As with other medicinal agents, the availability of a vitamin to the organism is determined by the extent to which it may be stored, absorbed, destroyed and excreted. Since all of these factors are closely interrelated, conclusions which are based on only one of them must be drawn with great reservation. Thus, the difference between the dose administered and excreted is not necessarily an indication of the basic requirement. For example, of a dose of 100 micrograms of pantothenic acid given to a rat, 7 micrograms were excreted. However, when 250 micrograms were given only 67 were excreted and with 3000 micrograms only 600 micrograms could be found in the urine. On the basis of these findings, the requirement would appear to be 93, 183 and 2400 micrograms respectively, while actually it is about 100 micrograms. The difference in the foregoing findings must be attributed either to storage, non-absorption or destruction; as a rule each of these factors plays a rôle.

TABLE 4

Oral and intravenous toxicity of B vitamins in rats

	INTRAVENOUS	PERORAL
	gm./kgm.	*gm./kgm.*
Thiamin	0.170	9.5
Riboflavin	0.560	more than 10
Nicotinamide	2.2	3.5
Pyridoxine	0.657*	5.5
Pantothenic acid	0.830	more than 10

* Weigand, Eckler and Chen, Proc. Soc. Exp. Biol. & Med., 44, 147, 1940.

Vitamins being essential food elements, they can obviously be effectively administered by mouth. However, under certain pathological conditions, such as severe gastro-intestinal disturbances, their absorption may be greatly delayed or even completely absent and in these cases parenteral administration is indicated.

Administration of vitamins by other than the peroral route influences the relative toxicity as well as the efficacy. As with most other drugs, the acute intravenous toxicity is generally considerably greater than the subcutaneous, and in turn, the peroral (table 4). Furthermore, the intravenous toxicity depends much upon the rate of injection (table 5). Whether the *intravenous* or *subcutaneous administration* of a vitamin is therapeutically more effective than its peroral administration depends upon several factors of which absorption from the gastrointestinal tract is only one. Intravenous injection produces not only (though temporarily) a much higher concentration of the vitamin in the blood, but probably also in the tissues. Thus, intravenous injection of 4 mgm. per kgm. of riboflavin to dogs in the form of its sodium salt raises the blood level 15 to 20 times above that obtained by feeding an excess dose by mouth; and the riboflavin content in the liver of rats increased from 35 micrograms per gram to 160 micrograms per gram after a dose of 500 mgm. per kgm.

The fact that parenteral administration can raise the concentration of a vitamin in the blood and tissues far above that obtainable by feeding of even the largest doses may explain certain therapeutic successes obtained by clinicians through parenteral administration of relatively enormous doses of vitamins. Furthermore, with

TABLE 5

Influence of rate of injection on intravenous thiamine toxicity

DOSE	NO. OF ANIMALS	VOLUME INJECTED	CONCENTRATION OF SOLUTION	RATE OF INJECTION	NO. DEAD
mgm./kgm.		*c.c.*	*per cent*	*cc./min.*	
90	10	0.25	0.72	0.1	1
90	10	0.25	0.72	0.2	5
90	10	0.25	0.72	0.4	7

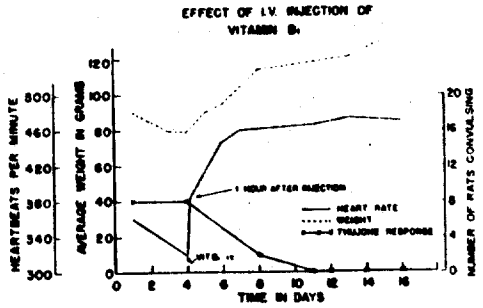

Fig. 2. Effect of intravenous injection of vitamin B_1.

this mode of administration the pharmacodynamic effect is likely to take place with great rapidity. Thus, the effect of thiamin on bradycardia due to deficiency of this vitamin can be observed within less than 1 hour (fig. 2). Still more dramatic is the life-saving effect of intravenously injected vitamins to animals in collapse from pantothenic acid or riboflavin deficiency or to beriberi patients suffering from acute cardiac decompensation. However, even when given by mouth, the therapeutic effect of most vitamins takes place very rapidly, provided the absorption from the gastro-intestinal tract is still normal.

While certain therapeutic effects may be more readily achieved by parenteral, particularly

intravenous administration, this procedure should probably be reserved for specific indications, since it introduces some of the hazards common to all injections made directly into the blood stream. One of these is anaphylactic shock. Rare cases of such incidents have been reported following the intravenous injection of thiamin. In our laboratory we have investigated the sensitizing effect of thiamin in dogs and guinea pigs. Ten dogs were divided into two groups. One group received on 10 consecutive days 50 mgm. per kgm. of thiamin hydrochloride intravenously, an amount sufficient to produce severe toxic reactions such as excessive salivation, nausea, vomiting and collapse. After 10 daily injections, an interval of 10 to 60 days was allowed before the final experiment was performed. The second group of dogs received no pretreatment. There was no significant difference between the two groups. However, upon autopsy it appeared that in some of the dogs which had received previously 10 doses of thiamin, the right heart was dilated and

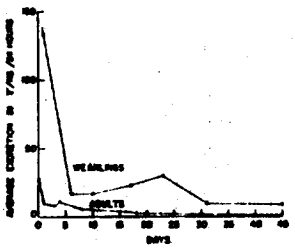

Fig. 3. Urinary excretion of pantothenic acid in weanling and adult dogs during depletion.

the abdominal organs showed greater congestion, while this was not observed in any of the controls. The outcome of the guinea pig experiments was not conclusive.

Normal as well as depleted animals excrete a certain amount of the administered vitamin through the urine. Both the absolute amount and the percentage of excretion (with or without administration of a test dose) have been used as a measure of the state of relative saturation and as indicator of the degree of deficiency. It is in the urine, where the first sign of vitamin depletion can usually be found (fig. 3). However, it must be remembered, that without the use of a test dose the urinary level may merely be a measure of the immediately preceding vitamin intake. In order to be significant, such "saturation tests" should therefore be conducted in fasting animals and after administration of a test dose.

In contrast to the wide fluctuations in the vitamin content of the urine, the concentration in the blood remains usually remarkably constant and such determinations offer therefore little help in the diagnosis of early vitamin deficiencies.

While the difference between the amount of a vitamin administered and excreted is not necessarily an indicator of the *maintenance* dose, it can nevertheless serve as a guide for the determination of the appropriate *therapeutic* dose. This is shown in the following experiment, in which the same dose of pantothenic acid was daily given to pantothenic acid depleted dogs and its urinary excretion determined. It may be seen that at least 4 to 16 times the maintenance dose (previously determined in actual feeding experiments) had to be given over a period of 1 to 2 weeks until saturation was accomplished. This again emphasizes the justification for apparently excessive vitamin doses in cases of advanced deficiencies.

Other factors which may considerably affect the dosage of vitamins are diet and age. For example, thiamin is necessary for the utilization of carbohydrates and consequently larger doses are needed when the carbohydrate intake is high.

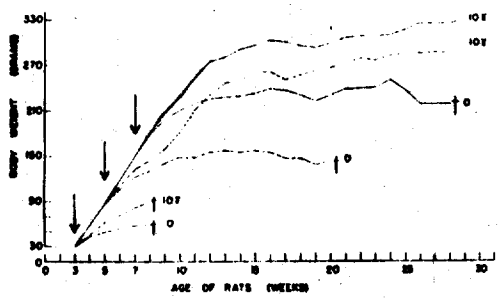

Fig. 4. Influence of age on pantothenic acid requirement. ↓ indicates when groups were changed from full diet to depletion diet. † indicates death of 50 per cent of the animals in group.

Similarly, it has been reported that the rat requirement for riboflavin or pyridoxine increases when a high fat diet is given.

The influence of age upon vitamin requirements depends upon the specific vitamin studied. Generally, a growing organism requires relatively larger amounts than an adult. This is particularly true for pantothenic acid where with increasing age a striking decrease takes place not only in the relative, but also in the absolute requirement (15) (fig. 4).

In the preceding paragraphs the general toxicity of vitamins and their absorption, storage and excretion have been discussed, but nothing has as yet been said about their pharmacodynamic properties. The reason for this is that in healthy animals vitamins in doses below the toxic range fail to exhibit such effects. Nevertheless, in a vitamin depleted animal they are capable of restoring to normal the pathological changes caused by lack of a specific vitamin and under

such conditions they compare in potency favorably with the most powerful medicinal agents, having a marked effect in doses within the milligram range.

The high specificity of vitamins can easily be demonstrated by comparing their effect upon pathological changes which, although closely resembling each other, are due in one instance to a specific vitamin deficiency and in the other to a non-specific cause. This is illustrated in the following experiment (fig. 5): Of two groups of rats one was placed on a thiamin deficient diet, while the other receiving a complete diet was restricted in its food intake to that of the depletion group. In both groups frequent records were taken of the weight, the heart rate and the sensitivity to a convulsing dose of thujone. It may be seen that in both groups convulsions could be produced more readily as the experiment progressed. However, only in the thiamin deficient group did the heart rate show the typical retarda-

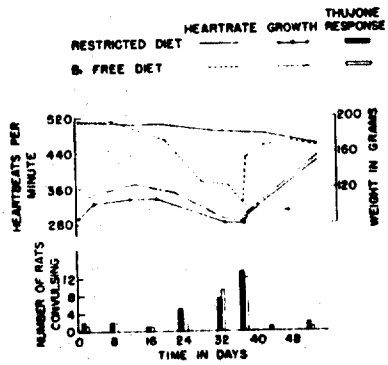

Fig. 5. The influence of thiamin on heart rate, growth and response to thujone.

tion, which responded immediately to thiamin administration. The increased sensitivity to the convulsant drug was, on the other hand, a result of the general state of inanition and responded therefore in both groups with equal promptness to an increased intake of food (16).

The observation of A. Keys (17) that administration of vitamins is without influence on the performance of athletes already living on an adequate diet, can be cited as further evidence that vitamins exhibit their effect only in specific deficiencies. However, if the subjects in Keys' experiment would have lived on a suboptimal vitamin intake, a beneficial effect of vitamins on the endurance might have been found. In our laboratory such an effect could be demonstrated in vitamin B_1 deficient rats. In experiments still in progress the endurance of rats in various stages of vitamin deficiencies is being measured by their ability to carry a given load while swimming. During thiamin deficiency, the work performance decreases to a very low level; administration of the vitamin improves it greatly, even if the food intake remains restricted (table 6).

A decrease in endurance due to a suboptimal intake of vitamins is but one of the many appar-

TABLE 6

Effect of thiamin-depletion on endurance

RAT NO.	WEIGHT	DATE	SWIMMING TIME	MEDICATION	REMARKS
	grams		*minutes*		
112	36	1/28	0	10γ Thiamin 4 gm. food	Fed and dosed after swimming
	35	1/29	12	4γ Thiamin 5 gm. food	
	36	1/30	14	4γ Thiamin 5 gm. food	
114	49	1/28	0	10γ Thiamin 4 gm. food	Fed and dosed after swimming
	49	1/29	16	4γ Thiamin 5 gm. food	
	50	1/30	60+	4γ Thiamin 5 gm. food	
119	52	1/28	0	10γ Thiamin 4 gm. food	Fed and dosed after swimming
	53	1/29	60+	4γ Thiamin 4 gm. food	
	56	1/30	60+	4γ Thiamin 4 gm. food	
SFE-1	53	3/21	6	10γ Thiamin food ad lib.	Control
	60	3/24	4	4γ Thiamin food ad lib.	
	62	3/25	4	4γ Thiamin food ad lib.	
SFE-2	62	3/21	11	10γ Thiamin food ad lib.	Control
	73	3/24	10	4γ Thiamin food ad lib.	
	76	3/25	16	4γ Thiamin food ad lib.	

ently non-specific conditions in which administration of vitamins may prove to be an effective therapeutic measure. While the clinical symptomatology of fully developed vitamin deficiencies, such as rickets, scurvy, beriberi and pellagra, is well known, there are undoubtedly a far greater number of subclinical deficiencies and of other

pathological conditions, in which vitamin deficiency is a complicating factor. The latter respond to vitamin therapy to the extent to which the deficiency is eliminated. Thus, it has frequently been stated that thiamin administration

TABLE 7

Pantothenic acid and riboflavin contents of livers of mice infected with influenza

INFECTED MOUSE	MOUSE WEIGHT	PANTOTHENIC ACID	RIBOFLAVIN	INANITION CONTROL	MOUSE WEIGHT	PANTOTHENIC ACID	RIBOFLAVIN
	grams	*γ per gram*	*γ per gram*		*grams*	*γ per gram*	*γ per gram*
No. 1	17.8	60	31	No. 21	15.5	76	28
2	17.3	71	33	22	17.5	92	29
3	14.4	59	40	23	13.3	79	36
4	15.5	69	34	24	18.5	86	29
5	19.2	60	28	25	18.7	96	32
6	9.3	61	30	26	9.1	80	32
7	14.6	62	29	27	10.3	79	29
8	14.6	56	30	28	10.5	63	24
9	19.5	69	32	29	18.9	83	25
10	15.4	58	25	30	12.1	87	29
11	12.5	59	26	31	10.8	65	27
12	11.9	51	30	32	10.3	79	36
13	16.0	46	21	33	12.6	66	28
14	15.2	49	28	34	12.9	104	30
15	15.5	49	33	35	13.0	94	32
16	17.3	58	33	36	12.2	88	31
17	17.1	58	27	37	15.9	77	25
19	13.8	54	31	39	10.5	96	34
20	11.9	53	32	40	9.5	69	26
Averages....		58	31			82	30

in diabetes mellitus permits a reduction in the necessary dosage of insulin. However, in strictly controlled experiments it can be shown that thiamin itself does not influence the course of the disease; its beneficial effect is probably due to the correction of a mild deficiency which is often produced by imposition of a rigid diet. Similarly, administration of vitamins to patients with gastric ulcers or on other restricted diets may be indicated and may improve the general condition by correcting a nutritional deficiency.

Under certain conditions, as in lactation, pregnancy, tropical environments (increased thiamin excretion through sweating) and infectious diseases, the vitamin requirements are considerably greater than normal. Thus, it has been reported by Mills (18) that the thiamin requirements of rats kept in a tropical climate are about twice those of the control animals. This is due primarily to a decreased food consumption in the hot room and can be corrected by doubling the thiamin concentrations in the diet. Mice on a sub-optimal riboflavin intake are less resistant to pneumococcus I infection (19); and in our own laboratory it has been found that during an infection with influenza virus the pantothenic acid content of the liver is significantly reduced, while the riboflavin content remains constant (table 7).

In summary, it may be stated that vitamins are pharmacological agents of extremely low toxicity which exhibit a pharmacodynamic effect only in an animal depleted of the specific vitamin. However, since mild stages of vitamin deficiencies are quite frequent and may accompany pathological conditions resulting from other causes, vitamins have a wide field of therapeutic application.

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Effects of Excess Thiamine and Pyridoxine on Growth and Reproduction in Rats

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INTRODUCTION

Although studies on vitamin interrelationships have shown little or no effect of excess thiamine or pyridoxine in growing rats (Scott and Griffith, '58; Morrison and Sarett, '59), the study of Richards ('45) and the report of Hunt et al. ('54) suggest that excess amounts of either of these vitamins may have deleterious effects in pregnancy. Richards ('45) found that administration of excess thiamine to female rats fed a white flour-casein diet, simulating a low-quality human diet, had no effect on growth, but adversely affected reproduction, as evidenced by high mortality and poor growth of the young. Convulsions similar to those found in pyridoxine deficiency were observed in some of the offspring, and could be prevented by giving the dams additional pyridoxine during lactation. However, signs of other deficiencies were also observed in the young rats. Hunt et al. ('54) reported that an infant, born to a mother who had received excess pyridoxine during pregnancy, exhibited convulsions which responded to pyridoxine. They postulated that excess pyridoxine intake during pregnancy may increase the pyridoxine requirement of the infant.

The present experiment was conducted to determine whether excess amounts of thiamine or pyridoxine or both affected growth, reproductive performance and vitamin stores in female rats fed an adequate diet, and to what extent these high levels of vitamins in the mothers' diet affected weight gain and vitamin stores in the young when raised on a pyridoxine-deficient diet.

MATERIAL AND METHODS

The basal 18% casein diet used in the experiment was similar to that of Sarett and Snipper ('54) except that sucrose was used as the carbohydrate, and ascorbic acid was omitted from the vitamin mixture. In addition, the levels of thiamine·HCl and pyridoxine·HCl were changed to 150 μg per 100 gm of diet. These levels are slightly greater than those listed by Brown and Sturtevant ('49) as the levels required by the growing rat. Four comparable groups of 12 female weanling rats each (McCollum-Wisconsin strain) were carefully selected from 19- to 21-day-old animals weighing approximately 50 gm. The animals were individually housed in screen-bottom cages and given the basal diet (diet 1), or this diet supplemented with excess thiamine (diet 2), excess pyridoxine (diet 3), or excess thiamine and pyridoxine (diet 4). Excess thiamine and pyridoxine were added at 50 times the levels used in the basal diet.

Records were kept of the amounts of food and water consumed by each rat, and the animals were weighed individually at weekly intervals. After the animals had been on experiment for 12 weeks, they were mated with stock males of proven fertility. As soon as possible after each litter was born, the number and weight of the young were recorded. When necessary, the number of young per litter was reduced to 8 at 5 days of age.

When the young had reached 21 days of age, the dams were sacrificed (without fasting) by intraperitoneal injection of Nembutal[1] solution and the livers, kidneys and adrenals were removed and weighed. The livers of the dams fed each diet were pooled and analyzed for solids, total lipid, thiamine, riboflavin, pyridoxine, pantothenate and vitamin B_{12}. The levels of solids and total lipid were determined by

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[1] Abbott.

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the method of Sarett and Jandorf ('47). Thiamine was determined by the thiochrome method (Pharmacopeia, '55), while riboflavin was assayed fluorometrically (Pharmacopeia, '55). Pyridoxine was extracted by autoclaving the samples at 121°C for 5 hours in 0.055 N HCl, according to the method of Rabinowitz and Snell ('47), and assayed microbiologically with *S. carlsbergensis*. Pantothenate was determined microbiologically with *L. plantarum* by the method of Toepfer et al. ('54), following release of bound forms of the vitamin by the double enzyme system of Novelli et al. ('49). Vitamin B_{12} was extracted with a buffered cyanide solution according to the procedure described by Gregory ('54), and assayed using *L. leichmannii* (Pharmacopeia, '55).

At weaning (19 to 21 days of age) representative young were killed and the livers of the animals from each group were pooled (sexes kept separate) and analyzed for solids, total lipid, thiamine and pyridoxine, by the methods outlined above. The remaining animals were individually housed in screen-bottom cages and were given a diet similar to diet 1, except that pyridoxine was omitted, and the level of thiamine was increased to 200 μg per 100 gm of diet. The weight gains of these animals were followed. After two and 5 weeks, one-third of the surviving males and females from each group were sacrificed. The livers were pooled as before and analyzed for solids, total lipid, thiamine and pyridoxine. The data on liver solids and total lipid levels in these animals showed no effect of maternal diet and are not reported herein. Most of the remaining rats died before the planned 8-week terminal analyses could be performed.

RESULTS AND DISCUSSION

Data on food and water intakes, weight gains and food efficiency values obtained before the animals were mated are summarized in table 1. In 12 weeks, the animals fed the control diet (diet 1) gained 145 gm, with an average food efficiency of 17.9 gm gain per 100 gm food intake. Addition of excess thiamine (diet 2) or excess pyridoxine (diet 3) had no significant effect on weight gain or food efficiency. However, supplementation with high levels of both thiamine and pyridoxine (diet 4) resulted in slightly greater weight gain and somewhat increased efficiency of food utilization. Although these differences were not significant, the results suggest that the thiamine and pyridoxine levels in diet 1 may have been borderline for maximum growth.

The data on reproductive performance (table 2) show that the average birth

TABLE 1

Data on weight gains, food and water intakes and food efficiencies of female rats fed diets containing various levels of thiamine and pyridoxine for 12 weeks

	Diet 1 Adequate thiamine, pyridoxine	Diet 2 Excess thiamine	Diet 3 Excess pyridoxine	Diet 4 Excess thiamine, pyridoxine
No. of survivors[1]	11	12	12	11
Initial weight, *gm*	49	49	49	49
6 weeks				
Weight gain, *gm*	106 ± 11[2]	109 ± 12	112 ± 9	116 ± 16
Food intake, *gm*	372	380	387	393
Water intake, *ml*	557	658	664	583
Food efficiency[3]	28.5 ± 2.1	28.8 ± 1.8	29.0 ± 2.6	29.5 ± 2.8
12 weeks				
Weight gain, *gm*	145 ± 19	146 ± 15	153 ± 14	158 ± 19
Food intake, *gm*	811	816	827	827
Water intake, *ml*	1354	1702	1620	1443
Food efficiency[3]	17.9 ± 1.4	18.0 ± 1.1	18.6 ± 1.8	19.2 ± 1.8

[1] Each group contained 12 animals initially.
[2] Standard deviation.
[3] Average grams gain per 100 gm food intake.

TABLE 2

Data on reproductive performance of female rats fed diets containing various levels of thiamine and pyridoxine

No. and description of diet	No. of animals	No. of litters	No. of young	Av. birth weight of young	Survival data				Av. weight of young, 21 days
					5 days		21 days		
				gm	no.	%	no.	%	gm
1. Adequate thiamine, pyridoxine	10	6	44	6.5	43	98	32	73	37
2. Excess thiamine	12	11	75	6.3	72	96	57	76	43
3. Excess pyridoxine	11	9	75	6.1	67	89	56	75	41
4. Excess thiamine, pyridoxine	10	9	87	6.2	78	90	56	64	39

weight, survival of the young to weaning, and average weight of the young at weaning were not significantly influenced by addition of excess thiamine or pyridoxine or both to the basal diet. However, the animals which received excess thiamine and pyridoxine (diet 4) had more young per litter than those fed the other diets. The lack of any deleterious effect of excess thiamine on reproduction observed in the present study is in contrast to the findings of Richards ('45). The discrepancy in the results may be due, in part, to the relative inadequacy of the diet used by Richards, since Morrison and Sarett ('59) have found that excess amounts of a single B vitamin may retard weight gain of animals fed diets low in several B vitamins, but usually have no effect in animals fed adequate diets.

The data on organ weights of the post-parturient females showed some hypertrophy due to pregnancy, but little effect of vitamin excess. The liver weights averaged 4.6 to 5.1% of the body weight, and the adrenal glands averaged 21.3 mg to 23.5 mg per 100 gm body weight. The kidney weights of the animals on diets 2, 3 and 4 averaged 0.73 to 0.78% of body weight, whereas those of the rats on diet 1 were slightly heavier, namely 0.82% of body weight.

The levels of solids and total lipid in the livers of the post-parturient females (table 3) were not significantly influenced by addition of excess amounts of thiamine or pyridoxine or both to the basal diet. The levels of riboflavin, pantothenate and vitamin B_{12} were also not significantly influenced by the diet fed. The addition of excess thiamine to the basal diet increased liver thiamine levels from 2.6 to 8.7 μg per gm. In contrast to the findings with thiamine, the addition of excess pyridoxine to the diet had no significant effect on liver pyridoxine levels. Other workers have also found that increasing the level of thiamine in the diet above that needed for maximum growth, increases tissue concentrations of the vitamins (Ochoa and Peters, '38; Byerrum and Flokstra, '51), whereas the concentration of pyridoxine in the liver of growing rats is not influenced by addition of pyridoxine to an adequate diet (Sheppard and McHenry, '46). Excess pyridoxine apparently had no effect on liver thiamine levels, nor did excess thiamine influence the level of pyridoxine in the liver.

The data on weight gains of the young on the pyridoxine-deficient diet are summarized in table 4. The young of mothers which received excess pyridoxine (diets 3 and 4) gained significantly more weight than did those of mothers which received the basal diet. These findings do not support the hypothesis of Hunt et al. ('54) that administration of excess pyridoxine to the mother during pregnancy increases the pyridoxine dependency of the young. The presence of excess thiamine in the maternal diet (diet 2) appeared to slightly increase weight gain in the young fed a pyridoxine-deficient diet.

The data on thiamine and pyridoxine levels in the livers of the young at weaning, and after two and 5 weeks on the pyridoxine-deficient diet, are summarized in table 5. Liver thiamine levels in the young at weaning reflected maternal thiamine intakes, in a manner similar to that found in the livers of the mothers. The pyridoxine levels of the livers of the young at weaning also reflected maternal pyridoxine intake, although those in the mothers showed no effect of excess pyridoxine (table 3). At weaning, the young of ani-

TABLE 3

Data on liver composition of post-parturient female rats fed diets containing various levels of thiamine and pyridoxine

	Diet 1 Adequate thiamine, pyridoxine	Diet 2 Excess thiamine	Diet 3 Excess pyridoxine	Diet 4 Excess thiamine, pyridoxine
Number of animals per group	6	11	8	9
Solids, %	30.5	29.4	29.3	29.4
Total lipid (ether extract), %	2.7	2.7	2.4	3.3
Thiamine, μg/gm	2.6	8.7	2.3	8.4
Riboflavin, μg/gm	33.3	31.5	28.8	29.1
Pyridoxine, μg/gm	11.2	10.2	10.4	11.0
Pantothenate, μg/gm	61.2	56.4	56.4	56.1
Vitamin B_{12}, mμg/gm	133.5	133.8	131.7	131.7

TABLE 4

Influence of maternal diet on weight gain of male and female weanling rats fed a pyridoxine-deficient diet for 5 weeks

	Maternal diet			
	Diet 1 Adequate thiamine, pyridoxine	Diet 2 Excess thiamine	Diet 3 Excess pyridoxine	Diet 4 Excess thiamine, pyridoxine
Males				
Two weeks				
No. of animals	12	12	12	12
Weight gain, gm	11	16	31	32
Five weeks				
No. of animals	5	8	8	8
Weight gain, gm	12 ± 7[1]	24 ± 8	56 ± 6	55 ± 7
Females				
Two weeks				
No. of animals	12	12	12	12
Weight gain, gm	11	14	33	30
Five weeks				
No. of animals	6	7	8	8
Weight gain, gm	14 ± 9	18 ± 7	50 ± 7	47 ± 3

[1] Standard deviation.

mals fed excess thiamine (diets 2 and 4) had higher liver thiamine levels than did their mothers. However, in the animals which received excess pyridoxine (diets 3 and 4), the levels of pyridoxine in the liver were lower in the young at weaning than in the mothers, suggesting limited placental transfer of this vitamin. In man, placental transfer of vitamin B_6 also occurs, as shown by the work of Wachstein et al. ('57), who found that administration of pyridoxine to pregnant women resulted in elevated pyridoxal phosphate levels in the umbilical cord blood at birth. During the 5-week period on the pyridoxine-deficient diet, the greater liver vitamin stores in the young of mothers fed excess thiamine or pyridoxine were decreased to the levels found in the young of mothers fed the basal diet.

SUMMARY

An experiment was conducted to determine whether excess thiamine or pyridoxine or both affect growth and reproduction in rats receiving an otherwise adequate diet. Groups of female weanling rats were fed a basal diet containing 150 μg of thiamine and of pyridoxine per 100 gm of diet, alone or supplemented with 50 times this level of thiamine or pyridoxine or both. After a 12-week growth period, the

TABLE 5

Influence of maternal diet on levels of thiamine and pyridoxine in the livers of male and female weanling rats fed a pyridoxine-deficient diet for 5 weeks

	Maternal diet			
	Diet 1 Adequate thiamine, pyridoxine	Diet 2 Excess thiamine	Diet 3 Excess pyridoxine	Diet 4 Excess thiamine, pyridoxine
Males				
Thiamine, μg/gm				
Initial	1.9(6)[1]	14.7(17)	1.8(9)	12.0(16)
Two weeks	2.5(4)	5.7(4)	2.6(4)	6.0(4)
Five weeks	1.6(2)	1.8(4)	2.1(4)	2.5(4)
Pyridoxine, μg/gm				
Initial	4.8(6)	5.8(17)	8.8(9)	8.7(16)
Two weeks	3.6(4)	4.0(4)	5.1(4)	5.0(4)
Five weeks	5.6(2)	4.0(4)	5.3(4)	4.8(4)
Females				
Thiamine, μg/gm				
Initial	2.0(9)	14.4(16)	1.7(15)	12.6(8)
Two weeks	2.6(4)	6.0(4)	2.7(4)	6.0(4)
Five weeks	1.6(3)	2.0(3)	2.0(4)	1.8(4)
Pyridoxine, μg/gm				
Initial	5.2(9)	5.5(16)	9.1(15)	9.7(8)
Two weeks	3.9(4)	4.0(4)	4.6(4)	5.1(4)
Five weeks	2.9(3)	3.1(3)	5.2(4)	4.2(4)

[1] Figures within parentheses indicate number of animals sacrificed to obtain pooled samples of liver.

animals were mated to stock males for study of reproductive performance. The mothers were sacrificed after the young were weaned, and liver vitamin levels were determined. The young were given a pyridoxine-deficient diet for 5 weeks, and growth and liver thiamine and pyridoxine levels were measured at intervals.

The results showed that excess thiamine or pyridoxine or both had no effect on weight gain, reproductive performance or the levels of solids, total lipid, riboflavin, pyridoxine, pantothenate and vitamin B_{12} in the livers of the animals after parturition and lactation. Liver thiamine levels in these animals, and in their young at weaning, were markedly increased by excess thiamine. Liver pyridoxine levels in the mothers were not increased by excess dietary pyridoxine, although pyridoxine stores in the livers of the young at weaning were greatly increased by excess pyridoxine intake of the mothers.

During 5 weeks on a pyridoxine-deficient diet, the young of mothers which had received excess pyridoxine gained significantly more weight than did those of mothers which had received the basal diet.

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ON THE MECHANISM OF THE NULLIFICATION OF CNS EFFECTS OF L-DOPA BY PYRIDOXINE IN PARKINSONIAN PATIENTS[1]

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Abstract—Administration of either Levodopa (L-DOPA) or pyridoxine increased the concentration of dopamine in the basal ganglia of rats. However, administration of pyridoxine to rats pretreated with L-DOPA for several days resulted in a reversal of the L-DOPA-induced elevation of dopamine. Pretreatment of rats with Ro 4-4602 (an inhibitor of peripheral aromatic amino acid decarboxylases) enhanced the L-DOPA-induced rise in the CNS level of dopamine. This effect was also reduced substantially after the administration of pyridoxine. We interpret these results to indicate that the antagonistic effect of pyridoxine on the beneficial effects of L-DOPA in the CNS is centrally mediated as a result of decreased formation of dopamine.

WITH the advent of Levodopa (L-DOPA) as a means of therapy in the treatment of Parkinson's disease, many attempts have been made to increase its therapeutic efficacy while minimizing its side effects. For this purpose DUVOISIN, YAHR and COTÉ (1969) gave pyridoxine to a group of Parkinsonian patients already receiving L-DOPA and found that pyridoxine antagonized the beneficial CNS effects of L-DOPA in these patients. This finding was quite unexpected since pyridoxal phosphate is a coenzyme for DOPA decarboxylase (EC 4.1.1.26) which converts L-DOPA to dopamine (see HORNYKIEWICZ, 1966 for review). The finding of Duvoisin and his collaborators (1969) has been substantiated by others (HUNTER, STERN and LAURENCI, 1970), but the basis for the antagonism between L-DOPA and pyridoxine is still a subject of discussion.

DUVOISIN *et al.* (1969) and COTZIAS and PAPAVASILIOU (1971) favour the hypothesis that the antagonism results from an increase in the decarboxylation of L-DOPA to dopamine peripherally, with a consequent decreased availability of L-DOPA to the brain. Since both pyridoxine and L-DOPA penetrate the blood-brain barrier very rapidly, this hypothesis does not provide an explanation for the selective enhancement in decarboxylation of L-DOPA outside of the CNS. Other investigators (e.g. EVERED, 1971) theorize that the antagonism reflects the formation of a Schiff base between pyridoxine and L-DOPA that results in a general biochemical and pharmacological inactivation of both L-DOPA and pyridoxine. Since no intermediary product(s) resulting from condensation of L-DOPA and pyridoxine has been isolated thus far, this theory remains to be tested experimentally.

Before the question of the mechanism could be properly answered, more study of the interrelationship between L-DOPA and pyridoxine seemed indicated. We report in this paper the effects of either L-DOPA or pyridoxine on enhancing dopamine in the

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basal ganglia when given individually, and the effects of L-DOPA and pyridoxine on reducing dopamine in the same area when administered concurrently. Our findings eliminate the increased peripheral decarboxylation of L-DOPA as the mechanism of antagonism of vitamin B_6 on the effect of L-DOPA and favour the concept of the formation of a Schiff base between L-DOPA and pyridoxine. A preliminary report has been presented (EBADI, PFEIFFER and JOHNSON, 1972).

MATERIALS AND METHODS

Male Sprague–Dawley rats (180–200 g) were purchased from Mid-Continent Research Animals, Inc. (Shawnee Mission, Kansas). The animals were caged comfortably and fed a diet of purina chow whose vitamin B_6 content remained constant throughout the experiments. Levodopa (L-β-3,4 dihydroxyphenylalanine) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Pyridoxine.HCl (2-methyl-3-hydroxy-4-5-bis [hydroxymethyl] pyridine hydrochloride) was obtained from Eli Lilly and Co. (Indianapolis, Ind.). Cellulose gum, type 7 HF, used to suspend Levodopa, was obtained from Hercules, Inc. (Wilmington, Del.). A generous supply of Ro 4-4602, an inhibitor of peripheral aromatic amino acid decarboxylase (BARTHOLINI, BURKARD and PLETSCHER, 1967; PLETSCHER and BARTHOLINI, 1970), was kindly donated by F. Hoffman LaRoche and Co., Ltd. (Basle, Switzerland). All purchased chemicals were of the purest grade available.

Dopamine was assayed in the basal ganglia according to the procedure of CHANG (1964), as elaborated and simplified by SPANO and NEFF (1971). The basal ganglia (caudate nucleus, putamen and globus pallidus), dissected according to KÖNIG and KLIPPEL (1963), were either frozen at −20°C for storage or kept on dry ice until assayed for dopamine.

Rats were divided into several groups. The *first* group was injected intraperitoneally with L-DOPA, 100 mg/kg per day for 4 days, given in divided doses every 8 h. After the administration of the last dose, the animals were decapitated and the basal ganglia were isolated and stored. The *second* group was injected intravenously with pyridoxine, 100 mg/kg, and were decapitated 1, 2, 6, 12, 24 and 36 h later. The *third* group was subjected essentially to the experimental procedures described for both the other groups: the rats were given L-DOPA as already described and were given 100 mg of pyridoxine per kg at the end of the 4-day period and then sacrificed at 1, 2, 6, 12, 24 and 36 h later, as already described.

In another series of experiments, the effect of pyridoxine on rats pretreated with Ro 4-4602 and L-DOPA was tested. These animals were also divided into several groups. The *first* group was injected intraperitoneally with Ro 4-4602 (50 mg/kg) and L-DOPA (75 mg/kg) at 90 and 60 min prior to decapitation, respectively. The *second* group was subjected to the same treatment and in addition received pyridoxine (250 mg/kg) intravenously, 45 min prior to decapitation. The *third* and *fourth* groups were treated exactly as groups one and two, respectively, except that the animals were decapitated at 6 h after drug treatment. In all cases the basal ganglia were removed and kept frozen until assayed for dopamine. Throughout the entire experimentation, the control animals were injected with appropriate vehicles and handled in the same fashion as experimental animals.

RESULTS

The intraperitoneal administration of a 100 mg/kg dose of L-DOPA per day in divided doses for 4 days increased the level of dopamine from 6·66 to 8·61 μg/g in the rat basal ganglia. The administration of a 100 mg/kg dose of pyridoxine (given intravenously) also increased the level of dopamine in the same region from 6·66 to 9·74, 8·09 and 8·73 μg/g at 6, 12 and 36 h, respectively, after pyridoxine administration. However, the administration of similar doses of pyridoxine in addition to the Levodopa administration rapidly reversed the L-DOPA-induced rises in the level of dopamine in the basal ganglia of the rats from 9·66 to 6·00 μg/g in 24 h (Table 1). Similarly the concomitant administration of pyridoxine reduced the dopamine-enhancing effects of L-DOPA and Ro 4-4602 after 6 h (Table 2).

DISCUSSION

The L-DOPA-induced rise in the brain levels of dopamine has been amply documented (CARLSSON, LINDQVIST, MAGNUSSON and WALDECK, 1958; HORNYKIEWICZ,

TABLE 1.—THE EFFECTS OF L-DOPA AND OF PYRIDOXINE (GIVEN SINGLY OR CONCOMITANTLY) ON THE LEVEL OF DOPAMINE IN THE BASAL GANGLIA OF RATS

Experimental conditions		Time after drug treatment to decapitation (h)		Dopamine	
Groups	No. of rats	After L-DOPA	After pyridoxine	μg/g of fresh brain tissue	P value
A. Control	19	—	—	6·66 ± 0·34	
B. L-DOPA—100 mg/kg i.p. per day for 4 days	16	96	—	8·61 ± 0·50	$P < 0·05$
C. Pyridoxine—100 mg/kg iv.	7	—	2	6·28 ± 0·55	
	8	—	6	9·74 ± 0·63	$P < 0·05$
	8	—	12	8·09 ± 0·47	$P < 0·05$
	8	—	24	7·48 ± 0·36	$P < 0·05$
	8	—	36	8·73 ± 0·55	$P < 0·05$
D. Pyridoxine—100 mg/kg iv. given to rats which had received a 100 mg/kg dose of L-DOPA/day for 4 days	6	96	1	9·66 ± 0·97	
	7	96	2	7·74 ± 1·06	$P < 0·05$
	6	96	24	6·00 ± 0·55	$P < 0·05$

The procedure for determination of dopamine has been described in the section on materials and methods. Values represent means ±S.E.M. for the number of animals indicated. Abbreviations: i.p. = intraperitoneally; i.v. = intravenously.

1966). However, a similar increase in the level of dopamine after the administration of pyridoxine has not been reported and deserves elaboration. The intravenous administration of pyridoxine. HCl 100 mg/kg increased the concentration of dopamine from 6·66 to 9·74 μg/g in 6 h. This higher level of dopamine most probably results from its increased synthesis by the accelerated activity of DOPA decarboxylase, which is a pyridoxal phosphate-dependent enzyme. Through the catalytic action of pyridoxal kinase (EC 2.7.1.35) (McCORMICK, GREGORY and SNELL, 1961), the administration of pyridoxine increases the available pool of pyridoxal phosphate in the brain (EBADI,

TABLE 2.—THE EFFECT OF L-DOPA (75 mg/kg i.p.) GIVEN ALONE; L-DOPA (75 mg/kg i.p.) AND Ro 4-4602 (50 mg/kg i.p.); OR L-DOPA (75 mg/kg i.p.), Ro 4-4602 (50 mg/kg i.p.) AND PYRIDOXINE (250 mg/kg i.v.) GIVEN CONCOMITANTLY ON THE LEVEL OF DOPAMINE IN THE BASAL GANGLIA OF RATS

Experimental conditions	No. of rats	Time after drug treatment to decapitation (h)	Dopamine μg/g wet wt. of brain tissue	P value
Control	19	—	6·66 ± 0·34	
L-DOPA	5	1·5	8·67 ± 0·50	$P < 0·05$
L-DOPA and Ro 4-4602	5	1·5	15·66 ± 1·61	$P < 0·05$
L-DOPA, Ro 4-4602 and pyridoxine	5	1·5	16·97 ± 2·22	$P < 0·05$
L-DOPA and Ro 4-4602	5	6	10·62 ± 0·74	$P < 0·05$
L-DOPA, Ro 4-4602 and pyridoxine	5	6	8·79 ± 0·87	

The schedule for drug administration and the procedure for determination of dopamine have been described in the section on Materials and Methods. Values represent means ±S.E.M. for the number of animals indicated. Abbreviations: i.p. = intraperitoneally; i.v. = intravenously.

McCoy and Kugel, 1970), which in turn activates DOPA decarboxylase, which is not saturated with coenzyme under physiological conditions (Awapara, Sandman and Hanly, 1962; Sourkes, 1966). This pyridoxal phosphate-induced activation of DOPA decarboxylase is compatible with the following observations. Blaschko (1950) reported the absence of DOPA decarboxylase in liver extracts of rats reared on a diet deficient in pyridoxine. Similarly, the activity of apocysteine sulfinate decarboxylase (EC 4.1.1.29) (Hope, 1955) decreased in vitamin B_6 deficiency states. In addition, the activity of L-aromatic amino acid decarboxylase (EC 4.1.1.26) was similarly inhibited by using either 5-hydroxytryptophan (Buxton and Sinclair, 1956; Weissbach, Bogdanski, Redfield and Udenfriend, 1957), DOPA (West, 1953; Sourkes, Murphy and Woodford, 1960), or histidine (Schayer, Rothschild and Bizony, 1959) as substrates. In all cases, the addition *in vitro* of pyridoxal phosphate restored the activity of the enzyme. By using biopsy material from the thalamus and pallidum of Parkinsonian patients, Metzel, Weinmann and Reichert (1969) have recently shown a low DOPA decarboxylase activity which could be substantially increased with pyridoxal phosphate.

Of great interest are the studies of Tapia, Pasantes and Massieu (1970) who measured the levels of pyridoxal phosphate and the activity of glutamate decarboxylase (EC 4.1.1.15) in brains of three vertebrate species – carp, pigeon and mouse. A direct relationship existed between the level of the coenzyme and the activity of the decarboxylase. This evidence suggests that the formation of dopamine may be enhanced by activating DOPA decarboxylase through saturation with its coenzyme as a result of increased 'bioavailability' of pyridoxal phosphate (Ebadi, 1970), which in turn increases as a result of the enhanced activity of pyridoxal kinase, the enzyme synthesizing the coenzyme (Ebadi, Russell and McCoy, 1968).

Since the symptoms of Parkinson's disease are generally thought to result from depleted levels of dopamine in the basal ganglia and substantia nigra (Barbeau, Murphy and Sourkes, 1961; Ehringer and Hornykiewicz, 1960), one would expect that the rise in levels of dopamine induced by pyridoxine would make it a useful drug in the treatment of Parkinson's disease, just like L-DOPA. Early reports indicated that pyridoxine was indeed of some value (Baker, 1941; Jolliffe, 1940; Spies, Hightower and Hubbard, 1940), but subsequent reports did not support this contention (Barker, Stein, Miller and Wintrobe, 1941; Zeligs, 1941) and the use of pyridoxine in the treatment of Parkinson's disease was abandoned.

If pyridoxine does not benefit the patient with Parkinson's disease, one might logically question how L-DOPA exerts its beneficial effect, since both L-DOPA and pyridoxine increase the levels of dopamine in the basal ganglia. There are at least two probable explanations for the apparent discrepancy. First, the effect of pyridoxine in elevating brain dopamine is too transient to be of any value to the Parkinson patient. Levodopa is beneficial only when large doses are given for a long period of time. Perhaps the pursuit of a similar therapeutic regimen with pyridoxine would be necessary to obtain clinical improvement. On the other hand, the beneficial effect of L-DOPA might not be solely attributable to elevation of dopamine levels. Formation of minor by-products (Calne, Karoum, Ruthven and Sandler, 1969) and the methionine-depleting effects of L-DOPA have been reported to contribute to its CNS effects in patients with Parkinson's disease (Cotzias, Papavasiliou, Stick and

The administration of pyridoxine to animals pretreated with L-DOPA for several days resulted in an initial transient rise followed by a precipitous drop in the cerebral levels of dopamine. These findings provide a possible basis for the clinically-observed abolition of the beneficial effect of L-DOPA in patients with Parkinson's disease. The extremely reactive pyridoxal phosphate undergoes formation of Schiff bases with many amines and amino acids, namely tryptophan, phenylalanine and phenylethylamine derivatives, histamine, dopamine and norepinephrine (SCHOTT and CLARK, 1952) and glutamic acid (TAPIA and AWAPARA, 1967). The resulting compounds (which are pharmacologically inactive) inhibit, among many, the activity of glutamic acid decarboxylase, GABA transaminase (TAPIA and AWAPARA, 1967) and tyrosine transaminase (BLACK and AXELROD, 1969) *in vitro* and *in vivo*. The formation of Schiff bases is usually reversible; however, these compounds may undergo a second but irreversible reaction at neutral pH. There is evidence that internal recyclization does occur between pyridoxal phosphate and L-DOPA (ZINKER, 1966) to reduce the concentration of both the coenzyme and the amine precursors. Furthermore, the resulting compound may inhibit the activity of pyridoxal kinase, the coenzyme-synthesizing enzyme, and essentially decrease the *de novo* synthesis of pyridoxal phosphate. It has been reported that condensation products formed between vitamin B_6 derivatives and hydroxylamine, O-substituted hydroxylamines, hydrazine and substituted hydrazines are the most potent inhibitors of pyridoxal kinase (MCCORMICK and SNELL, 1961; EBADI *et al.*, 1968). The degree of inhibition should be a function of the extent of the Schiff base formed. Indeed, YAHR, DUVOISIN, COTÉ and COHEN (1972) have shown that the antagonistic effect of pyridoxine toward L-DOPA in parkinsonian patients was dose-dependent, being more 'rapid, greater and sustained for a longer period' with higher dosages. Furthermore, KURTZ and KANFER (1971) have shown that oral administration of L-DOPA decreased the pyridoxal phosphate content of the brain and at the same time decreased the activity of glutamate decarboxylase in a dose-related fashion.

The hypothesis of increased peripheral decarboxylation of L-DOPA to dopamine held by DUVOISIN *et al.* (1969) and COTZIAS and PAPAVASILIOU (1971) as a mechanism for the antagonistic effect between pyridoxine and L-DOPA has certain shortcomings. This hypothesis is based on the assumption that pyridoxal phosphate activates DOPA decarboxylase primarily in the liver. Since pyridoxine passes across the blood-brain barrier very rapidly and is converted to pyridoxal phosphate, the hypothesis does not explain why DOPA decarboxylase in the brain does not become similarly activated. In defence of their hypothesis, COTZIAS and PAPAVASILIOU (1971) reported that the concomitant administration of pyridoxine and DL-α-methyl DOPA hydrazine, an inhibitor of peripheral DOPA decarboxylase that does not pass across the blood-brain barrier, did not produce any adverse effect in Parkinsonian patients receiving L-DOPA. Hence, they concluded that the effect of pyridoxine is peripherally mediated. Hydrazine and its derivatives are most avid chelators of pyridoxal phosphate (HOLTZ and PALM, 1964). In this case α-methyl DOPA hydrazine could compete with L-DOPA and become chelated with pyridoxine to spare the administered L-DOPA for conversion to dopamine.

In order to clarify this concept further, we repeated a group of experiments in the presence of an inhibitor of the peripheral L-aromatic amino acid decarboxylase. One such compound is Ro 4-4602 which has been shown by BARTHOLINI *et al.* (1967) to

inhibit the extracerebral decarboxylase preferentially. The intraperitoneal administration of a 50 mg/kg dose of Ro 4-4602 causes an almost complete inhibition of decarboxylase in the heart but has no effect on the brain decarboxylase. The administration of a 300 mg/kg dose of Ro 4-4602 inhibits the cerebral enzyme by more than 75 per cent (PLETSCHER and BARTHOLINI, 1970). The simultaneous administration of L-DOPA, Ro 4-4602 and pyridoxine resulted in decreased formation of dopamine in comparison to that obtained with L-DOPA and Ro 4-4602 (Table 2). Therefore, the therapeutic regimen consisting of pyridoxine, an inhibitor of the peripheral decarboxylase and L-DOPA is pharmacologically unsound and should be seriously questioned.

Our finding tends to favour the concept that the antagonistic effect of pyridoxine on L-DOPA reflects the decreased formation of dopamine resulting from a condensation product between L-DOPA and pyridoxal phosphate. The isolation and characterization of one such condensation product is under investigation.

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Vitamin B_6 Group. XV. Urinary Excretion of Pyridoxal, Pyridoxamine, Pyridoxine, and 4-Pyridoxic Acid in Human Subjects.*

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The earliest investigations of the excretion of vitamin B_6 by animals were made using the chlorimide reaction for analysis.[1,2] The method as then used was not specific for the determination of pyridoxine, but since the complex nature of vitamin B_6 was then unknown, all material found by this method was called *pyridoxine*. These early investigations did establish the fact that pyridoxine was rapidly absorbed from the digestive tract and rapidly cleared in the renal pathway. Although the recovery of the ingested vitamin in the rat was 50 to 76%,[1] only 10 to 20% of the dose was recovered when pyridoxine was fed to dogs or to human subjects.[2]

Subsequent refinement of the chlorimide method showed that small amounts of some substance other than pyridoxine were excreted in the urine of the dog and man after feeding a large dose of pyridoxine.[3] It was also shown that both pyridoxine and the unknown metabolite occurred in part as conjugated forms which did not react with 2, 6-dichloroquinone chlorimide, but which could be hydrolyzed with acid to give pyridoxine and the unknown metabolite.

The demonstration of the occurrence of *pseudopyridoxine* in human urine both before and after administration of pyridoxine[4] and the subsequent characterization of *pseudopyridoxine* as pyridoxal and pyridoxamine,[5] suggested the identity of the unknown metabolite of Scudi *et al.*[3] with pyridoxal or pyridoxamine.

The main metabolic product excreted after ingestion of pyridoxine was discovered by Huff and Perlzweig and identified as 4-pyridoxic acid.[6] This compound does not produce a color with the chlorimide reagent, and is inactive in promoting growth of microorganisms in vitamin B_6-free media.

Although pyridoxal and pyridoxamine are now known to be the forms of vitamin B_6 present in largest amounts in many foodstuffs and tissues,[7,8] no information is available concerning their metabolic fate. Development of a differential assay procedure for pyridoxal, pyridoxamine, and pyridoxine[8] makes such a study feasible. Results of such an investiga-

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tion conducted with normal human subjects are presented below.

Methods. Twenty-four hour samples of urine were collected from 3 adult male subjects and used as control samples to establish a base level for vitamin B_6 excretion by individuals on a normal diet. Individual subjects were then fed 100 mg of pyridoxine hydrochloride, pyridoxamine dihydrochloride, or pyridoxal hydrochloride in one dose with approximately 200 cc of water. Urine was collected 2, 5, 8, 12, 24, and 36 hours after ingestion of the test dose, and stored under toluene in amber bottles at 7° until analysis. Two months later the experiment was repeated using the same subjects but feeding each subject a different form of the vitamin than had been fed the first time.

The pyridoxal, pyridoxamine, and pyridoxine content of these samples was determined by the differential microbiological assay which uses *Lactobacillus casei, Streptococcus faecalis,* and *Saccharomyces carlsbergensis* as test organisms.[8] The pyridoxic acid content of the samples was determined by the fluorometric method of Huff and Perlzweig,[6] and is expressed in terms of the lactone.

Since a large part of the vitamin B_6 of natural materials is unavailable to the test microorganisms unless the samples are first autoclaved with acid,[9] control samples of urine were subjected to various conditions of acid hydrolysis to determine the optimal conditions for the liberation of vitamin B_6. The results (Table I) show that acid hydrolysis results in the liberation of increased amounts of vitamin B_6 in urine over that observed in unhydrolyzed samples. 0.055 N acid, the concentration commonly used for most samples[9,10,11] was found to be less effective than treatment with 0.11 N HCl or 0.55 N HCl. 0.11 N HCl was used instead of 0.55 N HCl in hydrolysis of the samples, since the amount of potassium chloride present in an assay tube after neutralization of samples treated with 0.55 N [illegible] reaches approximately 135 mg per 5 [illegible] medium, which is close to the highest level [illegible] potassium chloride tolerated by *Saccharomyces carlsbergensis* and lactic acid bacteria.

The samples collected were therefore treated in the following way prior to microbiological assay: 10 cc aliquots of each sample were autoclaved for 7 hours at 20 lb pressure in 180 cc of 0.11 N HCl. The samples were then neutralized with potassium hydroxide, diluted, and assayed.

TABLE I.
Liberation of Vitamin B_6 in Normal Urine by Acid Hydrolysis.

Treatment	Pyridoxal + HCl mγ/cc	Pyridoxamine + HCl mγ/cc
None	29	0
.055 N HCl*	78	133
.11 "	93	196
.55 "	93	210

* Autoclaved in 180 cc of acid at 20 lb pressure for 5 hr.

The limitations of the differential assay have been pointed out elsewhere,[8] especially in regard to the determination of the pyridoxine content of samples low in this form of vitamin B_6. In addition to these inherent limitations of the differential assay, it was found that the recovery of pyridoxine added to a normal urine sample was somewhat less than could be expected from purely analytical errors.[8] Urine seemed to contain a material toxic for *Saccharomyces carlsbergensis* but not for *Streptococcus faecalis* or *Lactobacillus casei*. The presence of this toxic material results in an underestimation of whatever pyridoxine may be present in the control sample of urine, but does not affect the determination of pyridoxal or pyridoxamine in this sample. None of the results are affected in other samples, which contained much more vitamin B_6 and were therefore assayed at dilutions such that the toxic material was without effect.

Results. The distribution of the 4 compounds determined in normal urine is shown in columns *b* and *c*, Table II. The levels

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TABLE II.
The Urinary Excretion of Pyridoxic Acid, Pyridoxal, Pyridoxamine and Pyridoxine.

Excretion products*	Mg excreted up to 24 hr after ingestion (a)	Mg excreted 24 hr before ingestion (b)	Mg recovered (c)	Mg excreted up to 24 hr after ingestion (d)	Mg excreted 24 hr before ingestion (e)	Mg recovered (f)
	Fed 70 mg pyridoxamine. Subject A			Subject C		
4-pyridoxic acid lactone	24.60	6.48	18.12	22.37	2.59	19.78
Pyridoxal	1.57	0.102	1.47	0.985	0.058	0.93
Pyridoxamine	1.83	0.028	1.80	1.18	0.149	1.03
Pyridoxine	0.00	0.00	0.00	0.209	0.00	0.21
Total			21.39			21.95
	Fed 82 mg pyridoxine. Subject B			Subject A		
4-pyridoxic acid lactone	28.40	1.97	26.43	25.27	3.91	21.36
Pyridoxal	1.69	0.074	1.62	1.38	0.052	1.33
Pyridoxamine	0.535	0.124	0.41	0.650	0.210	0.44
Pyridoxine	8.53	0.00	8.53	7.80	0.00	7.80
Total			36.99			30.93
	Fed 82 mg pyridoxal. Subject C			Subject B		
4-pyridoxic acid lactone	59.79	3.84	55.95	53.22	2.54	50.68
Pyridoxal	1.53	0.084	1.45	1.28	0.049	1.23
Pyridoxamine	0.021	0.083	0.00	0.241	0.097	0.14
Pyridoxine	0.039	0.00	0.04	0.132	0.00	0.13
Total			57.44			52.18

* All products are expressed in terms of the free bases. Pyridoxic acid is determined and expressed as the lactone. The molecular weight of the free bases are almost equal (pyridoxal = 167, pyridoxamine = 168, pyridoxine = 169, 4-pyridoxic acid lactone = 165). Expressed in this way, weights are directly comparable as molar quantities without significant error.

These compounds were not particularly constant. Of the 4, pyridoxic acid was quantitatively the most prominent, accounting for 91 to 98% of the total. Of the 3 forms of vitamin B_6, pyridoxamine was usually present in highest concentrations, although pyridoxal was sometimes present in higher concentrations. Some pyridoxal was always found in the urine. The presence of pyridoxine could not be demonstrated in any of the normal urine samples; but, as noted above, this result may be due to the limitations of the method of analysis and does not constitute satisfactory evidence that pyridoxine is not excreted under normal conditions.

When pyridoxamine was fed, a significant rise in the pyridoxal as well as in the pyridoxamine content of the urine was noted; in fact, the amount of the administered pyridoxamine recovered as pyridoxal was equal to the amount recovered as pyridoxamine. The amount of pyridoxine indicated as being formed was not significant. The principal excretion product was pyridoxic acid, which accounted for 85% of the measured excretion products for subject A and 90% for subject C.

After feeding pyridoxine, the major part of the vitamin B_6 excreted was in the form of pyridoxine, but 14 to 15% of the activity appeared as pyridoxal. The pyridoxamine of the urine accounted for only 4 to 5% of the vitamin B_6 activity of the urine. In this case 71 and 69% of the excretion products measured appeared as pyridoxic acid.

When pyridoxal was fed, larger amounts of pyridoxic acid were recovered than in either of the two preceding instances. However, the

total amount of vitamin B_6 in the urine was somewhat lower. 82 and 98% of this was pyridoxal.

Fig. 1 illustrates the fact that after feeding each of the 3 forms of vitamin B_6, a large increase in the level of the form fed occurs within 2 to 5 hours after administration of the

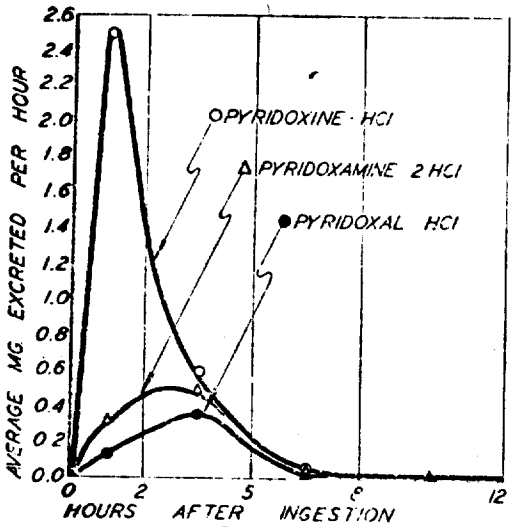

FIG. 1.

The Excretion of Vitamin B_6. The total amount of the indicated form of the vitamin found in the urine samples is plotted against the average time interval, after ingestion of the dose, during which the sample was collected.

○ Fed 100 mg of pyridoxine HCl.
△ Fed 100 mg of pyridoxamine 2HCl.
● Fed 100 mg of pyridoxal HCl.

The levels of the forms of the vitamin other than the one fed are not shown.

dose, and that this level returns to the normal value within 8 hours. Similar results, not shown in this graph, were also obtained in connection with the excretion of the forms of the vitamin other than the one fed. The pyridoxic acid levels of the urine, shown in Fig. 2, increase in a much more pronounced manner, and levels did not return to normal until 12 hours after ingestion of the dose.

The amount of the test dose recovered was characteristic of the form of the vitamin fed, judging from the agreement of the results obtained in the two experiments, in which different subjects were fed the same compound. The highest recovery of the test dose, 70% and 64%, was obtained after feeding pyridoxal. The amount of pyridoxine recovered as the 4 products measured was 45 and 38% while the smallest recovery, 31 and [illegible] was obtained after feeding pyridox[illegible] These results may be inferred from [illegible] since the major component of the [illegible] products is pyridoxic acid.

Discussion. The above comparison [illegible] amount of the test dose recovered as [illegible] products is of interest, since with pyri[illegible] and pyridoxamine, less than half of the [illegible]ministered dose was recovered. With pyr[illegible]al, the most highly oxidized form of the [illegible]min, the recovery was higher because [illegible] increased excretion of 4-pyridoxic acid w[illegible] resulted when this form was fed.

A number of explanations for these [illegible] recoveries might be postulated. It is pos[illegible] for example, that the absorption of the [illegible]ous forms of the vitamin from the trac[illegible] incomplete. This explanation, however, is [illegible] in accord with the observations of Scu[illegible] *al.*,[2] who showed with both dogs and hum[illegible] beings that the total urinary excretion of v[illegible]min B_6 following administration of la[illegible] doses of pyridoxine (50 to 100 mg) was [illegible]ly similar whether the vitamin was gi[illegible]orally or by intravenous injection. This in[illegible]cates rather conclusively that absorption [illegible] test doses of this size from the tract is c[illegible]plete. The amounts of "pyridoxine" (to[illegible] chlorimide reacting substances) which [illegible] found excreted following ingestion of 100 [illegible] of pyridoxine hydrochloride approxim[illegible] closely the amount of vitamin B_6 (pyrido[illegible] pyridoxamine and pyridoxine) found in [illegible] urine of our subjects. When limited amo[illegible] of the three forms of the vitamin are fed [illegible] deficient rats, chicks, or dogs apart from [illegible] diet, they show equal growth-promoting [illegible]tivities,[13,14] again indicating equal absorp[illegible]

A second possible explanation for low [illegible]coveries would be that the ingested dose w[illegible] being stored in the tissues. However [illegible] subjects used were ingesting normal diets [illegible] thus were presumably not deficient in vi[illegible]min B_6. Consequently, no great storage [illegible] the ingested vitamin would be expected. [illegible]

[13] Sarma, P. S., Snell, E. E., and Elvehj[illegible] C. A., *J. Biol. Chem.*, 1946, **165**, 55.

[14] Sarma, P. S., Snell, E. E., and Elvehj[illegible] C. A., PROC. SOC. EXP. BIOL. AND MED., 1946, 63 284.

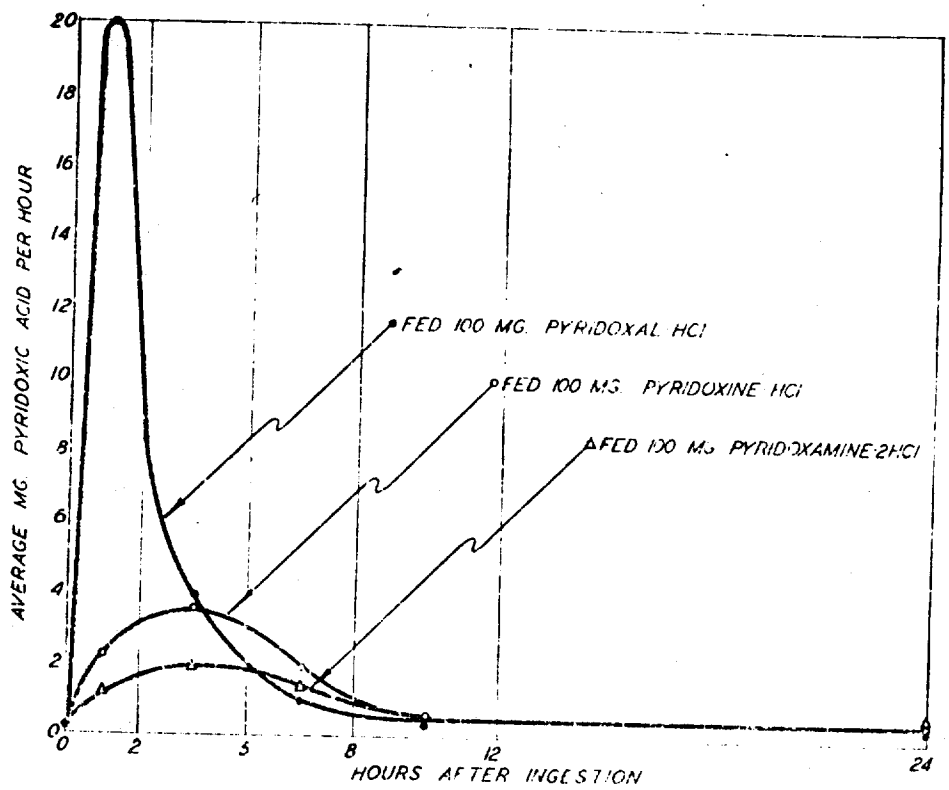

FIG. 2.
The excretion of pyridoxic acid (expressed as 4-pyridoxic acid lactone) following ingestion of various forms of vitamin B_6.

e fact that excretion levels rapidly returned basal levels indicates that no great storage curred. A third explanation for these low coveries would be that still unidentified tabolic products which are without vitamin tivity are formed from vitamin B_6 and reted. From the considerations outlined ove, this hypothesis seems most likely. It s additional experimental support in the ervation of Scudi, *et al.*[2] that following ction of 50 mg of pyridoxine hydrochloride dogs, only 18% of this dose could be reered in the urine. Yet, according to uff and Perlzweig,[15] dogs do not excrete pyridoxic acid. Unidentified excretion ducts, or complete oxidation within the y of considerable portions of the vitamin thus indicated.

Pyridoxal is the only form of the vitamin ich appears in the urine in greatly increased amounts as the result of feeding all 3 compounds. This further emphasizes the position of central importance indicated for this compound by direct assay of animal tissues,[8] by its universal availability to all microorganisms tested,[16] and by its occurrence in the only known catalytically active form of vitamin B_6, pyridoxal phosphate.[17]

Finally, it should perhaps be emphasized that the metabolic changes described above are those which occur when large amounts of vitamin B_6 are superimposed on a normal diet which already supplies the relatively small amounts of vitamin B_6 presumably required by man. Whether the small quantities normally ingested are metabolized in the same manner cannot be decided from these data.

Summary. The known metabolic products

[15] Huff, J. W., and Perlzweig, W. A., *Science*, 1944, **100**, 55.

[16] Snell, E. E., and Ramnefeld, A. N., *J. Biol. Chem.*, 1945, **157**, 475.

[17] Lichstein, H. C., Gunsalus, I. C., and Umbreit, W. W., *J. Biol. Chem.*, 1948, **161**, 311.

of vitamin B_6—pyridoxal, pyridoxamine, pyridoxine and pyridoxic acid—were measured in normal human urine and in the urine of human subjects each fed one of the 3 forms of the vitamin.

The chief product found, regardless of the form fed, was pyridoxic acid. Pyridoxal gave rise to significantly higher amounts of this product than did pyridoxine or pyridoxamine. No evidence could be obtained showing the conversion of pyridoxal or pyridoxamine to pyridoxine. When pyridoxal or pyridoxine was fed, the chief form in which the vitamin occurred in the urine was the form fed. However, when pyridoxamine was fed both pyridoxal and pyridoxamine were excreted in approximately equal amounts. Ingestion of pyridoxine also greatly increased the amount of pyridoxal and pyridoxamine excreted.

The excretion of all products was [illegible] rapid. The largest amounts of each of [illegible] compounds were found in samples colle[illegible] 2 and 5 hours after ingestion of the dose. [illegible] levels of pyridoxic acid returned to [illegible] values after 12 hours, while the vitamin [illegible] had returned to normal within 8 hours. [illegible] amount of the dose recovered varied with [illegible] form fed. The highest recovery, 70%, [illegible] obtained when pyridoxal was fed; 45% [illegible] the pyridoxine was recovered, while only [illegible] of the pyridoxamine could be recovered. [illegible] gether with published data which ind[illegible] that complete absorption of large dose[illegible] vitamin B_6 occurs, these findings suggest [illegible] a large proportion of the vitamin B_6 was [illegible] verted to products still unknown.

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Southern Med. Jour. 39(5): 405-407. 1946.

TREATMENT OF ROENTGEN SICKNESS WITH ORAL ADMINISTRATION OF PYRIDOXINE HYDROCHLORIDE (VITAMIN B_6)*

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The subject of roentgen sickness has been one of great concern to all radiation therapists. There have been many theories advanced as to its cause. Numerous and varied methods of treatment have been advocated for its prevention. No single method, however, has been found which is universally satisfactory. The barbiturates were found effective in that they were mildly hypnotic. Liver compounds are helpful in some cases. Other methods too numerous to mention have been advocated. Vitamin B_1 was reported by Moursund and Martin,[1] as being definitely beneficial to a larger group of patients. It was their belief at that time that roentgen sickness was a definite vitamin deficiency. With the use of B complex, the results seemed to be even better than with B_1. There were still a good number of cases who failed to respond.

Maxfield and co-workers,[2] in 1943, tried pyridoxine hydrochloride intravenously and found that a single dose of 25 mgs. daily would usually relieve the most obstinate sickness and frequently the nausea disappeared and injections could be discontinued and irradiation carried on without subsequent sickness. However, other medication was not excluded and liver extract, high-vitamin intake, and sedatives were used where indicated. Maxfield reported a series of over 50 cases where the results were most gratifying.

Willis and Massey,[3] in 1942, recommended the use of pyridoxine in the nausea and vomiting of pregnancy. They gave 20 mgs. intravenously and found it to be very effective. More consistent good results were obtained than with thiamin chloride.

Vitamin B_6 has been described in detail and

*Chairman's Address, Section on Radiology, Southern Medical Association, Thirty-Ninth Annual Meeting, Cincinnati, Ohio, November 12-15, 1945.

*From the Department of Radiology, Duke University School of Medicine.

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synthesized by Stiller and co-workers.[4] It was originally isolated from rice bran. The vitamin is stable to acids and alkalis. It has a definite absorption spectrum which changes markedly with change in hydrogen ion concentration. Stiller found the synthetic vitamin B_6 (pyridoxine) to be identical with the natural vitamin B_6. A single dose of 100 gamma of synthetic B_6 in rats on a B_6 deficient diet gave a curative effect which paralleled that of the natural vitamin B_6.

A statement has been made that pyridoxine may be a carcinogenic agent. Extensive studies have been made to determine its effect on tissue growth. Kline and co-workers,[5] implanted the Flexner-Jobling carcinoma into rats partially depleted of pyridoxine and found the percentage of takes lower, the number of regressions higher and the size of the tumors smaller than in control animals receiving the same number of calories. Similar results were observed with two types of transplantable mouse tumors: the Yale carcinoma I and a fibrosarcoma originally induced by ultra violet. A low percentage of tumors in each of the deficient groups grew as well as the average for the corresponding group on the complete diet. The production of skin tumors in mice painted with methylcholanthrene was definitely retarded on a diet low in pyridoxine. They could not prove that sarcomas were more active in mice on pyridoxine deficient diets.

Miner, Miller and Bauman,[6] also reported extensive studies on the relation between vitamin B diets and cancer. They found the production of tumors to be more difficult in animals raised to maturity on a diet consisting of fortified milk than in animals raised on a diet relatively low in the B vitamins.

We find no evidence that pyridoxine is a definite carcinogenic agent. It is not surprising that tumors were found to grow less readily in pyridoxine deficient rats as pyridoxine is a vitamin and consequently is a growth factor. Workers at the Merck Institute for Therapeutic Research[7] have studied the effect of the chronic administration of massive doses of pyridoxine to mice and no carcinogenic effect was found.

It has been found that there is a maximum absorption after oral administration of pyridoxine hydrochloride in 3 to 4 hours. The fact that the drug, when given intravenously, proved so satisfactory led the author to try oral administration because of its simplicity, and also because of slower absorption and excretion. A total of 100 cases who developed roentgen sickness have have been followed since March, 1944, to the present date. A dosage of 50 mg. of pyridoxine hydrochloride was given orally in the mornings, about 4 hours before the patient received irradiation. Another 25 mg. was given at noon and 25 mg. before the evening meal, making a total daily dose of 100 mg. This routine was started at the onset of the roentgen sickness and continued as long as nausea persisted. It is of interest to note that some patients were relieved of roentgen sickness and the drug discontinued before irradiation was completed, while in other instances sickness reappeared after stopping the drug, but cleared up when B_6 was again administered.

Of the series of 100 cases treated, 45 were over the pelvis for carcinoma of the cervix, rectum or bladder. These cases received an average of 8,000 to 10,000 r. distributed over the pelvis. The field of irradiation was usually 10 x 15 centimeters. The average daily dose was started at 150 r. to each of two opposing parts daily and increasing this 50 r. daily until 300 r. was delivered to each of two opposing parts. Only 21 patients in this group had actual vomiting following irradiation. The results obtained in this group are shown in *Table 1.*

Two of the cases who received no benefit from oral administration of B_6 responded readily to a single dose of 25 mg. given intravenously. In four cases the drug was discontinued, sickness returned but cleared up after further adminis-

Total cases	Nausea and vomiting	Nausea	Results		
			Good	Moderate	No benefit
45	24	21	25	16	4

Table 1

tration of B_6. Ten patients had a second series of irradiation with varying degrees of nausea, but responded to B_6.

Of the larger group of 55 cases treated over the thorax and upper abdomen, consisting chiefly of postoperative carcinoma of the breast, lymphoblastoma, polycythemia and Marie-Strumpell arthritis, the results are tabulated in *Table 2*.

Because of a certain psychic element it is difficult to evaluate systemic reactions in some individuals. We again found that cases who did not respond favorably to oral administration of B_6 would respond to 25 mg. of the drug.

ADDENDUM

The third group of cases were furnished by Dr. Paul C. Swenson of Jefferson Hospital, Philadelphia. He reported treatment of 35 patients with pyridoxine. The dosage was essentially the same as ours. One-half of the group were treated over the abdomen. They were either abdominal Hodgkin's disease, carcinoma of the cervix, or other abdominal tumors. The other half were cases where considerable irradiation was taking place over the torso, either the chest for malignancy of the lung or breast, or over the back because of malignancy in the skeleton or retroperitoneal region. This latter group seemed to be especially benefited. The cases receiving irradiation over the abdomen were divided into two groups. One group did not receive pyridoxine until symptoms developed. The other group was treated prophylactically with 25 mg. orally, three times a day at the beginning of irradiation. The latter group never developed any degree of irradiation sickness, nothing to compare with other patients receiving no medication. Dr. Swenson states.

"It is my conclusion that B_6 is of definite benefit in helping to control roentgen sickness. No controls were run other than semi-controls in giving one group the vitamin prophylactically and in the other, only when symptoms were complained of. It was of interest that once symptoms developed, it was more difficult to eliminate them, but when the drug was given prophylactically the symptoms were less severe."

Total cases	Nausea and vomiting	Nausea	Results		
			Good	Moderate	No benefit
55	13	42	52	2	1
Above cases second series irradiation					
32	12	9	28	3	1

Table 2

SUMMARY

Pyridoxine hydrochloride was given in daily amounts of 100 mg. and was found to be definitely more effective in treatment of irradiation sickness than many other drugs. The routine of 50 mg. after breakfast, around 7 o'clock in the morning, 25 mg. at noon and 25 mg. after dinner seemed to work better than 25 mg. three times daily. Oral administration is advocated because of its simplicity, ease of administration, and effectiveness. The occasional case which does not respond to the oral administration responds readily to intravenous injection. Failures from the use of this method were partly explained since the factors of nutritional balance and general care play a part in the effectiveness of any treatment.

The author is indebted to Merck and Company for the Vitamin B_6 (pyridoxine hydrochloride) used in the treatment of most of these cases, and to Dr. Paul C. Swenson, Jefferson Hospital, Philadelphia, Pennsylvania, for allowing us to use his material.

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Byull. Exptl. Biol. i Med. 40:58-60. 1955. Bib No. 2153

EFFECT OF PANTOTHENIC ACID AND VITAMIN B_6 (PYRIDOXINE) ON THE REGENERATION OF CORNEAL EPITHELIUM

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Having read the data in the literature on the medicinal effect of pantothenic acid in marginal infiltration, burns, and traumatic corneal erosion, we conducted a series of experiments on rabbits for a comparative study of the effect of pantothenic acid and pyridoxine (vitamin B_6) on the regeneration of the epithelium of the corneal membrane.

The rabbits were kept, from the moment of operation until the cornea was completely healed, in special partitioned areas with an opening in one wall for the animal's head. ½% Dicaini solution was dropped 3 times into both the animal's eyes as an anesthetic. In the center of the corneal membrane of both eyes, a 4X4 mm was inserted using a Filatov trephine, with which the epithelium was subsequently scraped off with a scalpel. The portion of erosion was somewhat greater than the tracer, usually 5X6 mm. To stain the eroded portion, we dropped 1% fluorescein solution in the eyes and then the calcium salt of pantothenic acid, $(C_9H_{16}O_5)_2Ca$. The calcium pantothenate was dropped in the right eye of all the rabbits every 3 hours; at night (12 o'clock), an ointment of calcium pantothenate (in the same concentration) was used to cover the eye. The left eye served as a control. The next day the eroded portion was measured every 6 hours.

The calcium pantothenate (0.5% solution in 1-2 drops) in the right eye every 3 hours hastened healing of the erosion - it began at 52 hours - by 5-6 hours before healing of the left eye erosion (Table 1).

Table 1.

Effect of 0.5% Calcium Pantothenate Solution on the Healing of Corneal Erosions

Rabbit #	After operation until healing Dec. 13, in 10 hr 30 min		After healing Dec. 15, in 14 hr 30 min	
	right eye	left eye	right eye	left eye
	Eroded Portion (in mm)			
1	5X6	5X6	Localized erosion	4X6
2	5X6	5X6	Localized erosion	1X1.5
3	5X6	5X6	Localized erosion	1X2
4	5X5	5X6	1X2	2X4
5	4X5	4X5	Complete epithelialization	Localized erosion
6	5X6	5X6	Complete epithelialization	Localized erosion

Use of a higher concentration of calcium pantothenate -1% - resulted in a more active effect: the disappearance of the corneal erosion in the right eye occurred 9 hours earlier than in the left eye (control) (Table 2).

Table 2.

Effect of 1% Calcium Pantothenate Solution on Corneal Erosion Healing

Rabbit #	After operation until healing Mar. 15, in 19 hr 30 min		After healing Mar. 18, in 9 hr	
	right eye	left eye	right eye	left eye
	Eroded Portion (in mm)			
1	5X5	5X5	Localized erosion	2X5
2	4X4	5X5	Complete epithelialization	1X2
3	5X5	5X4	Complete epithelialization	1X2

Administration of 0.1% calcium pantothenate solution to 6 rabbits did not yield a similar effect and healing was accelerated in only one animal.

The following experiments on 15 rabbits were conducted to study the action of vitamin B_6 (pyridoxine B_6 - $C_8H_{11}O_3N$). In introducing 0.5% pyridoxine solution into the right eye 3 times a day (in the form of an ointment at night), corneal epithelium regeneration proceeded significantly more quickly and was completed after 94 hours - 10-12 hours earlier than in the control eye (Table 3).

Table 3.

Effect of 0.5% Vitamin B_6 (Pyridoxine) Solution on Corneal Erosion Healing

Rabbit #	Before healing Sept. 15, in 12 hr		After healing Sept. 17, in 9 hr	
	right eye	left eye	right eye	left eye
	Eroded Portion (in mm)			
1	6X4	4X4	Complete healing	1.5X1
2	4.4X4	4.5X4	Complete healing	2X3
3	4.5X5	4.5X4	Complete healing	Corneal edema, linear erosion
4	4.5X4	4.5X4	1X0.5	3X4
5	4X4	4X4	Complete healing	2X3
6	4.5X4	4.5X4	Complete healing	1X2
7	6.5X4	4X4	3X3	3X3

In only one rabbit (#7) was healing inhibited: healing of both eyes began only after 76 hours.

Thus, pantothenic acid and pyridoxine in appropriate doses hasten the regeneration of epithelium in corneal erosions.

The data set forth are evidence of the medicinal action of these vitamins.

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ВЛИЯНИЕ ПАНТОТЕНОВОЙ КИСЛОТЫ И ВИТАМИНА B_6 — ПИРИДОКСИНА НА РЕГЕНЕРАЦИЮ ЭПИТЕЛИЯ РОГОВИЦЫ

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(Поступила в редакцию 16/XI 1954 г. Представлена действительным членом АМН СССР А. Л. Мясниковым)

Учитывая литературные данные о лечебном действии пантотеновой кислоты при краевой инфильтрации, ожогах, травматической эрозии роговицы, мы поставили серию опытов на кроликах для сравнительного изучения влияния пантотеновой кислоты и пиридоксина (витамин B_6) на регенерацию эпителия роговой оболочки.

Кролики находились с момента операции и до полного заживления роговицы в специальных стенках с отверстием в одной стенке для головы

животного. Животным троекратно закапывали в оба глаза S. Dicaini ($^1/_2$%) для анестезии глаз. В центре роговой оболочки обоих глаз трепаном Филатова наносили метку (4×4 мм), по которой затем скальпелем соскабливали эпителий. Участок эрозии был несколько больше, чем метка, чаще 5×6 мм. Для окрашивания участка эрозии мы закапывали 1% раствор флюоресцина, а затем кальциевую соль пантотеновой кислоты — $(C_9H_{16}O_5)_2Ca$. Пантотенат кальция закапывали в правый глаз всем кроликам каждые 3 часа; на ночь (на 12 часов) накладывали мазь с пантотенатом кальция (той же концентрации). Левый глаз служил для контроля. На следующий день каждые 6 часов измеряли участок эрозии.

Введение пантотената кальция (0,5% раствор по 1—2 капли) в правый глаз каждые 3 часа ускоряло заживление эрозии — оно наступало через 52 часа — на 5—6 часов раньше, чем заживление эрозий левого глаза (табл. 1).

Таблица 1

Влияние 0,5% раствора пантотената кальция на заживление эрозии роговицы

№ кролика	После операции до лечения 13/XII в 10 часов 30 минут		После лечения 15/XII в 14 часов 30 минут	
	правый глаз	левый глаз	правый глаз	левый глаз
	участок эрозии (в мм)			
1	5×6	5×6	Точечная эрозия	4×6
2	5×6	5×6	То же	1×1,5
3	5×6	5×6	» »	1×2
4	5×5	5×6	1×2	2×4
5	4×5	4×5	Полная эпителизация	Точечная эрозия
6	5×6	5×6	То же	То же

Применение более высокой концентрации пантотената кальция— 1%—оказывало более активное влияние: исчезновение эрозии роговицы правого глаза наступало на 9 часов раньше, чем в контрольном левом глазу (табл. 2).

Таблица 2

Влияние 1% раствора пантотената кальция на заживление эрозии роговицы

№ кролика	После операции до лечения 15/III в 19 часов 30 минут		После лечения 18/III в 9 часов	
	правый глаз	левый глаз	правый глаз	левый глаз
	участок эрозии (в мм)			
1	5×5	5×5	Точечная эрозия	2×5
2	4×4	5×5	Полная эпителизация	1×2
3	5×5	5×4	То же	1×2

Введение 6 кроликам 0,1% раствора пантотената кальция не давало подобного эффекта и ускорило заживление эрозии только у одного животного.

Следующие опыты на 15 кроликах были поставлены для изучения действия витамина B_6 — пиридоксина B_6 — $C_8H_{11}O_3N$. При введении в правый глаз 0,5% раствора пиридоксина 3 раза в день (на ночь в виде мази) регенерация эпителия роговицы происходила значительно быстрее и полностью заканчивалась через 44 часа — на 10—12 часов раньше, чем в контрольном глазу (табл. 3).

Таблица 3

Влияние 0,5% раствора витамина B_6—пиридоксина на заживление эрозии роговицы

№ кролика	До лечения 15/IX в 12 часов		После лечения 17/IX в 8 часов	
	правый глаз	левый глаз	правый глаз	левый глаз
	участок эрозии (в мм)			
1	6×4	4×4	Полное заживление	1,5×1
2	4,4×4	4,5×4	То же	2×3
3	4,5×5	4,5×4	» »	Отек роговицы, линейные эрозии
4	4,5×4	4,5×4	1×0,5	3×4
5	4×4	4×4	Полное заживление	2×3
6	4,5×4	4,5×4	То же	1×2
7	6,5×4	4×4	3×3	3×3

Только у одного кролика (№ 7) заживление правого глаза задержалось: заживление обоих глаз наступило только через 76 часов.

Таким образом, пантотеновая кислота и пиридоксин в соответствующих дозах ускоряют регенерацию эпителия при эрозиях роговицы.

Изложенные данные подтверждают лечебное действие этих витаминов.

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The Function of Vitamin B_6 in Fat Metabolism[1]

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VITAMIN B_6 is one of the B vitamins which are essential for human nutrition. The requirement of the vitamin for man is believed to be 2–3 mg per day (1). Structurally related pyridine derivatives, pyridoxine, pyridoxal, and pyridoxamine represent the members of the vitamin B_6 group (Figure 1). Pyridoxine is mainly found in plant tissue whereas pyridoxal and pyridoxamine are largely found in animal tissue (2). The existence of pyridoxal 5-phosphate (3) and pyridoxamine 5-phosphate (4) in natural sources has been proved; the former phosphate is known as codecarboxylase, a coenzyme form of vitamin B_6, which is actively engaged in protein and amino acid metabolism. The various forms of vitamin B_6 and its phosphates are biochemically interconvertible (Figure 1). They are excreted from the body as such (5), as 4-pyridoxic acid (5, 6), and as a complex of unknown structure (7, 8).

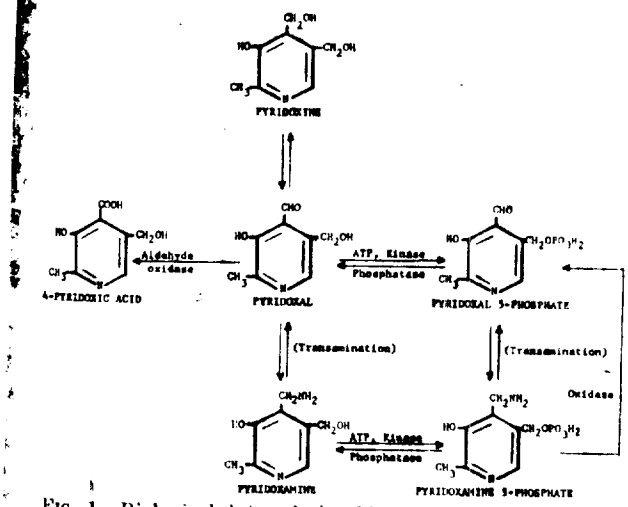

FIG. 1. Biological interrelationship among the members of vitamin B_6 and 4-pyridoxic acid.

Vitamin B_6 and Oxidation of Fats. Vitamin B_6 is known to be unstable in the presence of oxidizing agents (9). Burr and Barnes have suggested that vitamin B_6 might eventually be destroyed when it is brought into contact with oxidized or rancid fats (10). The 4-hydroxymethyl group of the pyridoxine molecule is reactive, and pyridoxine is converted to pyridoxal upon mild treatment with manganese dioxide or potassium permanganate (Figure 2) (11, 12). Further oxidation with the latter oxidizing agent leads to the formation of 4-pyridoxic acid, which is lacking in vitamin activity and constitutes the main excretory end-product of the vitamin in humans (6). By means of spectrophotometric techniques it has been shown that pyridoxal and its structurally related 5-deoxypyridoxal can also exist in the resonance form, a semiquinone structure (13, 14). When pyridoxal or the deoxypyridoxal was treated with hydrogen peroxide in an alkaline condition, the resulting product was an *ortho* dihydroxy compound (Figure 2) (13, 14). It is unknown however whether pyridoxine and pyridoxal undergo such reactions when they come in contact with oxidized fats. Vitamin B_6 contains a hydroxyl group at the 3-position which shows typical phenolic reactions, and structurally it belongs to a group of hindered phenols. It is therefore not surprising to find antioxidative activity in the vitamin. Indeed Hove and Harris reported that pyridoxine, in the form of a free base, stabilized vitamin A in fish oils (15). However an attempt to find possible antioxygenic properties in the fat-soluble derivative of pyridoxine, pyridoxine 5-monopalmitate (16), failed to succeed (17). It was also noted that the 4,5-bisdeoxy analog of pyridoxine, 2,4,5-trimethyl-3-pyridinol, was much less effective than the corresponding benzene derivative, 2,3,6-trimethylphenol, in protecting lard from oxidation at 37°C. (17). This indicates that the nitrogen in the ring probably deactivates pyridoxine as an antioxidant despite the favorable arrangement of the substituents.

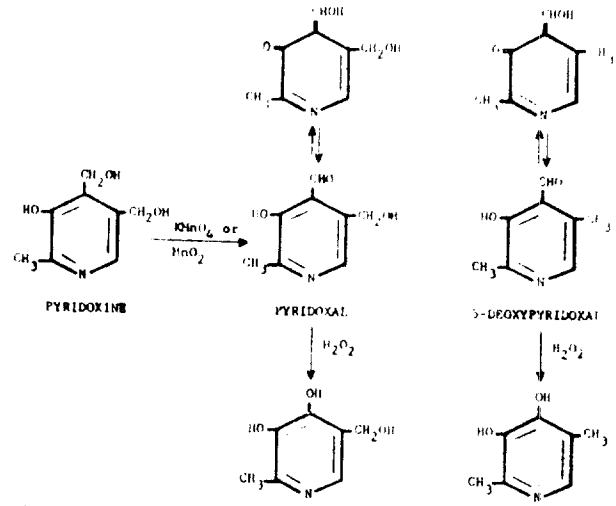

FIG. 2. Oxidation of pyridoxine and its related compounds.

A well-known physiological antioxidant, α-tocopherol, has been reported to spare essential fatty acids in rats (15). Because of the properties superficially similar to tocopherol Hove and Harris postulated that vitamin B_6 might also serve as a physiological antioxidant (15). It was noted that, in plant cells, pyridoxine prevented the oxidation of the unsaturated fatty fractions *in vitro* as well as *in vivo* (18). Abnormal excretion of allantoin and creatine by rats which were deficient in both vitamin E and B_6 was eliminated upon administration of either one of the vitamins (19). Such possible antioxygenic properties of vitamin B_6 *in vivo* have been considered as an explanation for the effective cure of radiation injuries with pyridoxine (20). Although no direct evidence has been obtained to show that vitamin B_6 is involved in the biological oxidation-reduction systems, Tulpule and Williams suggested that the vitamin was essential for the utilization of linoleic acid in maintaining the phosphate esterification system which is closely associated with the oxidation of reduced cytochrome c

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(21). Depression in the activity of liver glutamic dehydrogenase in pyridoxine-deficient rats has also been reported (22). How vitamin B_6 contributes to the maintenance of the enzyme function remains obscure. Witting *et al.* observed that the poor growth of rats kept on a diet containing "used" oils could be partially overcome upon increasing the dietary level of pyridoxine (23) although the mechanism of reversal remains unknown.

Vitamin B_6 and Fat Metabolism. It has been claimed that vitamin B_6 plays a vital function in general fat metabolism, including the conversion of carbohydrate and proteins to fats (24, 25), although the studies are open to alternative interpretation. It has been reported that vitamin B_6 is indispensable (26) for the conversion of linoleic to arachidonic acid.

An ingenious method has become available for the preparation of carboxy-labelled essential fatty acids, the synthesis of which is not otherwise readily achieved (27). The principle involves decarboxylation of fatty acids, followed by regeneration of the carboxyl group with radioactive carbon dioxide. By means of tracer techniques Mead and his coworkers investigated the mode of *in vivo* conversion of arachidonate from linoleate or γ-linolenate and other highly unsaturated fatty acids. When linoleate-1-C^{14} was fed to rats, the arachidonate fraction isolated from the tissue contained radioactivity, and 74.7% and 24.5% of the total activity were found to be located at the carboxyl carbon and the 3-position, respectively. Only a trace of activity was detected at the 2-position, and no activity was found in the 4-position or in the rest of the chain (28). This indicated that linoleic acid was a precursor of arachidonic acid. The findings also indicated that a part of the radioactive linoleate underwent β-oxidation *in vivo*, and some of the two-carbon fragments thus freed were then condensed at the carboxyl of the linoleate. Condensation of linoleate and a two-carbon fragment to form an arachidonate skeleton has also been confirmed by using radioactive acetate and unlabelled linoleate (29). Similar tests proved the absence of formation of arachidonate from linolenate (30). Mead *et al.* also prepared γ-linolenate-1-C^{14} and found that this acid served exclusively as a source of arachidonate (31). The results led them to postulate that linoleate was transformed to arachidonate most likely through γ-linolenate. This reaction involved removal of four hydrogen atoms from the molecule and condensation with a two-carbon fragment at the carboxyl end of the linoleate (Figure 3). No evidence however has yet been obtained to show that γ-linolenate is actually formed from linoleate *in vivo*. It is suggested that an intermediate other than γ-linolenate may also be involved in arachidonate formation (31).

Although it is clear that vitamin B_6 is in some way closely connected with the formation of arachidonate and possibly a hexaenoate from linoleate and linolenate, respectively (26), the mechanism is totally unknown. It remains to be investigated whether vitamin B_6 is indispensable for the synthesis or for the maximum function of enzymes involved in metabolism. For the conversion of palmitic acid to palmitoleic acid and of stearic acid to oleic acid, vitamin B_6 is believed to be required as a cofactor (32).

Four sources of evidence have shown a close biological interrelationship between vitamin B_6 and essential fatty acids. a) In rats the skin lesions produced by vitamin B_6 deficiency and those produced by essential fatty acid deficiency show superficial resemblance; b) onset of vitamin B_6 deficiency symptoms is facilitated by removing essential fatty acids from the diet and *vice versa;* c) deficiency symptoms of one of the nutrients can be partially or temporarily cured upon administration of the other, and most efficiently cured with both; and d) changes in the composition of polyunsaturated fatty acids in the tissue take place when vitamin B_6 is removed from the diet (33). The two deficiency symptoms however are histologically quite distinct (33).

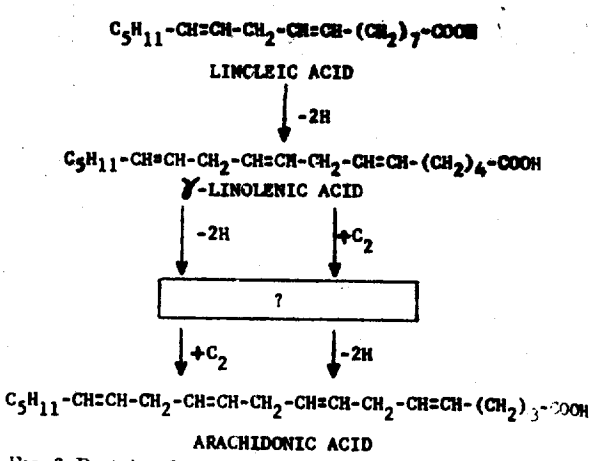

FIG. 3. Postulated conversion mechanism from linoleic acid to arachidonic acid *in vivo* (31).

As early as 1938 Birch postulated that vitamin B_6 might combine with unsaturated fatty acids and phosphoric acid to form a phospholipide type of compound as an essential cell constituent (34). Although the existence of such vitamin B_6 complexes in nature is still unknown, it is of interest that both vitamin B_6 and essential fatty acids are now known to be vitally involved in the maintenance of normal cell functions and the regulation of cell permeability (35).

Sinclair postulated that destruction of cell structure in essential fatty acid deficiency and in vitamin B_6 deficiency was caused by an inability to synthesize phospholipides and an interference with protein metabolism (36). Beaton *et al.* however reported that in the regenerating liver tissues of partially hepatectomized normal as well as vitamin B_6 deficient rats, essential fatty acid deficiency did not affect the synthesis of cellular protein or the maintenance of liver enzyme protein (37). It is of interest to note that either linoleate or pyridoxine is able to protect animals from radiation injury (38, 39). It has also been suggested that a complex of pyridoxine and arachidonate may constitute an essential component of skin tissue (40). Another link of the function of vitamin B_6 to fat metabolism has been amply demonstrated in seborrheic dermatitis. In human subjects vitamin B_6 deficiency, which was often produced experimentally with the aid of an antivitamin B_6, induced seborrheic dermatitis of the "sicca" type. A typical symptom involves abnormal deposition of fatty material on the skin. This can be effectively cured upon topical application of pyridoxine (41).

In conjunction with the effect of vitamin B_6 on the maintenance of cell structure it seems of interest to mention the observation of Holden and Holman.

strain of lactic acid bacteria, *Lactobacillus arabinosus* 17-5, is rod-shaped with a round end. When the culture of this organism was transferred successively in a vitamin B_6-deficient medium, all the cells began to show a swollen elliptical shape (42, 43). The microorganism might therefore serve as a convenient tool for the study of vitamin B_6 and its relationship to cell structure.

Rinehart and Greenberg observed that the rhesus monkeys which had been kept on a vitamin B_6-deficient diet for a prolonged period of time tended to produce more advanced arteriosclerotic lesions than those found in the control animals (44). They later reported that pyridoxine deficiency in monkeys regularly led to the formation of sclerotic lesions in the blood vessels, which was also accompanied by high blood steroid levels (45). The results obtained by other workers from similar lines of study were not always in agreement with the findings of Rinehart *et al.* (46, 47, 48). It is however not surprising to find some correlations between the deposition of fatty materials in the circulating systems and vitamin B_6, for the vitamin is indispensable for the normal metabolism of fats. If essential fatty acid is directly involved in the mobilization and deposition of cholesterol, as has been postulated (49), it is understandable that vitamin B_6 would indirectly but actively affect the metabolism of the steroid. It may also be implied that vitamin B_6 stimulates the *in vivo* conversion of tryptophan to nicotinic acid, which, in turn, may depress serum cholesterol levels (50). The effect of the vitamin in preventing the deposition of fatty materials, as observed by Rinehart *et al.*, may not be a direct one. They found that the initial lesion was characterized by the accumulation of a mucous substance in the intima and in the media of the arteries involved (51). This material was not fatty in nature and was found to exhibit reactions characteristic of mucopolysaccharides. Deposition of lipide material takes place in the later stage of atherosclerosis. The findings suggest that vitamin B_6 deficiency might impair the functions of enzymes in the cells, and the degenerated cells, in turn, would be susceptible to the deposition of fatty material along the damaged walls.

Conclusion

It is evident that vitamin B_6 serves as a physiological antioxidant. The mechanism cannot be dependent solely on the chemical structure of the vitamin. Despite the favorable arrangement of the substituents, pyridoxine and its derivatives show no, or only slight, antioxidative activity *in vitro*. The inactivation apparently results from the nature of the pyridine ring.

Vitamin B_6 is one of the B vitamins which are required for the normal metabolism of the fats and essential fatty acids. However it still remains to be investigated whether the vitamin is directly associated with fat metabolism or whether the vitamin itself is responsible for the maximum function or the synthesis of enzymes involved.

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Effect of High Intakes of Thiamine, Riboflavin and Pyridoxine on Reproduction in Rats and Vitamin Requirements of the Offspring [1]

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ABSTRACT Female rats were fed during pregnancy and lactation a control diet containing 0.25 mg pyridoxine, 0.4 mg thiamine, and 0.4 mg riboflavin/100 g, or a high pyridoxine, high thiamine, or high riboflavin diet containing the specified vitamin at 25 times the level of the control diet. The levels of these vitamins in the carcasses of the young at birth and in the liver at weaning were determined. The effect of maternal vitamin intake on the vitamin requirements of the young was tested in 2 ways: 1) by comparing the rate of depletion of the young of females fed the high vitamin diets with the rate of depletion of the control group, and 2) by the growth response of the depleted young to graded levels of the vitamin fed in excess in the maternal diet. It was concluded that high intakes of thiamine, riboflavin, or pyridoxine during the reproductive period had no effect on the young, as shown by litter size at birth, growth until weaning, or their vitamin requirements after weaning.

Relatively little attention has been given to the effects of high intakes of B vitamins on reproduction. Massive doses of thiamine have been reported to interfere with lactation, produce cannibalism, and decrease fertility in rats (1, 2). Richards (3) also reported that excess thiamine fed to female rats increased mortality and decreased the weights of the young at weaning. However, Morrison and Sarett (4) reported normal reproduction in rats fed high levels of thiamine. An abnormally high need for pyridoxine was reported in a newborn human infant whose mother had received large doses of pyridoxine intramuscularly during the first trimester of pregnancy (5). It was suggested that excess pyridoxine intake during gestation may have increased the pyridoxine requirement of the infant. Later, Hunt (6) reported that the pyridoxine intake of the dam during gestation did not affect the rate of vitamin B_6 depletion or increase the occurrence of convulsions in young rats after birth. Morrison and Sarett (4) also reported that a high intake of pyridoxine during pregnancy did not affect reproduction or increase the rate of vitamin B_6 depletion in young rats in the period from 3 to 5 weeks of age.

The preceding studies have shown that high levels of pyridoxine in the maternal diet did not appear to increase the vitamin B_6 requirement of young rats as indicated by rate of vitamin B_6 depletion. If this had been the case, then vitamin B_6 deficiency should have developed more rapidly in the young from the dams fed the high pyridoxine diets. No test was made, however, of the pyridoxine requirement of the young rats after they had been depleted of pyridoxine. This point is important, since an increased need for pyridoxine in the young during the depletion period may have been masked by the higher liver stores of the young from the dams fed the high pyridoxine diets. Greater liver stores were reported by Morrison and Sarett (4).

The following experiment tested the effect of maternal intake of pyridoxine, thiamine, or riboflavin on the rate at which the young rats were depleted of each vitamin, as well as on the subsequent growth response of the depleted young to graded levels of the vitamins.

METHODS

Animals. Thirty female rats (about 300 g) of the Long-Evans strain, were used for each of 3 experiments. In each experi-

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ment, 15 females were fed the basal diet.[2] The other rats were divided into 3 groups of 5 rats each. One group was fed the high pyridoxine diet, the second group the high thiamine diet, and the third group the high riboflavin diet. The high vitamin diets contained 25 times the level of pyridoxine, thiamine, or riboflavin used in the basal diet. The basal diet contained: (in mg/100 g) pyridoxine, 0.25; thiamine, 0.4; and riboflavin, 0.4 (7). The high vitamin diets contained: (in mg/100 g) pyridoxine, 6.25; thiamine, 10; and riboflavin, 10. Food and water were provided ad libitum. Weight gain and food intake were recorded 3 times weekly.

The females were mated after they had been fed the experimental diets for 2 weeks, and these diets were continued during gestation and lactation. The number and average weight of the newborn were recorded as soon as possible after the birth of each litter. Some of the newborn from each diet group were decapitated, the stomachs and intestinal tracts removed, and the carcasses weighed and frozen. The remaining young were nursed for 21 days, when they were weaned. The young had access to the maternal diet during the nursing period. At weaning, representative young from each group were decapitated, the livers removed, weighed and frozen. A number of the male weanlings, weighing from 40 to 50 g, were saved for the growth study.

The growth study was divided into a depletion period and a supplementation period. The rats were housed individually in galvanized, screen-bottom cages. Food and tap water were given ad libitum. Weight gains and food intakes were recorded 3 times weekly. Four to five animals from the high vitamin maternal groups were fed the basal control diet, with the omission of the vitamin which had been fed in excess in the maternal diet. Weanlings from the dams fed the basal diet were also fed the vitamin-deficient diets. The remaining rats from each maternal group served as controls and were fed the complete basal ration. All of the diets were fed until the deficient groups ceased to gain (weight plateau) or until their rate of gain was significantly less than that of the appropriate control group.

Once depletion was established, the animals were fed the appropriate vitamin at a below-minimal or minimal level. The minimal level for pyridoxine was 15 μg/rat/day (4);[3] for thiamine, 10 μg/rat/day (8); for riboflavin, 24 μg/rat/day (7). The below-minimal levels were one-half of the minimal level. Negative controls (without pyridoxine, thiamine, or riboflavin) also continued to be fed the deficient diets.

Each level of the vitamin was made up in a 20% ethyl alcohol solution. Two milliliters of this solution were fed 3 times a week. The total supplement over a period of one week was equivalent to 7 times the daily amount indicated.

Vitamin analyses. The carcasses of the newborn and the livers of the weanlings were homogenized and analyzed for pyridoxine, thiamine, and riboflavin. Pyridoxine was determined microbiologically with *Saccharomyces carlsbergenesis* in a modification of the method of Rabinowitz and Snell (9). Thiamine was determined by the thiochrome method and riboflavin was determined fluorometrically (10).

RESULTS

Reproduction. Reproduction data are shown in table 1. The reproduction of the control group and the high thiamine group was considerably better (68% for both groups) than that of the high riboflavin or high pyridoxine groups (38 and 47%, respectively). The value of 68% was slightly less than that usually found for the stock colony females of this laboratory (70 to 75%).

The average birth weight, number of young per litter, and average weight of the young at weaning were not signi-

[2] Composition of basal diet: (g/100 g) vitamin free casein, 18; sucrose, 61.8; USP Salts 14, 4; Cellu Flour (Chicago Dietetic Supply House, Chicago), 5; cottonseed oil, 9; vitamin-fortified cottonseed oil, 1; vitamin mix in sucrose, 1; choline chloride, 0.15. The fortified oil provided per 100 g diet: vitamin A, 17,000 IU; vitamin D_2, 100 IU; α-tocopheryl acetate, 6.7 mg. The vitamin mixture in sucrose provided: (mg/100 g diet) thiamine, 0.4; riboflavin, 0.4; pyridoxine, 0.25; pantothenate, 2.0; inositol, 10.0; biotin, 0.01; folic acid, 0.1; niacinamide, 1.0; vitamin B_{12} (0.1% triturate in mannitol), 0.02; menadione, 0.5.

[3] The minimal requirement for pyridoxine for normal growth in the rat beyond 6 weeks of age has recently been shown to be greater than 30 μg/day (Beaton, G. H., and M. C. Cheney, Federation Proc. 24: 624, 1965; Williams, M. A., Federation Proc. 24: 624, 1965). However 15 μg/day will produce nearly maximal growth in the rat for the first 2 weeks after weaning or the first 2 weeks of repletion of previously depleted rats (Williams, M. A., unpublished results).

TABLE 1

Reproduction data on animals receiving a diet containing levels of thiamine, riboflavin or pyridoxine 25 times that of the control level [1]

Maternal diet	No. of mothers	No. of litters	No. of young	Average birth wt	Survival	Average wt at 21 days
				g	%	g
Control [1]	40	27 [2] (68%)	295	6.2	78.6 (214) [3]	45.2
+ thiamine (10 mg/100 g diet)	19	13 (68%)	105	6.6	89.4 (96)	45.6
+ riboflavin (10 mg/100 g diet)	13	5 (38%)	51	6.2	60.0 [4] (40)	47.9
+ pyridoxine 6.25 mg/100 g diet)	15	7 (47%)	64	6.1	83.0 (53)	45.8

[1] Control diet contained: (mg/100 g diet) thiamine, 0.4; riboflavin, 0.4; pyridoxine, 0.25.
[2] Cumulative figure; each experiment included a control group of approximately 10 animals.
[3] Numbers in parentheses refer to number of observations on which percentage is based.
[4] Ten offspring that did not survive in this group were all of one litter; therefore the apparent difference loses significance.

TABLE 2

Effect of the level of thiamine, riboflavin or pyridoxine in the maternal diet on the storage of the vitamin in the fetal carcass and in liver of offspring at weaning

Maternal diet	Fetal carcass storage			Liver storage		
	Thiamine	Riboflavin	Pyridoxine	Thiamine	Riboflavin	Pyridoxine
	μg/g	μg/g	μg/g	μg/g	μg/g	μg/g
Control [1,2]	0.93 ± 0.27 [3] (5) [4]	3.43 ± 0.28 (8)	0.79 ± 0.15 (7)	5.81 ± 0.81 (8)	21.46 ± 2.53 (5)	7.43 ± 1.84 (10)
+ thiamine (10 mg/100 g diet)	0.95 ± 0.11 (5)	—	—	10.13 ± 0.75 [5] (8)	—	—
+ riboflavin (10 mg/100 g diet)	—	3.26 ± 0.19 (10)	—	—	22.41 ± 3.64 (5)	—
+ pyridoxine (6.25 mg/100 g diet)	—	—	1.23 ± 0.11 [5] (6)	—	—	7.12 ± 0.52 (10)

[1] Control diet contained: (mg/100 g diet) thiamine, 0.4; riboflavin, 0.4; pyridoxine, 0.25.
[2] Each vitamin analysis had its own control group.
[3] Mean ± SE.
[4] Numbers in parentheses indicate number of samples analyzed.
[5] Difference is statistically significant ($P < 0.01$).

cantly influenced by increasing the levels of pyridoxine, thiamine, or riboflavin in the maternal diet. The number of offspring per litter from the dams fed the high thiamine ration was somewhat smaller than that from the other maternal groups. The average birth weight, however, was somewhat greater in the high thiamine group. The higher mortality (birth to weaning) in the high riboflavin group reflected the complete loss of one litter (10 young). Two control litters were also lost because the mothers failed to nurse. More data are needed to determine whether the high riboflavin diet increased mortality.

Tissue vitamin storage. High levels of thiamine or riboflavin in the maternal diet did not increase the levels of vitamins in the newborn (table 2). The level of pyridoxine in the newborn from the dams fed the high pyridoxine diet was significantly higher than the level for the control group ($P < 0.01$). At weaning, the offspring of the females fed the high thiamine diet had nearly twice as high a level of liver thiamine as did the controls. In contrast with the effect of thiamine, high levels of riboflavin or pyridoxine in the maternal diet did not influence the storage of these 2 vitamins in the livers of the weanlings. The difference caused by maternal diet in the tissue concentration of pyridoxine at birth did not appear at weaning (21 days after birth).

Growth studies — depletion and supplementation. Figure 1 shows the growth curves of the different groups when fed diets deficient in pyridoxine, thiamine, or riboflavin, as well as the control groups. The only difference observed during depletion was that the onset of thiamine deficiency was delayed slightly in the rats from the dams fed the high thiamine diet. Maternal diet did not affect the development of riboflavin deficiency, in which both groups reached a plateau in body weight by day 21. The depletion period of vitamin B_6 was arbitrarily ended on day 28 when the weight gains of the deficient groups were significantly less than the gains of the groups fed the control diet. Maternal pyridoxine intake did not affect pyridoxine depletion in the young (fig. 1). Neither did the maternal diet affect the growth response of any of the groups to the control diet.

Figure 2 shows the growth response of the vitamin-depleted rats to graded levels of the corresponding vitamin. All groups received the supplements for 14 days. Growth reflected the level of supplementa-

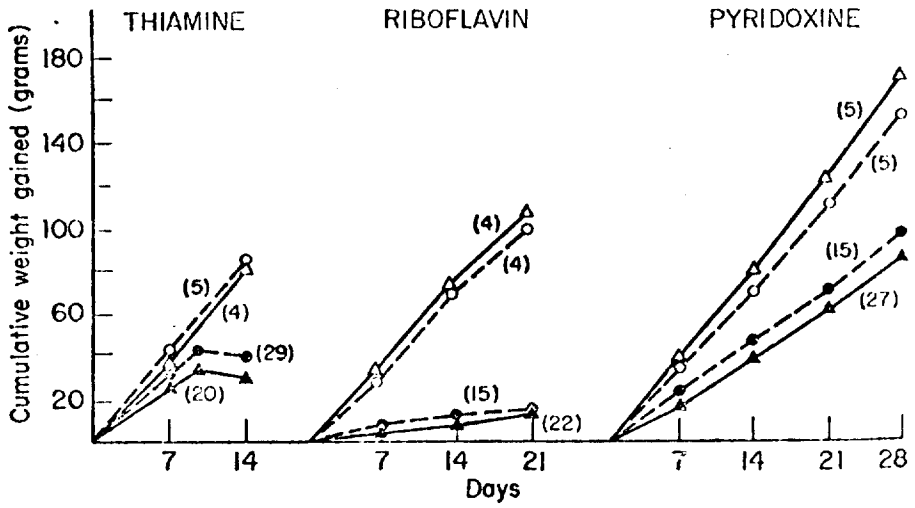

Fig. 1 Influence of excess thiamine, riboflavin, or pyridoxine in maternal diet on weight gain of male weanling rats fed a diet deficient in the respective vitamin. △——△, control young fed adequate diet; ○--○, experimental young fed adequate diet; ▲——▲, control young fed depletion diet; ●---●, experimental young fed depletion diet. Numbers in parentheses indicate number of rats in the group.

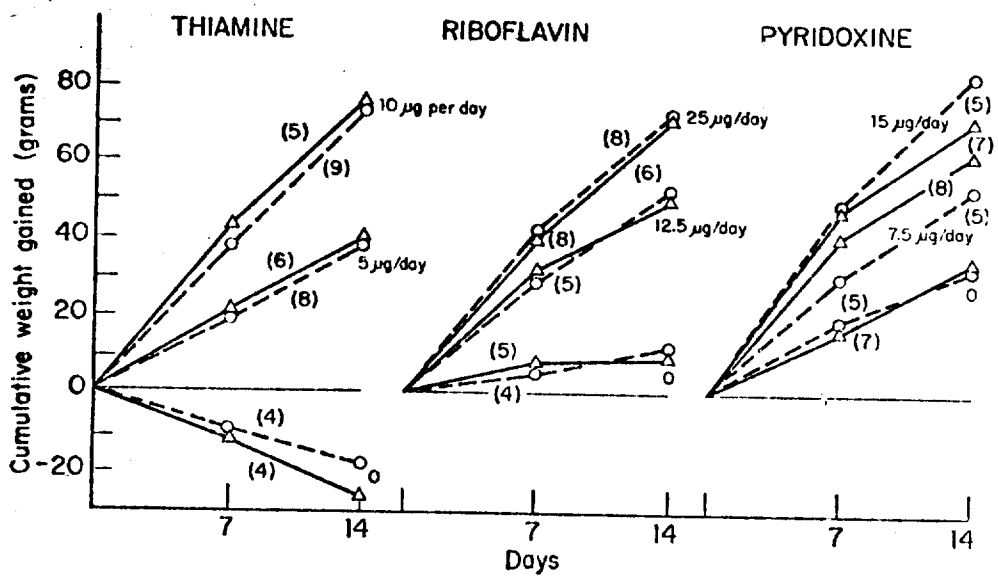

Fig. 2 Influence of excess thiamine, riboflavin, or pyridoxine in maternal diet on weight gain of previously depleted male rats fed graded levels of that vitamin. △——△, control young; ○- - -○, experimental young. Numbers in parentheses indicate number of rats in the group.

tion for each vitamin, and maternal diet had no significant effect. The greater variability in the growth of both pyridoxine-depleted groups to supplements of 7.5 or 15 µg pyridoxine is typical of the response of pyridoxine-depleted rats fed at these levels of the vitamin.[4] Nevertheless, the rats from the dams fed the high pyridoxine diet made weight gains similar to those from the control dams. If the need for pyridoxine had been greater in the young from the high pyridoxine dams, then their weight gains at both levels of pyridoxine would have been less than those of the control young. This lack of a difference in pyridoxine need between the young from the high pyridoxine dams and the control group is supported by the similar weight changes in the pyridoxine-depleted groups that continued to be fed the pyridoxine-deficient diet.

DISCUSSION

The reduced number of litters for the high riboflavin and high pyridoxine groups may be related to the high maternal intake of these vitamins. More data are needed to establish this point. With respect to the number of young per litter and the average birth and weaning weights, all of the high vitamin groups and the control group compared favorably in performance with the laboratory breeding colony. The results from the high thiamine diet confirm the conclusions of Morrison and Sarett (4) that high intakes of thiamine did not affect the overall reproductive performance, in contrast with the report of Richards (3).

The level of thiamine (0.93 µg/g) in the tissues of the newborn from either the control or the high thiamine group was less than that reported by Barrett and Everson (11) and by Brown and Snodgrass (12). Barrett and Everson reported a value of 3.0 µg/g with a maternal diet containing 1.32 mg/100 g. Brown and Snodgrass reported a level of approximately 2 µg/g with a maternal diet containing 0.25 or 0.50 mg/100 g. There is no explanation for these differences except that the analyses were not made on comparable tissues. In the present study, the vitamin analyses were made on the exsanguinated carcass, with the head, stomach, and intestinal tract removed. The other reports imply that the analyses were made on the entire animal. Fetal storage of riboflavin (3.34 µg/g) was similar to

[4] Williams, M. A., unpublished results.

the values of Barrett and Everson (2.5 μg/g). No values for fetal storage of pyridoxine were found in the literature.

At weaning, liver storage of thiamine reflected the thiamine level of the maternal diet although the maternal diet did not affect the carcass thiamine of the newborn. The higher level at weaning may reflect the amount of maternal diet eaten by the young before weaning. The level in the control weanlings (5.8 μg/g) is higher than the value (1.9 μ/g) observed by Morrison and Sarett whose control diet contained only 0.15 μg thiamine/100 g, in contrast with 0.4 mg/100 g in the present study. Ochoa and Peters (13) and Byerrum and Flokstra (14) had reported previously that increasing the dietary thiamine above that needed for maximal growth increased the tissue concentrations of thiamine.

High levels of riboflavin or pyridoxine in the maternal diet did not influence the liver storage of these 2 vitamins at weaning. The value for riboflavin (21.5 μg/g) was similar to the value observed by Decker and Byerrum (15) in rats 30 days after birth (24.5 μg/g). The similarity of values for liver pyridoxine in both the high pyridoxine and the control groups differs from the results of Morrison and Sarett, who observed increased levels of pyridoxine in the livers of weanlings from dams fed high pyridoxine diets (7.5 mg/100 g) in contrast with a control diet containing 0.15 mg/100 g. Their values were 8.8 μg/g for the high pyridoxine group and 4.8 μg/g for the control group. In the present paper the values were 7.1 μg/g for the high pyridoxine group and 7.4 μg/g for the control. Perhaps the level of pyridoxine in the diet of Morrison and Sarett (4) was too low for maximal tissue storage, although this is above the stated requirement (16).

The growth studies gave no evidence for any beneficial effect in the young resulting from high maternal intakes of these vitamins during pregnancy and lactation. In addition, there was no evidence that the vitamin requirements of the offspring were increased, that is, the rats were made more vitamin-dependent, as a result of high maternal intakes. The slight delay in the development of thiamine deficiency in the rats from the high thiamine maternal diet probably reflects the increased tissue thiamine storage at weaning.

In conclusion, high levels of thiamine, riboflavin, or pyridoxine ingested during the reproductive period had no effect on the young, as shown by litter size at birth, growth until weaning, or vitamin requirements after weaning.

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A STUDY OF THE URINARY EXCRETION OF VITAMIN B_6 BY A COLORIMETRIC METHOD

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Chemical methods for the determination of biological substances, in contrast with animal assays, offer a considerable saving in time, and the analytical data are usually more precise and reproducible. For these reasons, a study of the vitamin B_6-indophenol color reaction has been in progress in these laboratories for some time (1). In the course of this work a procedure has been devised whereby solutions containing a minimum of 2 micrograms of the vitamin per cc. can be analyzed with precision (2). The general applicability of this method, the nature of interfering substances, and the specificity of the test are under investigation. Details of this work will be presented elsewhere.

Method

It has been found that this method may be used in a very simple form to measure the urinary excretion of vitamin B_6 administered orally or parenterally. This application of the method depends upon the analysis of samples of urine obtained before and after the administration of the vitamin. Both analyses are necessary because interfering substances present in the preadministrative or control sample give rise to a small concentration of colored reaction products. This concentration, measured in the Evelyn colorimeter with a No. 660 filter to reduce the influence of extraneous colors, is subtracted from the concentration found in the postadministrative sample. The difference is the vitamin B_6 output. This is based on the assumption, however, that the interfering substances are the same in both samples of urine, which seems likely under the conditions employed.

In general, the conditions used are such as might be used clinically in a saturation test of vitamin B_6 deficiency. A fasting subject was given water to flush the urinary tract. The urine was discarded. After 1 hour, the control sample was taken and the bladder was emptied. At this time additional water was

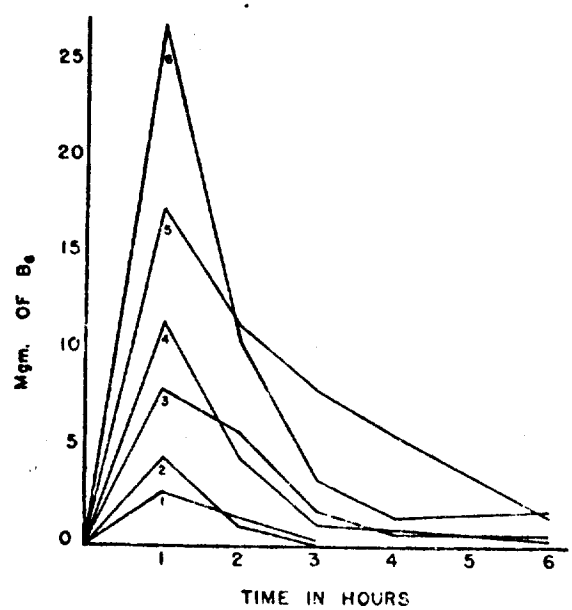

FIG. 1. Excretion of vitamin B_6 following oral administration. Curve 1, Dog 51, dose 50 mg.; Curve 2, Dog 58, dose 50 mg.; Curve 3, Dog 31, dose 100 mg., 6 hour output 16.8 per cent; Curve 4, Dog 97, dose 100 mg., 6 hour output 18.4 per cent; Curve 5, Dog 92, dose 200 mg., 6 hour output 22.0 per cent; Curve 6, Dog 31, dose 200 mg., 6 hour output 22.1 per cent, Dog 31, dose 500 mg., 6 hour output 20.3 per cent, Dog 97, dose 500 mg., 6 hour output 18.7 per cent. For Curves 1, 2, and 5 the urine samples were taken by catheter.

given to provide an adequate diuresis and the vitamin was injected intravenously or given orally. Postadministrative samples were then taken at measured intervals of time. Under these conditions, interfering substances should be essentially the same in both samples, although the concentration might be slightly lower in the postadministrative samples. This would tend to

give low, rather than high results. Urine samples taken from test subjects were analyzed both by the chemical method and by the curative assay with vitamin B_6-deficient rats (3) and the results were found to be in excellent agreement. Furthermore,

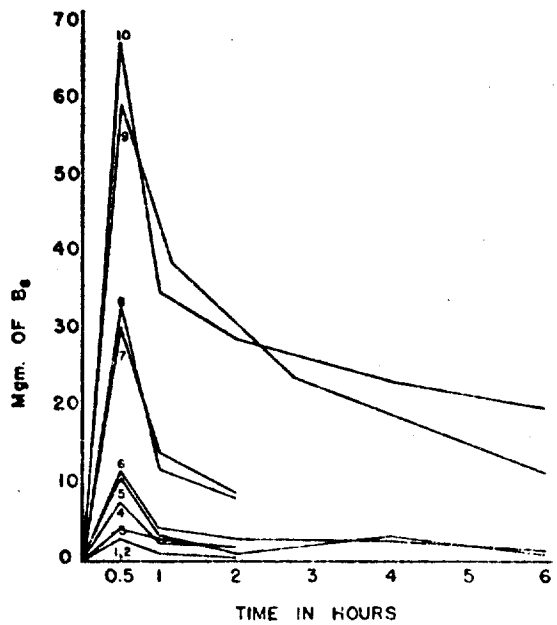

FIG. 2. Rate of excretion following the intravenous administration of vitamin B_6. Curve 1, Dog 31, dose 25 mg., 1 hour output 17.2 per cent; Curve 2, Dog 97, dose 25 mg., 1 hour output 16.8 per cent; Curve 3, Dog 97, dose 50 mg., 1 hour output 15.0 per cent; Curve 4, Dog 31, dose 50 mg., 1 hour output 20.8 per cent; Curve 5, Dog 97, dose 100 mg., 1 hour output 14.4 per cent; Curve 6, Dog 97, 100 mg., 1 hour output 16.5 per cent; Curve 7, Dog 31, dose 250 mg., 1 hour output 18.0 per cent; Curve 8, Dog 97, dose 250 mg., 1 hour output 18.0 per cent; Curve 9 (urine by catheter), Dog 66, dose 500 mg., 1 hour output 20.8 per cent; Curve 10, Dog 97, dose 500 mg., 1 hour output 20.5 per cent.

100 per cent recovery was obtained upon the addition of vitamin B_6 to samples of both dog and human urine. Nevertheless, in order to eliminate further the possibility of variations in urine samples, both the control and postadministrative samples of urine were diluted equally until the control urine gave a blank, or

very faint color reaction. The concentration of the postadministrative sample was then reckoned directly from a calibration curve, or by difference as the case required. This dilution reduces the sensitivity of the test, and may be a handicap if doses lower than 50 mg. are required in the development of a saturation test in man.

Experiments in Dogs—Six dogs, weighing 7 to 15 kilos and maintained on a diet of Purina Dog Chow, were used. Control

TABLE I

Vitamin B_6 Output in Human Subjects after Intravenous and Oral Administration

Subject No.	After intravenous administration of 50 mg. vitamin B_6					Total output 4 hrs. after oral administration of 100 mg. vitamin B_6
	15 min.	30 min.	45 min.	90 min.	Total output	
	mg.	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*
1	3.3	1.0	0.2	0.0	9.0	7.6
2	3.1	0.7	0.2	0.1	8.1	8.7
3	3.9	1.2	0.2	0.4	11.4	9.4
4	3.6			2.2	11.6	7.6
5	2.4			0.9	6.6	5.1
6	2.9			0.7	7.2	7.5
7	3.7			0.3	8.0	9.4
8	4.3			0.3	9.2	7.6
9	3.1			0.5	7.2	4.5
10	5.2			0.7	11.8	8.8
11	4.5			0.3	9.6	7.2
12	3.1			0.0	6.2	
13	3.5			0.1	7.2	
Average					8.7±1.6	7.6±1.1
Maximum deviation					3.1	3.1

samples of urine and postadministrative samples were taken either by catheterization or through a bladder fistula at the time intervals indicated in Figs. 1 and 2. A point-line method of plotting was used for clarity in presenting these data.

Fig. 1 shows the urinary output of fasting dogs following the oral administration of the vitamin. Maximum excretion occurred within 1 hour, indicating rapid absorption from the gastrointestinal tract. In fed dogs (recoveries shown in the legend

under Fig. 1) maximum excretion was delayed 2 to 3 hours. In all experiments 20 per cent of the vitamin (±1, maximum deviation 3.2 per cent) was recovered within 6 hours.

After the intravenous injection of doses of the vitamin varying from 25 to 500 mg. per dog, the maximum output was attained within 30 minutes (Fig. 2). Within 1 hour, 18 per cent of the vitamin was excreted, regardless of the weight of the animal. This approximates the 6 hour output following oral administration.

Experiments in Man—A group of thirteen non-fasting, apparently healthy, adult male subjects weighing 60 to 85 kilos were given 50 mg. of the vitamin intravenously. Postadministrative samples were then taken at the intervals shown in Table I. The results obtained are more variable than those found in our study with dogs. This may be due to the non-fasting condition of the subjects.

After the oral administration of 100 mg. of the vitamin to a group of eleven non-fasting male subjects, an average recovery of 7.6 per cent of the vitamin was obtained at the end of 4 hours.

DISCUSSION

It has been reported (2) that 50 to 70 per cent of test doses of vitamin B_6 was recovered in normal rat urine regardless of the mode of administration of the vitamin. In fasting dogs, urinary excretion of the vitamin is essentially complete within 6 hours after oral administration, but there was only 20 per cent recovery. An almost equal recovery (18 per cent) was obtained in dogs within 1 hour after intravenous administration of the vitamin. These results were remarkably constant, regardless of the weights of the animals and the doses (25 to 500 mg.) administered. In non-fasting human subjects only 8.7 per cent of a 50 mg. intravenous dose of the vitamin was recovered within 1 hour, although excretion appeared to be practically complete by this time.

In earlier experiments (2) it was observed that the fraction of the vitamin excreted in the urine of deficient rats was smaller than that excreted by normal animals. However, the amounts of the vitamin given (100 micrograms per rat, or approximately 2 mg. per kilo) were too small to permit a quantitative interpretation of the results obtained. Nevertheless, it was believed that

at this same dose level (2 mg. per kilo) or even smaller doses, the urinary output of the vitamin could be measured quantitatively in larger animals. This is amply substantiated by the present work. The fraction of the vitamin excreted following doses of 2 to 3 mg. per kilo has been measured in the dog, and the output following less than 1 mg. per kilo has been quantitatively measured in human subjects.

SUMMARY

1. A colorimetric method based on the indophenol reaction has been used to study the excretion of test doses of vitamin B_6 in the normal dog and man.

2. The short interval between oral administration of the vitamin and its appearance in the urine indicates that it is rapidly absorbed from the gastrointestinal tract and readily excreted.

3. After the intravenous administration of the vitamin in doses ranging from 25 to 500 mg., 18 per cent of the vitamin was recovered from the urine of dogs within 1 hour. After oral administration, these dogs excreted 20 per cent of the vitamin within 6 hours.

4. In a group of apparently healthy human subjects, an average of 8.7 per cent of a 50 mg. intravenous dose of the vitamin was recovered within 1 hour, while 7.6 per cent of a 100 mg. oral dose was recovered within 4 hours.

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Metabolic Significance of B-Vitamins

The following four papers were presented in a symposium of the American Institute of Nutrition held April 8, 1953, during its annual meeting at Chicago, Ill.

Since they were prepared as symposium papers they are reviews of present concepts of the metabolic functions of the B-vitamins, and of relatively recent work in the field, emphasizing that from the authors' own laboratories.

Summary of Known Metabolic Functions of Nicotinic Acid, Riboflavin and Vitamin B_6

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THAT SYMPTOMS OF VITAMIN DEFICIENCY result from partial failure of one or more metabolic functions for which the missing vitamin is essential is a truism. It is an interesting commentary on the present state of our knowledge, therefore, that in almost no instance is it possible in higher animals to correlate the presence of a given symptom of vitamin deficiency with hypofunction of a known enzymatic reaction for which that vitamin is essential. Until this becomes possible, our knowledge of the relationship of these bodily catalysts to physiological function is incomplete.

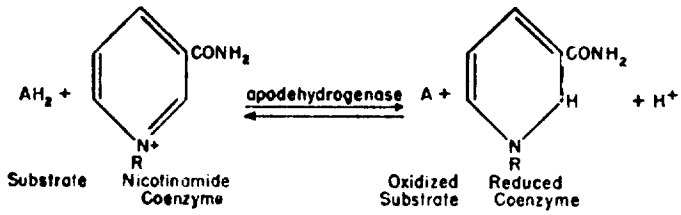
It is desirable, therefore, that the nutritional research worker consider from time to time what is known of the details of vitamin function. In this paper, a brief summary of the enzymatic roles played by nicotinic acid, riboflavin and vitamin B_6 will be given. With nicotinic acid and riboflavin in particular, much of this information is now common knowledge; their treatment will therefore be abbreviated and reference will be made frequently to appropriate monographs rather than to the original literature. Our knowledge of vitamin B_6 is more recent; more extensive reference to recent literature is thus appropriate.

NICOTINIC ACID

Nicotinic acid is known to occur in biological materials in the free form, as the amide, and in three coenzymatically active forms illustrated in figure 1. DPN and TPN have been known for many years (1); it now appears definite that the third phosphate residue of TPN is present on the 2'-carbon of the adenosine moiety of the molecule, as pictured (2, 3). Coenzyme III, a recent addition to the nicotinamide coenzymes, was discovered as an essential cofactor for the oxidation of cysteine sulfinic acid to cysteic acid and has the *tentative* structure indicated (4).

These coenzymes, together with appropriate apoenzymes, serve as hydrogen

acceptors (oxidizing agents) in the dehydrogenation of a great variety of metabolically important compounds, according to the equation:



Each of the coenzymes thus exists in an oxidized and a reduced form; the latter forms may also serve as hydrogen donors (reducing agents) for the reduction of appropriate substrates, A, to more reduced compounds, AH_2 (cf. 1, 5, 6).

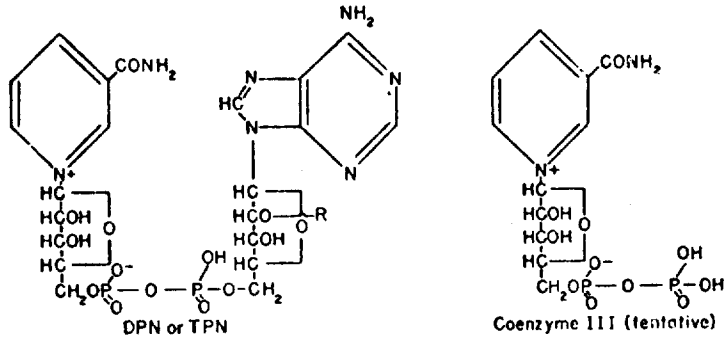


FIG. 1. STRUCTURES OF DIPHOSPHOPYRIDINE NUCLEOTIDE (DPN, Coenzyme I, R = H), triphosphopyridine nucleotide (TPN, Coenzyme II, R = PO_3H_2) and Coenzyme III.

A list of partially purified enzymes known to function in this way with the nicotinamide coenzymes is given in table 1, together with the substrates oxidized. The list is already long and is certain to be extended as more and more is learned of the enzymatic transformations undergone by the various components of living tissues. For example, nicotinamide coenzymes are required for various metabolic conversions among the steroids, including the oxidation of testosterone to androstenedione by liver and kidney tissues (29, 30), for the rapid oxidation of estrogens (31), and for the enzymatic degradation of the cholesterol side chain (31a). Details of their participation in oxidation of fatty acids have been clarified by the finding that DPN is essential for the reversible conversion of β-hydroxyacyl coenzyme A derivatives to the corresponding β-ketocompounds (table 1).

Since the nicotinamide coenzymes are present in catalytic quantities, the reduced coenzymes formed during oxidation of a given substrate must be continually regenerated by oxidative processes if substrate oxidation is to continue. Present knowledge of how this is accomplished is well summarized elsewhere (5, 6). Briefly it may be accomplished anaerobically by the coupling of two dehydrogenase systems having a common requirement for a single coenzyme, as indicated in *equations* 2

$$\text{(a)}\quad AH_2 + DPN^+ \xrightleftharpoons{\text{dehydrogenase 1}} A + DPNH + H^+$$

$$\text{(b)}\quad A' + DPNH + H^+ \xrightleftharpoons{\text{dehydrogenase 2}} A'H_2 + DPN^+$$

$$\text{(c)}\quad AH_2 + A' \rightleftharpoons A'H_2 + A$$

TABLE 1. REPRESENTATIVE DEHYDROGENASES THAT REQUIRE NICOTINAMIDE COENZYMES FOR ACTIVITY[1]

Enzyme	Substrate (AH_2)	Product (A)	Coenzyme	Ref.
A. Reaction type: R(R')CHOH + coenzyme ↔ R(R')C=O + reduced coenzyme				
Alcohol dehydrogenase	Ethanol	Acetaldehyde	DPN	(6)
	Vitamin A	Retinene	DPN	(9)
Isocitric dehydrogenase	Isocitrate	Oxalosuccinate (or α-ketoglutarate + CO_2)	TPN	(6, 10)
Isocitric dehydrogenase[2]	Isocitrate	α-ketoglutarate + CO_2	DPN	(11)
α-Glycerophosphate dehydrogenase	α-Glycerophosphate	Dihydroxyacetone phosphate	DPN	(6, 12)
β-Hydroxybutyric dehydrogenase	β-Hydroxybutyrate	Acetoacetate	DPN	(6, 12a)
β-Hydroxyacyl coenzyme A dehydrogenase	β-Hydroxyacyl coenzyme A derivatives	Corresponding β-ketocompounds	DPN	(12a)[3]
Lactic dehydrogenase	L-Lactate	Pyruvate	DPN	(6)
Malic dehydrogenase	L-Malate	Oxaloacetate	DPN	(6)
Malic enzyme	L-Malate	Pyruvate + CO_2	TPN	(13, 14)
Choline dehydrogenase	Choline	Betaine aldehyde	DPN	(15, 16)
B. Reaction type: H_2O + RCHO + coenzyme → RCOOH + reduced coenzyme				
Formic dehydrogenase	Formate	CO_2	DPN	(6)
Triosephosphate dehydrogenase	D-Glyceraldehyde phosphate + H_3PO_4	1,3-Diphosphoglycerate	DPN	(6, 17)
Glucose dehydrogenase	D-Glucose	D-Gluconate	DPN	(6)
Glucose-6-phosphate dehydrogenase[4]	Glucose-6-phosphate	6-Phosphogluconate	TPN	(6)
Betaine aldehyde dehydrogenase	Betaine aldehyde	Betaine	DPN	(15, 19)
C. Reaction type: $RCHNH_2COOH$ + coenzyme → RC(=NH)COOH + reduced coenzyme; RC(=NH)COOH $\xrightarrow{H_2O}$ RCOCOOH + NH_3				
Glutamic dehydrogenase	Glutamate	α-ketoglutarate + NH_3	DPN or TPN	(6)
D. Reaction type: RSSR + reduced coenzyme → 2 RSH + coenzyme				
Glutathione reductase	Oxidized glutathione	Reduced glutathione	TPNH	(20, 21)
Cystine reductase	Cystine	Cysteine	DPNH	(21a)

[1] Other enzymes that are not dependent upon nicotinamide coenzymes for the dehydrogenation of certain of these substrates may also exist. Examples are α-glycerophosphate dehydrogenase (7) and glucose dehydrogenase (8). Unless marked with a footnote 2, each of the enzymes listed has been detected in animal tissues.

[2] These enzymes have not so far been detected in animal tissues.

[3] Details of fatty acid oxidation, in which this enzyme is an important link, were divulged during the April 1953 meeting of the American Society of Biological Chemists by F. Lynen and co-workers and by D. E. Green, H. R. Mahler and their co-workers. See also Lehninger and Greville (12a) and Lardy (12b).

[4] A similar enzyme that dehydrogenates both glucose-6-phosphate and ribose-5-phosphate occurs in rat liver (18).

TABLE 1—*Continued*

E. Miscellaneous

Enzyme	Substrate	Product	Coenzyme	Ref.
Ketoglutaric dehydrogenase[a]	Ketoglutarate	Succinyl CoA + CO_2	DPN	(22–24)
Pyruvic dehydrogenase[a]	Pyruvate	Acetyl CoA + CO_2	DPN	(25, 26)
Quinone reductase	*p*-Benzoquinone	Hydroquinone	DPNH or TPNH	(27)
—[a]	Nitrate	Nitrite	TPNH	(28)
Cysteine sulfinate dehydrogenase	L - Cysteine sulfinate	L-Cysteic acid	Coenzyme III	(4)

[a] The action of DPN with these enzymes probably belongs to reaction type D, involving the oxidation by DPN of the dithiol form of lipothiamide pyrophosphate to the disulfide form (23).

and *b*. The sum of these equations is *c* in which one metabolite has been oxidized at the expense of another, DPN serving only as a catalyst, although participating in both the oxidation and the reduction. Well-known examples of this type are the coupling of triosephosphate dehydrogenation with pyruvate reduction in the production of lactate by muscle, or with ethanol production by yeast. A great number of other similar systems are possible (5).

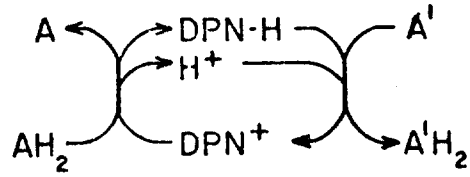


FIG. 2. SCHEMATIC REPRESENTATION OF PARTICIPATION of nicotinamide coenzyme in anaerobic oxido-reductions. A = substrate 1, A' = substrate 2.

This type of coupling can be depicted more simply by the diagram of figure 2, made familiar by Baldwin (5). The similar diagram of figure 3 indicates how the aerobic reoxidation of the reduced coenzymes may be accomplished through a series of hydrogen carriers that lead ultimately to oxygen. Riboflavin coenzymes, considered later, are an important link in this chain. Oxidized nicotinamide coenzymes may thus be regenerated continually, and catalytic amounts suffice for oxidation of much larger amounts of substrate.

The nicotinamide coenzymes are not concerned solely with these dehydrogenations, however. In the dehydrogenation of triosephosphate by purified triosephosphate dehydrogenase, DPN is required not only for the oxidative step per se, but also for the phosphorylation by inorganic phosphate which results in formation of an energy-rich phosphate bond (17). Furthermore, during the oxidative regeneration of DPN from DPNH (or TPN from TPNH) up to 3 moles of adenosine triphosphate (ATP) can be formed from adenosine diphosphate (ADP) for each mole of DPN regenerated (5, 32). The mechanism of this coupling of DPNH oxidation to phosphorylation is not known, a fact that emphasizes the many gaps in our knowledge of this process, now roughly depicted in figure 3. It is clear, however, that a major portion of the phosphate bond energy that becomes available to the body from the aerobic oxidation of carbohydrate is formed during reoxidation of the reduced nicotinamide coenzymes.

It is known that the level of nicotinamide coenzymes present in many tissues of pellagrins and of animals suffering from niacin deficiency is reduced to a degree

dependent upon the severity of the deficiency state (1, 33–35). In view of the many essential metabolic reactions dependent upon these coenzymes, it cannot be doubted that deficiency symptoms result from a lowering in the rate of one or more of these reactions below a level compatible with the well-being of the organism. Since the various dehydrogenases vary in their affinity for their coenzymes, concentrations of these that permit optimal function of one dehydrogenase may be insufficient for that of another. In any given organism maintained under constant conditions, interference with these reactions should occur, therefore, in a definite and reproducible order. The first of these to be affected is not known; when it becomes so, a beginning can be made in explaining the observed physiological derangements in terms of chemical reactions.[1]

Riboflavin

Riboflavin occurs naturally in the free form, as riboflavin-5′-phosphate and as flavin-adenine-dinucleotide. One or the other of the latter two compounds serves as a component of each of the enzymes currently known to depend upon riboflavin for activity. Formulas of these compounds are given in figure 4. These coenzymes combine with their apoenzymes—by and large more firmly than is true of the nicotina-

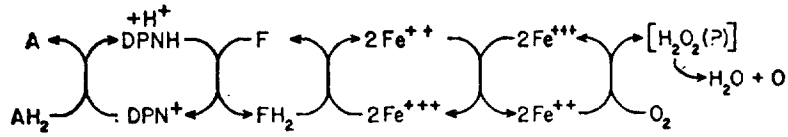
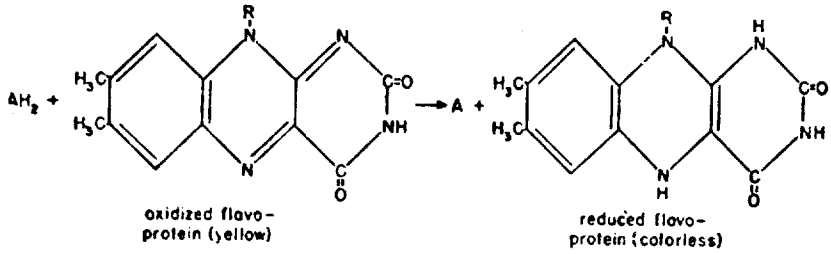


FIG. 3. SCHEMATIC REPRESENTATION of the oxidation of a substrate *via* nicotinamide coenzymes (cf. 5). DPN is used as an example of the latter. The exact number and sequence of hydrogen carriers between DPN and oxygen is not clear and may not be constant. F and FH_2 represent oxidized or reduced flavoproteins, respectively. Fe^{++} and Fe^{+++} represent the various cytochromes and cytochrome oxidase.

mide coenzymes—to form enzymatically active *flavoproteins*. The latter serve as hydrogen carriers in metabolism, dehydrogenating a variety of substrates according to the equation:



[1] Perhaps the most penetrating approach to this problem was made with dogs by Handler and Dann (34) who showed that death from blacktongue resulted from dehydration and electrolyte imbalance, suggesting a possible adrenal insufficiency. In this connection, the imperfectly understood relationship of DPN to steroid metabolism (29–31) is suggestive, but whatever the cause it is apparent that the impaired function of which dehydration was the indirect result required a high concentration of nicotinamide coenzyme relative to other systems. When the dehydration and electrolyte imbalance were corrected by forced administration of saline, symptoms of blacktongue cleared, and the animals lived until tissue concentration of nicotinamide fell to much lower levels, i.e. to levels at which additional essential functions requiring nicotinamide coenzymes failed.

The reduced flavoprotein may in some cases be reoxidized by oxygen, as indicated by the diagram of figure 5*a*, but is more commonly linked to oxygen through the cytochrome system, as indicated in figures 5*b* and 3. In some instances, also, flavoproteins may catalyze entirely anaerobic oxido-reductions between two different metabolites in a manner analogous to that indicated for nicotinamide coenzymes in figure 2.

A list of metabolic reactions known to require flavoproteins as catalysts is given in table 2. Again, the number of oxidative reactions actually involving flavoproteins probably is much larger. Recent evidence indicates that the fatty acid dehydrogenases (47), or, more correctly, the fatty acyl coenzyme A dehydrogenases, are flavo-

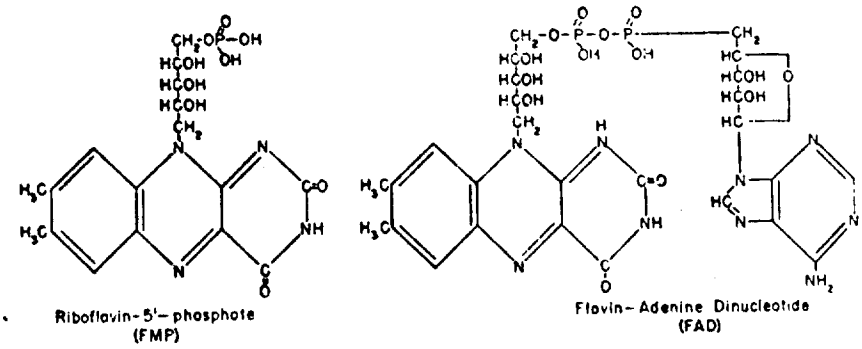


FIG. 4. STRUCTURES of the known coenzymes derived from riboflavin.

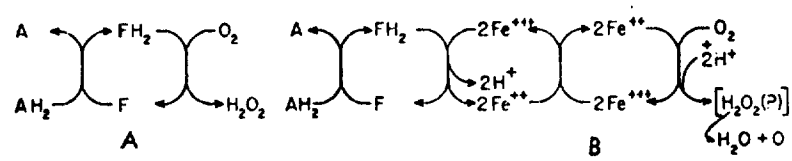
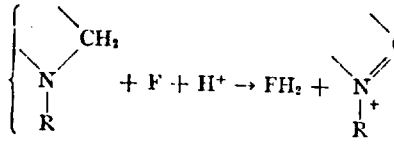
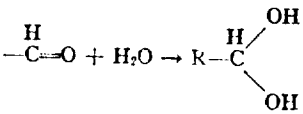
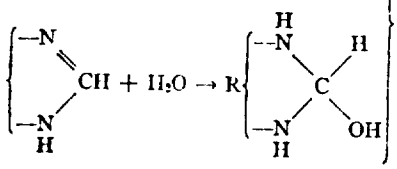
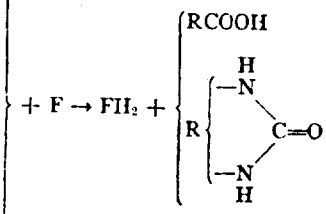


FIG. 5. SCHEMATIC REPRESENTATION of the catalysis of oxidations by flavoproteins. F = oxidized flavoproteins; FH_2 = reduced flavoproteins. The iron atoms indicated are bound to porphyrins in the cytochromes and cytochrome oxidase (5, 6).

proteins (table 2); these are among the most important enzymes concerned in degradation and synthesis of fatty acids. Other enzymes that dehydrogenate the —CH_2—CH_2— linkages to yield —CH=CH— linkages (e.g. succinic dehydrogenase) may also prove to be flavoproteins; suggestive of this is the observed reduction in activity of succinic dehydrogenase during riboflavin deficiency (48). The flavoproteins, like the nicotinamide coenzyme proteins, are obligatory for a great variety of oxidation-reduction reactions in metabolism.

Riboflavin deficiency is readily established upon feeding riboflavin-low diets to all animals so far investigated. The excretion of riboflavin is greatly reduced, falling to as low as two per cent of 'normal' values; such decreases occur before other symptoms of the deficiency become apparent (1). Those flavoproteins so far determined (D-amino acid dehydrogenase (1, 49), xanthine oxidase (50)) are markedly lowered in tissues when deficiencies of riboflavin sufficient to produce symptoms are induced. Such effects are undoubtedly responsible, directly or indirectly, for the deficiency

TABLE 2. REPRESENTATIVE METABOLIC REACTIONS FOR WHICH FLAVOPROTEINS ARE REQUIRED

Enzyme	Substrate	Product	Coenzyme	Ref.
A. Reaction type: $RCHNHR'\cdot COOH + F \rightarrow FH_2 + RC\cdot = NR'COOH \xrightarrow{H_2O} RCOCOOH + H_2NR'$				
D-Amino acid dehydrogenase	Most D-amino acids[1]	Corresponding keto acids + NH_3	FAD	(6)
L-Amino acid dehydrogenase[2]	Most L-amino acids	Corresponding keto acids + NH_3	FMP	(6, 36)
Glycine dehydrogenase	Glycine	Glyoxylic acid	FAD	(6)
D-Aspartic dehydrogenase	D-Aspartate	Oxaloacetate (→ oxalate)	FAD	(38)
B. Reaction type: $RCHOHCOOH + F \rightarrow FH_2 + RCOCOOH$				
L-Hydroxyacid dehydrogenase[3]	Several L-hydroxy acids	Corresponding α-keto acids	FMP FAD	(6, 39) (40)
Glycollic acid dehydrogenase	Glycollate	Glyoxylate		(41)
C. Reaction type: DPNH / TPNH =  = TPN / DPN				
DPN-Cytochrome *c* reductase	DPNH	DPN	FAD	(6)
TPN-Cytochrome *c* reductase	TPNH	TPN	FMP FAD	(6, 42) (42)
D. Reaction type: (a) 				
(b)  + F → FH_2 + 				
Notatin (glucose dehydrogenase)[4]	D-Glucose	D-Gluconate	FAD	(43, 44)
Aldehyde dehydrogenase	Several aldehydes	Corresponding acids	FAD	(6)

[1] All of the D-amino acids corresponding to the naturally-occurring L-amino acids are attacked (although at varying rates) except glutamic acid, lysine and cystine. Glycine is not attacked.

[2] Several closely related L-amino acid dehydrogenases that differ somewhat in specificity and in the ultimate electron (hydrogen) acceptor have been reported in various organisms. For a more complete listing and an account of relative specificities see (6) and (37). The enzyme from rat liver does not oxidize glycine, threonine, serine, the dibasic amino acids or the diamino acids.

[3] This enzyme may be identical with the L-amino acid dehydrogenase of rat liver (6).

TABLE 2—*Continued*

Xanthine dehydrogenase	Xanthine Hypoxanthine Aldehydes Xanthopterin	Uric acid Xanthine (uric acid) Corresponding acids Leucopterin	FAD	(6, 45)
Quinone oxidase[5]	Aldehydes Quinoline Cinchona alkaloids N′-Methylnicotinamide	Corresponding acids Carbostyril Carbostyril analogues 6-Pyridone of N′-Methylnicotinamide	FAD	(46)
E. Reaction type: $-CH_2-CH_2- + F \rightleftharpoons FH_2 + -CH=CH-$				
Fatty acyl coenzyme A dehydrogenase	Fatty acyl coenzyme A derivatives	α,β-unsaturated fatty acyl coenzyme A derivatives	FAD	—[6]
Fumaric reductase	Fumarate	Succinate	FAD	(46a)

[4] This enzyme has not been detected in animal tissues.

[5] Possibly identical with the aldehyde dehydrogenase of liver. However, preparations of the latter dehydrogenase from pig liver do not oxidize quinine or other nitrogen bases (46).

[6] See footnote (3) to table 1.

symptoms, but which of the many systems dependent upon riboflavin are first affected is unknown. Again, the enzymatic malfunctionings leading to pathological symptoms have not been elucidated in any experimental animal.

VITAMIN B_6

Pyridoxal, pyridoxamine and pyridoxine are naturally occurring free forms of vitamin B_6; the vitamin also occurs naturally as pyridoxamine phosphate and pyridoxal phosphate, the latter compound showing coenzymatic activity with all vitamin B_6-dependent enzymes so far discovered (1, 5, 6). Formulas for these compounds are given in figure 6; in view of current practices in nomenclature, it should perhaps be emphasized that pyridoxine is less plentiful in most tissues than are other forms of vitamin B_6 (51).

Pyridoxal phosphate is now recognized as the coenzyme for a great variety of enzymes involved in catalyzing various transformations of amino acids. Many of these transformations are summarized in table 3. They include decarboxylation of amino acids, transamination between amino acids and keto acids, racemization of amino acids, the dehydration (deamination) of hydroxyamino acids and the desulfhydration of sulfur amino acids, and a variety of miscellaneous transformations. So great is the number and variety of these reactions that pyridoxal phosphate can be considered as almost generally required for enzymatic reactions involving the non-oxidative degradation and interconversion of free amino acids.

The catalytic activities of vitamin B_6-containing enzymes are mirrored in the chemical behavior of pyridoxal itself (cf. 84). Thus pyridoxal undergoes reversible transamination with many amino acids to form pyridoxamine and keto acids (85, 86), a reaction which appears to be a model of enzymatic transamination reactions (87), and which is catalyzed by ions of iron, aluminum or copper (86). The detailed

mechanism of the reaction is unknown; however, it may be visualized as proceeding approximately as pictured in figure 7. The participation of vitamin B_6 in enzymatic transamination was first predicted from a consideration of this reaction (85, 88). At elevated temperatures, pyridoxal (or pyridoxal phosphate) also decarboxylates histidine to histamine in low yield (58), racemizes optically active amino acids (90), dehydrates serine to pyruvate and ammonia, and cysteine to pyruvate, hydrogen sulfide and ammonia (91), cleaves threonine reversibly to acetaldehyde and glycine (92) and serine to formaldehyde and glycine, and cleaves cystathionine at the thioether linkage to yield a variety of products (92).

The striking similarity of these purely chemical reactions to the enzymatic reactions catalyzed by vitamin B_6 proteins leaves no doubt that the chemical reactivity of the prosthetic group determines the type of reaction that these proteins promote.

Investigation of the chemical prerequisites for these non-enzymatic reactions has shown the formyl group, the phenolic group and the ring nitrogen of pyridoxal to be essential, together with an appropriate metal ion (Cu^{++}, Fe^{+++} or Al^{+++} (86, 90, 91, 92, 93)). It appears probable that the common feature of these reactions lies

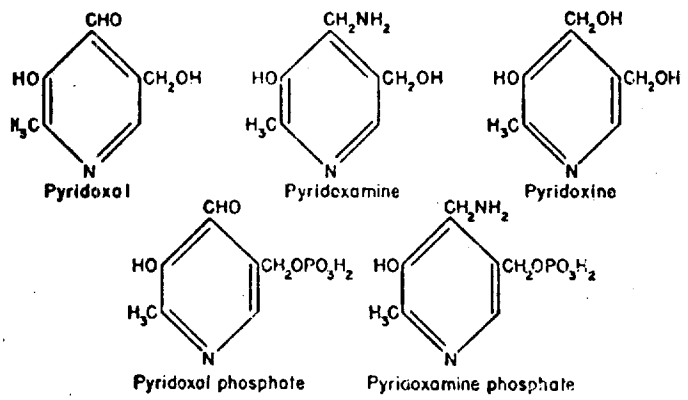


FIG. 6. CHEMICALLY IDENTIFIED free and bound forms of vitamin B_6.

in formation of a Schiff base between pyridoxal and amino acid, stabilized by chelation with a metal ion as indicated in figure 7, and resulting in an activation of the amino acid residue so that it may undergo any of a variety of reactions in groupings immediately surrounding the α-carbon atom. The predominant nature and rate of these reactions is determined by the structure of the amino acid (93). It appears probable that metal ions will also prove essential for the analogous enzymatic reactions. The vacant coordination positions about the metal ion of figure 7 might well serve to tie coenzyme (and substrate) to apoenzyme; in non-enzymatic reactions, these coordination positions are undoubtedly occupied by water or additional molecules of the reactants.[2]

With certain lactic acid bacteria, cultural conditions can be arranged such that the rate or extent of growth will be determined by the activity of known single enzymes, dependent in turn for their activity upon the supply of vitamin B_6. This subject has been discussed elsewhere (84) and will be considered only briefly here.

[2] Baddiley (89) has isolated chelates of Schiff bases containing two moles of pyridoxal and two of amino acid per metal ion.

TABLE 3. SOME ENZYMATIC REACTIONS CATALYZED BY PYRIDOXAL PHOSPHATE ENZYMES

A. Amino acid decarboxylases

General reaction: $RCHNH_2COOH \rightarrow RCH_2NH_2 + CO_2$

Amino Acid	Product	Ref.
L-Arginine	Agmatine	(6, 52, 53)
L-Ornithine	Putrescine	(6, 52, 53)
L-Lysine	Cadaverine	(6, 52, 53)
L-Phenylalanine	Phenylethylamine	(6, 52)
L-Tyrosine[1]	Tyramine	(6, 52, 54)
β-(3,4-Dihydroxyphenyl)-L-alanine (dopa)[1]	3,4-Dihydroxyphenylethylamine (dopamine)	(6, 52, 54)
L-Glutamic acid[1]	γ-Aminobutyric acid	(6, 52, 55)
β-Hydroxyglutamic acid	γ-Amino-β-hydroxybutyric acid	(56)
L-Aspartic acid[2]	β-alanine	(57)
L-Cysteic acid[1,2]	Taurine	(6, 52, 54)
L-Histidine[1,2]	Histamine	(6, 52, 54, 58)
L-Tryptophan[2]	Tryptamine	(52, 54)
α,ε-Diaminopimelic acid	Lysine	(59)

B. Transaminases

General reaction: $RCHNH_2COOH + R'COCOOH \rightleftharpoons RCOCOOH + R'CHNH_2COOH$

$RCHNH_2COOH$	$R'COCOOH$	Reference
22 different α-amino acids, L-series[3]	α-Ketoglutaric acid	(60, 61)
L-Leucine, L-Phenylalanine, L-Methionine, L-Histidine, L-Tyrosine	Pyruvic acid	(62)
L-Ornithine	Pyruvic acid	(63)
Cysteic Acid	Pyruvic acid	(64)

C. Dehydrases and desulfhydrases

General Reaction: (A=O or S)

$$RCH(AH)-CH(NH_2)COOH \xrightarrow{-H_2A} [RCH=C(NH_2)COOH] \xrightarrow{+H_2O} RCH_2COCOOH + NH_3$$

Enzyme	Substrate	Products	Ref.
L-Serine dehydrase	L-Serine	Pyruvate + NH_3	(65)
D-Serine dehydrase	D-Serine	Pyruvate + NH_3	(66, 67)
L-Threonine dehydrase	L-Threonine	α-Ketobutyrate + NH_3	(65)
L-Cysteine desulfhydrase	L-Cysteine	Pyruvate + NH_3 + H_2S	(68–70)

D. Racemases

Alanine racemase	D or L-Alanine	DL-Alanine	71
Glutamic racemase	D or L-Glutamate	DL-Glutamate	72

[1] These enzymes have been demonstrated in animal tissues (6, 53); all decarboxylases except cysteic acid decarboxylase have been demonstrated in bacteria.

[2] The requirement of these enzymes for pyridoxal phosphate has not been proved but appears likely by analogy (cf. also (58) for evidence that histidine decarboxylase requires this coenzyme).

[3] These include each of the L-amino acids commonly found in proteins and several that do not. The amount of transamination observed with serine, threonine, histidine, glycine, lysine and cysteine, was, however very low (60). Because of differences in distribution in different rat tissues, the authors (60) believe each transamination reaction is due to a different enzyme. A large number of these transaminases also have been observed in bacteria (81–83).

TABLE 3—*Continued*

E. Miscellaneous

Homocysteine desulfhydrase[4]	Homocysteine	α-Ketobutyrate + NH_3 + H_2S	(69, 73)
Cystathionase[4]	Cystathionine	a) Cysteine + ketobutyrate + NH_3 b) Homocysteine + pyruvate + NH_3	(73–75)
Kynureninase	a) Kynurenine	Anthranilic acid + L-alanine	(76–78)
	b) 3-Hydroxy-kynurenine	3-Hydroxyanthranilic acid + L-alanine	78
Tryptophanase	L-Tryptophan	Indole + pyruvate + NH_3	79
Tryptophan-synthesizing enzyme	Indole + L-serine	Tryptophan + H_2O	80

[4] These are enzymatic activities of relatively crude extracts, and may in some instances represent more than a single enzymatic reaction. Pyridoxal phosphate appears required in all cases, however.

Streptococcus faecalis R, for example, grows in the absence of added vitamin B_6 if all of the L-amino acids of a complete protein are supplied together with D-alanine (94). Under these conditions vitamin B_6 synthesis by the cells cannot be detected with certainty. If it is desired to make synthesis of D-alanine by the alanine racemase the growth-limiting reaction, it is necessary only to omit D-alanine from the basal medium. Growth now fails for lack of this amino acid but resumes if *either* D-alanine *or* vitamin B_6 is added, the vitamin B_6 functioning by conversion to pyridoxal phosphate and activation of the alanine racemase, which then furnishes the essential D-alanine by synthesis from L-alanine present in the medium. Similarly, if one wishes to make growth dependent upon transamination of α-ketoisovaleric acid to valine, the keto acid may be substituted in the growth medium for the essential amino acid, valine. As shown in figure 8, growth with valine occurs without added vitamin B_6; growth with α-ketoisovaleric acid occurs only if vitamin B_6 is added. It has been shown with resting cells that the keto acid is converted to valine in the latter case by a vitamin B_6-dependent transaminase in the cells, the activity of which can be controlled by controlling the supply of vitamin B_6 (82, 84, 95).

By use of similar systems, it can be readily shown that amounts of vitamin B_6 sufficient fully to activate one B_6-dependent enzyme will not suffice to activate another. For example, tyrosine decarboxylase is inactive in cells of *Streptococcus faecalis* grown with low levels of vitamin B_6 which suffice to permit full activity of the glutamic-aspartic transaminase (96). Both enzymes are active in cells liberally supplied with vitamin B_6 (96); neither is active in cells grown without vitamin B_6 (97).

The situation described above is also observed in animals. Thus, the B_6-enzyme concerned in transfer of sulfur from homocysteine to serine (i.e. in formation and cleavage of cystathionine) is severely lowered in even the early stages of vitamin B_6 insufficiency (75). The activity of cysteine desulfhydrase is reduced to very low levels in livers of vitamin B_6-deficient animals (98) at a time when the activity of the glutamic-aspartic transaminase, though somewhat lowered, is still appreciable,[3]

[3] The glutamic-aspartic transaminase of animal tissues is lowered only to about one-half to one-third of its initial concentration even in animals showing severely impaired growth as a result of vitamin B_6 deficiency (98–100).

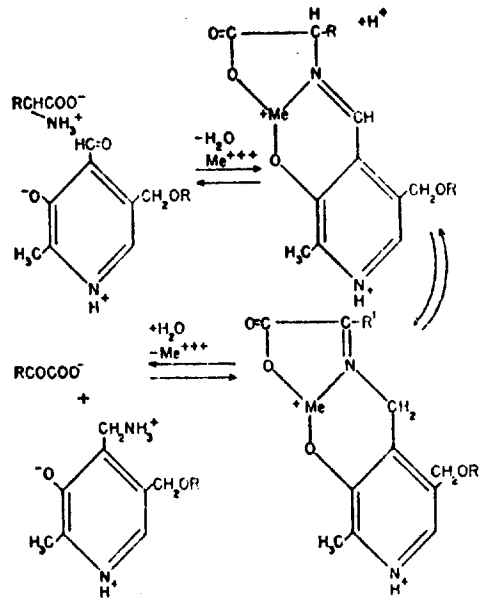


FIG. 7. A POSSIBLE MECHANISM of transamination between pyridoxal and amino acids.

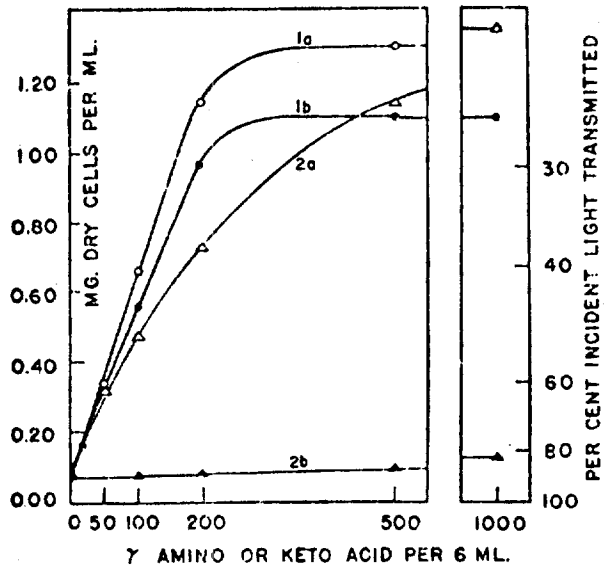


FIG. 8. RESPONSE OF *S. f. calis* TO VALINE or the corresponding keto acid in the presence and absence of vitamin B_6. Curves 1a and 1b, DL-valine; 2a and 2b, α-keto-isovaleric acid; a, with 1γ pyridoxamine; b, no pyridoxamine. Incubated at 30° for 24 hours.

and that of the threonine-splitting enzyme (threonine → glycine) is unimpaired (101).[4] It appears certain that symptoms of vitamin B_6 insufficiency result from such a lowering in activity of one or more vitamin B_6-dependent enzymes below an e-

[4] It is assumed here that the threonine-splitting enzyme, which is sensitive to carbonyl reagents, will prove to be a pyridoxal phosphate protein, since the reaction catalyzed by it is apparently exactly analogous to that obtained by heating pyridoxal and metal salts with threonine (92).

sential threshold value, but it is not currently known which of the many possible enzymes are thus involved.[5] An important corollary of this viewpoint is that different enzymatic deficiencies may be the primary result of vitamin B_6 deficiency under different dietary conditions. For example, the symptoms of vitamin B_6 deficiency observed when diets high in methionine are fed (102) are not necessarily due to insufficiency of the same enzymatic reactions responsible for deficiency symptoms observed when vitamin B_6 deficiency is induced upon diets of customary methionine content.

From this discussion and the summarized reactions of table 3, the great importance of vitamin B_6 in catalyzing the degradation of amino acids is evident. The role of this vitamin in synthesis of amino acids also should be emphasized. The transamination reaction provides one important means of transforming suitable keto acids into amino acids. The tryptophan-synthesizing enzyme, and the α,ϵ-diamino-

TABLE 4. ESSENTIAL ROLE OF VITAMIN B_6 IN SYNTHESIS OF AMINO ACIDS AS REFLECTED IN GROWTH OF *Lactobacillus arabinosus*[1]

AMINO ACID OMITTED[2]	VITAMIN B_6 ABSENT	VITAMIN B_6 PRESENT	AMINO ACID OMITTED[2]	VITAMIN B_6 ABSENT	VITAMIN B_6 PRESENT
	ml. 0.1 N acid produced per 10 ml. of medium			ml. 0.1 N acid produced per 10 ml. of medium	
None	8.7	11.5	Alanine	0.6	8.3
Lysine	0.82	11.2	Arginine[3]	1.0	10.6
Phenylalanine[3]	1.76	10.0	Histidine	2.0	12.2
Tyrosine[3]	1.26	8.4	Threonine	0.4	5.5
Aspartic acid[3]	3.2	10.0	Cystine	1.3	7.9
Serine	2.0	11.6			

[1] Data are taken principally from Lyman *et al.* (104); others have made similar findings with fewer amino acids in other organisms of this same group (82, 95, 105, 106). Acid production correlates closely with total growth; it is assumed that the ability of *either* an amino acid *or* vitamin B_6 to permit growth under the same conditions means that when vitamin B_6 is supplied, the missing amino acid is synthesized by the cells (cf. 84).

[2] The complete basal medium contained each of the L-amino acids that occur in complete proteins.

[3] Both vitamin B_6 *and* an increased carbon dioxide tension were necessary to permit growth in the absence of these amino acids.

pimelic acid decarboxylase provide examples of the participation of vitamin B_6 in additional types of synthetic reactions leading to amino acids. Growth experiments with lactic acid bacteria, summarized in table 4, reveal the participation of vitamin B_6 in the synthesis of every one of the amino acids examined. The nature of the vitamin B_6-dependent reactions involved in many of these syntheses remains to be established.

[5] Such lowered enzymatic capabilities can be observed indirectly in living animals. Dalgleish, for example, fed rats a diet low in vitamin B_6 and observed the excretion of many intermediate products of tryptophan metabolism, including kynurenine, not present in appreciable amounts in the urine of normal animals fed similar amounts of tryptophan. It is known that the level of liver kynureninase, a pyridoxal phosphate enzyme, is decreased in vitamin B_6 deficiency (76). Thus, hypofunction of a given enzyme may result not only in failure of a given product to be formed in amounts adequate for other reactions, but may also result in accumulation of the substrate of the deficient enzyme in amounts harmful per se or following conversion through other metabolic pathways to other products. Once an essential portion of the metabolic machinery becomes hypofunctional, many possibilities exist for an altered metabolism and production of deficiency symptoms, some of which may be far removed from the original metabolic defect.

In addition to its primary catalytic role in reactions of amino acids, much nutritional evidence (reviews, 107, 108) indicates that vitamin B_6 is in some way concerned in fat metabolism. Vitamin B_6-deficient animals show a lowered capacity to transform protein into fat, a finding that may have its explanation in the obligatory participation of the transaminases or other vitamin B_6 enzymes in the deamination of the amino acids preliminary to transformation of the nitrogen-free residues into fatty acids. No ready explanation of the development of fatty livers in animals with long-standing vitamin B_6 deficiency can yet be given. Vitamin B_6 and unsaturated fatty acids show mutual sparing effects in the alleviation of the acrodynia observed in rats on some vitamin B_6-deficient diets. Witten and Holman (109) have recently demonstrated conclusively the role of vitamin B_6 in enhancing formation of certain unsaturated fatty acids in rats, and interpret their results to indicate that "pyridoxine is involved in the synthesis of very highly unsaturated acids (arachidonic and hexaenoic acids) from linoleic and linolenic acids." If this involvement is a direct one, it indicates a fundamental role for vitamin B_6 in addition to and apparently quite different from its role in the activation of amino acids.

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MODIFICATIONS IN THE GENITAL TRACT REACTIVITY AND SEXUAL CYCLE EVOLUTION AFTER PROLONGED ADMINISTRATION OF PYRIDOXINE

by

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In research on pubescent rats, the authors studied the non-specific pharmacodynamic action of pyridoxine in the genital tract. The pyridoxine was administered intraperitoneally for 66 days, in doses comparable to the therapeutic doses. The authors determined stimulation of the hypophyseo-utero-ovarian axis, manifested by reduction of the sexual cycle, prolongation of estrus (O) and appearance of proestrus (P) or a transient (P-O), and disappearance of diestrus (D) in most cases. There was also hypertrophy of the ovaries and uterine cornea persisting in other phases, hypertrophy of numerous glands in the chorion, thickened and greatly vascularized musculature, a lumen filled with a secretion liquid that sometimes distends the walls of the uterus, numerous yellow bodies in the ovaries. Also noted was an increase in the parasympathomimetic reactivity (RP-mim) in relation to acetylcholine (Ach) of the myometer in the P phase and its persistence in other phases, in contrast to the controls in the same phase.

Since 1948, Nelson and Evans [4] have shown that the harmful effect of vitamin B_6 deficiency on the reproductive system is rapidly reversed if this vitamin is added to the animal's food.

In the ovary too, the harmful effect of vitamin B_6 deficiency on the R in contrast to the gonadotropin stimulation is partially compensate for in 5 days. The uterus of rats with this deficiency is, in this regard, even more reactive to this stimulation than that of the controls.

The decrease in reproduction is apparently due to the deficiency of ovarian hormones because, in 90% of the rats with pyridoxine deficiency, pregnancy can be maintained if doses of estrone and progesterone (5) are administered. These two have proved to be effective in rats ovariectomized

after copulation [10].

Pyridoxine serves as a co-enzyme for numerous enzymes involved in the protein metabolism, among them the transaminases.

L.I. Grebnik et al. [3] have shown that the descendants of rats that received a diet poor in vitamin B_6 develop normally for the first 9-15 days, but that subsequently their growth is arrested and most of them die 15-20 days after birth.

These are of interest in the problem of the effect of pyridoxine deficiency of mothers on the growth of their young.

The studies of Benjamin Eckstein [1] show that the efficiency of the diet deficient in B_6 can be deduced from the fact that the weight of the animals remains stationary. This concerns above all the weight of the uterus and the ovaries. The enzyme activity in the ovary of these animals is reduced by about one-half in comparison with the controls, while the activity of the GOT (glutamnic-oxalacetic transaminase) in the uterus does not differ much from that of the controls.

Albert Einstein [2] concludes that in the case of B_6 deficiency, the cortico-adrenal function is not altered, despite the hypertrophy of this gland.

Than et al. [8], [9], using pyridoxine exclusively in the treatment of pregnancy toxicoses in 53 hypoacidic or anacidic females, with vomiting and whose gastric secretion was examined using the acido-test, determined that, concomitantly with the disappearance of the vomiting, the gastric acidity is modified, becoming normal or hyperacid. The authors conclude that vitamin B_6 does not exercise its effect directly on the gastrosecretic cells, but rather indirectly, through the hypophyseo-cortico-adrenal system, and that the ovarian estrogens probably play a role in the appearance of illness.

An explanation of the effect of pyridoxine in the treatment of pregnancy toxicoses when they appear or in prophylaxis of them requires additional

information, especially as the study of its effect is an actual problem of gynecology which studies, among other things, its effect on the vaginal pH.

Besides the important role of pyridoxine in the metabolism of the nerve cell and its properties that cause clear changes in the organism, its non-specific pharmacodynamic action, that is, its mechanism of action, is still relatively unexplained.

Just as in our preceding research concerning the influence of thiamine on the R of the genital tract [7], the changes obtained with this vitamin are most manifest after prolonged administration.

The present studies intend to explain the role of pyridoxine in the equilibrium of the sexual functions.

Materials and Methods

Our studies concern two groups of pubescent rats (30 in all), one group serving as controls. In this latter group, we took samples of vaginal scrapings each day for 29 successive days, in order to establish the characteristics of the sexual cycles of each animal. The other group was treated with pyridoxine hydrochloride ($C_8H_{12}O_3N.HCl$) BDH England -- 400 μg/100 g body weight/day, for three weeks. Since this dosage did not alter the sexual cycles significantly, the dose was doubled (800 μg/100 g), and the treatment was continued for an additional 66 days.

The doses were comparable to therapeutic doses. They induced no toxic phenomena or signs of intolerance. Beyond the modifications in the sexual cycle, we examined the behavior of the animals, their weight, and their appearance. Finally, the animals were killed in various phases of the cycle and we compared the R of uterine cornea in relation to the Ach in a tyrode solution, at the animal's temperature (38°C) and under constant aeration, to that of the controls.

For the histological examination, we took samples of the ovaries and

the remaining uterine cornea in respective phases. The organs were fixed in formol at 10%, imbedded in paraffin and stained with hematoxylin-eosin and Van Gieson. The vaginal scrapings were stained with the method of Postelnicu.

Results and Discussion

By analyzing the evolutionary aspect of the rats' sexual cycles under the effect of prolonged treatment with pyridoxine and by comparing them with the controls, we find

a) modification of the appearance of the vaginal scrapings, notably reduction of the sexual cyclic period, disappearance of D, prolongation of the O phase and appearance of a transient P or of a P-O.

b) hypertrophy of the uterus and ovaries, most evident in the O phase and persisting in the other phases of methestrus (M) or M-D, since the D disappeared in all the cases treaded with pyridoxine.

c) the myometer reveals spontaneous, rhythmic contraction, regular and abundant in all the phases. The RP-mim in relation to the Ach is more increased in O as compared with the controls in the same phase. Here are some representative experiments:

Exp. 1. Rat no. 9. (Fig. 1). Sacrificed in O 48 hours ago. In the course of the pyridoxine treatment, the rat no longer showed the D phase after 20 days, and the O lasted 5 days. Ovaries large with points of congestion. Uterine cornea hypertophied, congested, filled with secretion liquid. In a Tyrode solution bath of 38°C, with constant aeration, the left uterine cornea shows large spontaneous, regular contractions. 1 γ Ach intesifies the rhythmic contractions. 5 γ, 10 γ and 50 γ Ach produce clear hypertonia, related to the dose administered.

Hist. exam. Ovaries and uterus appearance of O. Follicles of varying degrees and numerous corpora lutea (30) of different sizes. Uterine

wall thickened, large, scalloped lumen. Large chorion, with many glands.

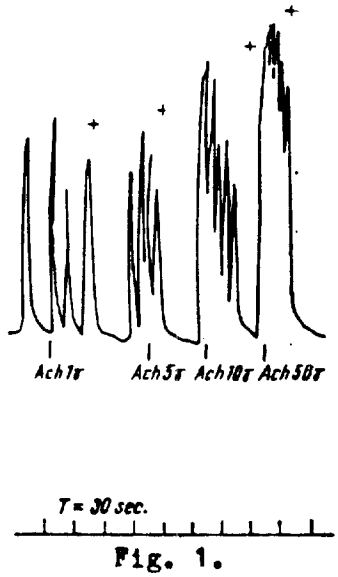

Fig. 1.

Exp. 2. Rat no. 11, sacrificed in O after 24 days, with no D phase. In the course of the treatment, it revealed prolonged O and M. Uterine cornea hypertrophied, congested, without fluid in the lumen, showing spontaneous, large, irregular contractions. The RP-mim towards Ach is modified. 1 γ Ach increases the amplitude of the spontaneous contractions. 5 γ, 10 γ, and 100 γ Ach cause hypertonia, depending upon the dose. Pyridoxine administered in progressive doses (160 γ, 360 γ, 0.0016 g), encourages the P-mim effect of standard doses of Ach.

Hist. exam. Uterus with thick wall, free, scalloped lumen, chorion thick in the interior, with numerous glands. Thickened and vascularized musculature. Ovary with many corpora lutea [21] of various sizes and follicles of varying degrees.

Exp. 3. Rat no. 2, sacrificed in O. Reveals one D in the course of 34 days. during the treatment. Cornea hypertrophied, congested, full of secretion fluid. Characteristic spontaneous contractions. 1 γ, 5 γ Ach clearly increase the amplitude of the spontaneous contractions. After 160 γ pyridoxine , the P-mim effect of the standard dose of 5 γ Ach is clearly encouraged (fig. 2).

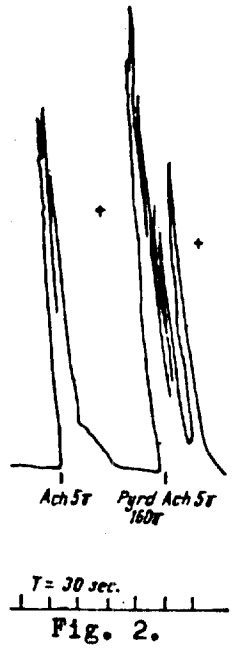

Fig. 2.

Exp. 4. Rat no. 18 (fig. 3), sacrificed in O. During the treatment, it had no D for 20 days. Uterine cornea large, dilated, full of secretion fluid. 1γ, 5γ, 10γ, and 50γ Ach raise the myometer tonus, in relation to the dose. 160γ pyridoxine slightly encourages the P-mim effect of the dose of 5γ Ach.

Hist. exam. Ovaries large, with follicles of various degree and numerous corpora lutea [21] of different dimensions. Unequal size of uterus, musculature developed, chorion large, with numerous glands.

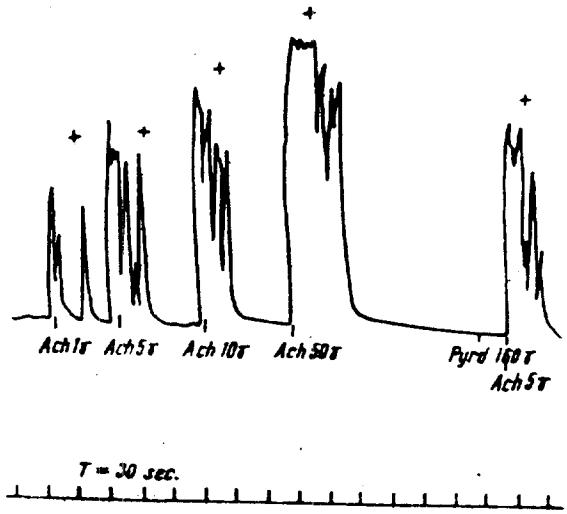

Fig. 3.

Exp. 5. Rat no. 8, sacrificed in O. During the entire treatment, it manifested periods with prolonged O of up to 4 days and a single D in 22 days. Uterine cornea more developed, congested. Spontaneous, regular, large contractions. 0.1 - 0.5γ Ach are isomimetic doses. 1γ and 5γ Ach increase the amplitude of the contractions. 10γ and 50γ Ach cause clear hypertonia. 160γ and 360γ pyridoxine promote the P-mim effect of the dose of 10γ Ach.

Hist. exam. The ovaries have follicles of the first, second and third degree, and numerous corpora lutea [21] of various sizes. The uterus has a thickened, much-vascularized wall, the chorion is large, loose, with numerous glands.

In conclusion, in the O phase, the myometer shows increased RP-mim towards Ach, since we know that in the controls in this phase, small doses of this mediator (0.1γ and 0.5γ, 1γ and even 5γ sometimes) produce a sympathomimetic effect (S-mim), that is to say, a reduction in the size of the spontaneous contractions, sometimes to the point of disappearance, and since we know that doses of 5γ, 10γ, and even 50γ sometimes, produce evident hypertonia (fig. 4).

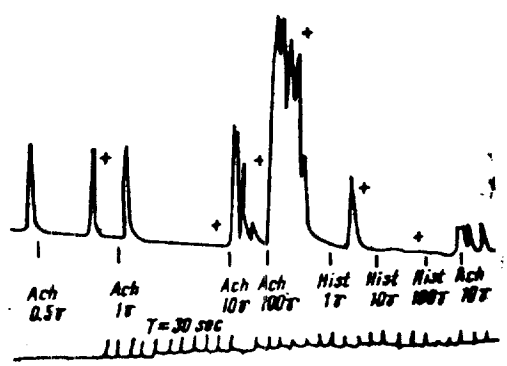

Fig. 4.

These aspects of the R of the uterus and the histological aspect persist in the other phases of M or M-D in rats treated with pyridoxine. For example:

Exp. 6. Rat no. 13 (fig. 5) sacrificed in M moving towards D. Had

rare D's during treatment. Uterine cornea moderately hypertrophied, manifesting spontaneous rhythmic contractions. 1 γ Ach has no evident effect. 5 γ, 10 γ and 50 γ Ach clearly increase the myometer tonus.

Hist. Exam. Ovaries with follicles of the first, second and third degrees, corpora lutea rather numerous [17] and of various sizes. The wall of the uterus is of average thickness, with well-developed musculature with numerous vessels, a necrotized (2/3) cubic epithelium. Lax chorion, with glands.

In conclusion, though it is in the M-D phase, the histological aspect of the uterus still has a well-developed musculature, numerous vessels and a constant RP-mim towards Ach.

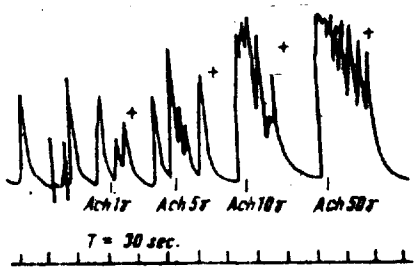

Fig. 5.

Thus we see that most of the animals treated for a long term with pyridoxine generally reveal an increase in the R (reactivity) of the genital tract, due to the hypophyseo-utero-ovarian stimulation, and consequently to folliculino-progesterone stimulation. This stimulation is manifested by:

- disappearance of the D (20-24 days) in the great majority of cases. Sometimes prolongation of the O phase, appearance of a transitory P or P-O, tendency to shortening of the sexual cycles.

- hypertrophy of the ovaries and uterine cornea with accentuated hyperemia. Histologically, the ovaries reveal numerous follicles of all degrees, and above all numerous corpora lutea of various sizes (fig. 6, rat no. 9). The uterine cornea have an over-developed and vascularized musculature (fig. 7, rat no. 11), the chorion is large with numerous glands and secretion

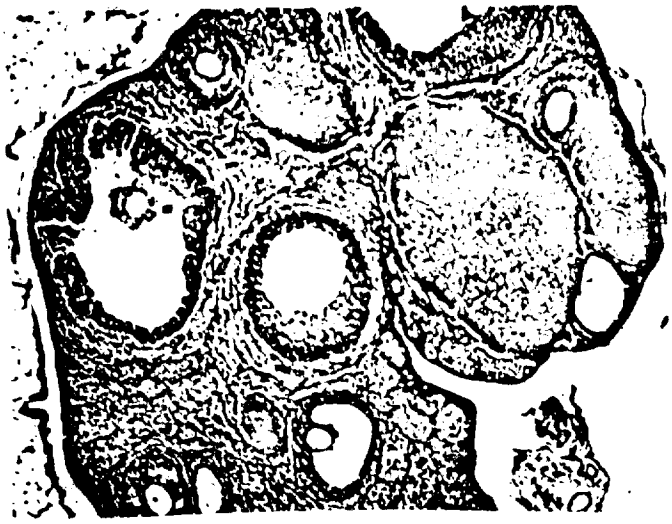

Fig. 6.

Fig. 7.

fluid most abundant in the lumen, features that appear in the P as well, and persist in M until the following cycle.

- The RP-mim towards Ach of the uterus is most increased in O and O in the case of the treated animals, remaining high in M as well, in comparison with the characteristic aspects of the same phases in the case of the controls. In the great majority of experiments, the various doses of pyridoxine encouraged the P-mim effect of Ach on the isolated rat uterus, in the O phase. In the other phases of the cycle, their action is not evident.

Taking into account the earlier studies of authors who demonstrated the co-enzyme role of pyridoxine for many of the enzymes involved in the protein metabolism, among them the transaminases, and taking into account also the gonadostimulative effect and the stimulating effect on the nervous system, as well as the encouraging action on the effect of Ach (role in the cholinergic mechanism), we can assume an activating role on the part of this vitamin (from the standpoint of non-specific pharmacodynamics).

In contrast to the results of our previous studies [7] on thiamine administered chronically for a long term, in which its anti-cholinergic action (curarimimetic type) was predominant and which inhibited the genital tract (physiological as well as histological), pyridoxine used in the same conditions, always in therapeutic doses, stimulated the hypophyseo-utero-ovarian axis.

These effects are similar to those described in the earliest publications [6] concerning the study of the effect of activating substances on the neuro-endocrine-effecting mechanism. By analyzing the non-specific pharmacodynamic action of eserine, we have shown the reactivating effect of this substance on the genital tract, due to its encouragement of the action of Ach and the hypophyseo-utero-ovarian stimulation. Thanks to its quality as an excitory activating substance of these systems, eserine has increased the R (reactivity) of the endocrine glands and centers (hypophysis and ovaries), causing an increase in the estral cycles, prolongation of O and M,

and even disappearance of D in certain animals, so that they enter directly into M or P, or even O, without passing through the phase of sexual rest (D). The excitory activating mechanism of action of exerine is manifested both by its anticholinesterase action (thus enouraging Ach), and by folliculin stimulation, the anticholinesterase, and thus cholinergic effect of which is well known.

Taking into account the cumulative effect of chronic treatment with pyridoxine and the length of administration, we could explain the morpho-physiological modifications in the genital tract and the development of the sexual cycle.

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MODIFICATIONS DE LA RÉACTIVITÉ DU TRACTUS GÉNITAL ET DE L'ÉVOLUTION DU CYCLE SEXUEL APRÈS L'ADMINISTRATION PROLONGÉE DE PYRIDOXINE*

par SILVIA SIMIONESCU, AURELIA BORDEIANU, BEATRICE SLĂVESCU, CARMEN STANCU et VANTIȚA NECULAU-PINTILESCU

Dans des recherches faites sur des rates pubères, les auteurs ont étudié au niveau du tractus génital l'action pharmacodynamique non spécifique de la pyridoxine administrée durant 66 jours par voie intrapéritonéale, à des doses rapportées à celles thérapeutiques, sur le tractus génital. On a constaté la stimulation de l'axe hypophyso-utéro-ovarien, traduite par : réduction du cycle sexuel, prolongement de l'œstrus (O) et apparition d'un pro-œstrus (P) ou d'un (P—O) fugace et disparition du diœstrus (D) dans la plupart des cas; hypertrophie des ovaires et des cornes utérines qui persiste dans les autres phases aussi, de nombreuses glandes dans le chorion, musculature épaissie et très vascularisée, lumière remplie d'un liquide de sécrétion qui distend parfois les parois de l'utérus, de nombreux corps jaunes dans les ovaires; augmentation de la réactivité parasympathomimétique (RP-mm) envers l'acétylcholine (Ach) du myomètre dans la phase de P et sa persistance dans les autres phases, par rapport aux témoins dans la même phase.

Dès l'année 1948, Nelson et Evans [4] avaient montré que l'effet nuisible du déficit en vitamine B_6 sur le système de reproduction du rat est rapidement inversé si cette vitamine est ajoutée à l'alimentation de l'animal.

Dans l'ovaire aussi, l'effet nuisible du déficit en vitamine B_6 sur la R envers la stimulation gonadotropinique est partiellement compensé en 5 jours. L'utérus des rates avec ce déficit est, sous cet aspect, encore plus réactif envers cette stimulation que celui des témoins.

La diminution de la reproduction est due, en apparence, au déficit d'hormones ovariennes, parce que, chez 90% des rates avec déficience en pyridoxine, la grossesse peut être maintenue si l'on administre des doses d'œstrone et de progestérone (5) qui se sont avérées efficientes chez les rates ovariectomisées après l'accouplement [10].

La pyridoxine sert de co-enzyme pour nombreux enzymes impliqués dans le métabolisme protéique, parmi lesquels les transaminases.

L. I. Grebnik et coll. [3] ont montré que les descendants provenant de rates ayant reçu un régime pauvre en vitamine B_6 se dévelop-

* Reçu le 1er décembre 1970

pent normalement les 9—15 premiers jours, mais qu'ensuite leur croissance s'arrête et la plupart d'entre eux meurent 15—20 jours après la naissance.

Ces résultats présentent un intérêt pour le problème de l'effet du déficit en pyridoxine des mères sur la croissance de leurs petits.

Les recherches de Benjamin Eckstein [1] montrent que l'efficience de la diète déficitaire en B_6 peut être déduite du fait que le poids des animaux demeure stationnaire. Ceci intéresse surtout le poids de l'utérus et des ovaires. L'activité enzymatique dans l'ovaire de ces animaux est réduite environ de moitié par rapport aux témoins, alors que l'activité de la GOT (transaminase glutamique-oxalacétique) dans l'utérus ne diffère trop de celle des témoins.

Albert Eisenstein [2] conclut qu'en cas de carence en B_6, la fonction cortico-surrénalienne n'est pas altérée, en dépit de l'hypertrophie de cette glande.

Than et coll. [8] [9], en utilisant exclusivement la pyridoxine dans le traitement des toxicoses gravidiques chez 53 femmes hypoacides ou anacides, avec des vomissements gravidiques, chez lesquelles la sécrétion gastrique a été examinée à l'aide de l'acido-test, constatent que concomitamment à la disparition des vomissements, l'acidité gastrique s'est modifiée, devenant normale ou hyperacide. Les auteurs concluent que la vitamine B_6 n'exerce pas son action directement sur les cellules gastrosécrétrices, mais indirectement, par la voie du système hypophyso-cortico-surrénalien et que les œstrogènes d'origine ovarienne jouent vraisemblablement un rôle dans l'apparition de la maladie.

L'élucidation de l'action de la pyridoxine dans le traitement des toxicoses gravidiques de début ou dans leur prophylaxie nécessite un surcroît d'informations, d'autant plus que l'étude de son action est une préoccupation actuelle de la gynécologie qui étudie, entre autres, son effet sur le pH vaginal.

En dehors du rôle important de la pyridoxine dans le métabolisme de la cellule nerveuse et de ses propriétés qui déterminent de nettes modifications dans l'organisme, son action pharmacodynamique non spécifique, c'est-à-dire son mécanisme d'action, demeure encore incomplètement élucidé.

Tout comme dans nos recherches précédentes concernant l'influence de la thiamine sur la R du tractus génital [7], les modifications obtenues avec cette vitamine deviennent plus manifestes après une administration prolongée.

Les recherches présentes visent à élucider le rôle de la pyridoxine dans l'équilibre des fonctions sexuelles.

MATÉRIEL ET MÉTHODE

Nos recherches portent sur deux lots de rates pubères (en nombre de 30), dont l'un a servi de témoin; chez ce lot on a prélevé chaque jour, 29 jours de suite, des frottis vaginaux pour établir les caractéristiques des cycles sexuels de chaque animal. L'autre lot a été traité par pyridoxine hydrochloride ($C_8H_{12}O_3N.HCl$) BDH England — 400 μg/100g

poids/jour, durant 3 semaines. Vu que ce dosage n'a pas modifié sensiblement les cycles sexuels, on a doublé la dose (800 μg/100 g) et on a continué le traitement encore 66 jours.

Les doses ont été rapportées à celles thérapeutiques ; elles n'ont pas induit de phénomènes toxiques ou de signes d'intolérance. En dehors des modifications du cycle sexuel, on a examiné le comportement des animaux, le poids, l'aspect. Ensuite, les animaux ont été sacrifiés dans diverses phases du cycle et on a étudié, comparativement aux témoins, la R des cornes utérines envers l'Ach en solution Tyrode, à la température de l'animal (38°C) et aération constante.

Pour l'examen histologique on a prélevé les ovaires et les cornes utérines restantes, dans les phases respectives ; les organes ont été fixés en formol sublimé 10%, inclus en paraffine et colorés par hématoxyline-éosine et Van Gieson. Les frottis vaginaux ont été colorés par la méthode de Postelnicu.

RÉSULTATS ET DISCUSSIONS

En analysant le tableau évolutif des cycles sexuels des rates sous l'influence du traitement prolongé par pyridoxine et en le comparant avec celui des témoins, on constate :

a) modification de l'aspect des frottis vaginaux, notamment : réduction de la période cyclique sexuelle, disparition du D, prolongement de la phase de O et apparition du P fugace ou d'un P—O ;

b) hypertrophie de l'utérus et des ovaires, plus évidente dans la phase de O et persistante dans les autres phases de methœstrus (M) ou de M—D, vu que le D a disparu chez tous les cas traités par pyridoxine.

c) le myomètre présente des contractions spontanées, rythmiques, régulières et amples dans toutes les phases. La RP-mim envers l'Ach est plus augmentée en O par rapport aux témoins de la même phase. Voilà quelques expériences démonstratives :

Exp. 1. Rate n° 9 (fig. 1) sacrifiée en O depuis 48 heures. Au cours du traitement par pyridoxine, elle n'a plus présenté la phase de D depuis 20 jours et le O a duré parfois 5 jours. Ovaires grands avec points de congestion. Cornes utérines hypertrophiées, congestionnées, remplies de liquide de sécrétion ; dans le bain de solution Tyrode à 38°C à aération constante, la corne utérine gauche présente des contractions spontanées amples, régulières. 1 γ Ach intensifie les contractions

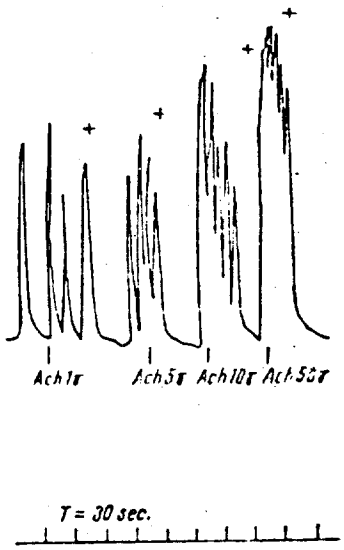

Fig. 1

rythmiques; 5γ, 10γ et 50γ Ach produisent une hypertonie nette, en rapport avec la dose administrée.

Ex. histologique. Ovaires et utérus à aspect de O. Follicules de divers degrés et nombreux corps jaunes (30) de dimensions différentes. Paroi utérine épaissie, lumière grande, festonnée; chorion gros, avec glandes ramifiées.

Exp. 2. Rate n° 11, sacrifiée en O depuis 24 jours, sans aucune phase de D. Au cours du traitement elle a présenté des O et des M prolongés.

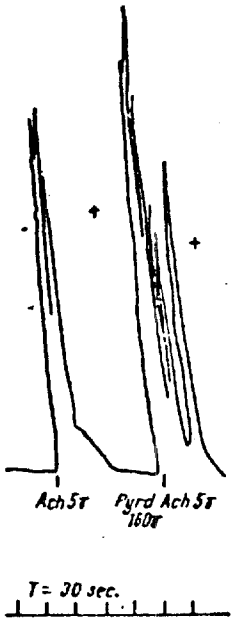

Fig. 2

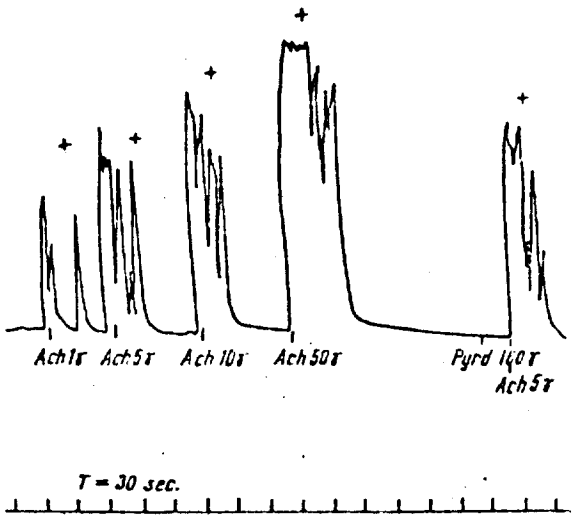

Fig. 3

Cornes utérines hypertrophiées, congestionnées, sans liquide dans la lumière, présentant des contractions spontanées, amples, irrégulières. La RP-mim envers l'Ach modifiée. 1 γ Ach augmente l'amplitude des contractions spontanées. 5 γ, 10 γ, 100 γ Ach provoquent hypertonie, en rapport avec la dose. La pyridoxine (Pyrd.), en doses progressives (160 γ, 360 γ, 0,0016 g), favorise l'effet P-mim des doses étalon d'Ach.

Ex. histologique: utérus à paroi grosse, lumière libre, festonnée, chorion dense à l'intérieur, avec de nombreuses glandes, musculature en couche épaisse, très vascularisée. Ovaire avec beaucoup de corps jaunes [21] de diverses dimensions et follicules de divers degrés.

Exp. 3. Rate n° 2, sacrifiée en O; a présenté un seul D au cours de 34 jours, pendant le traitement. Cornes hypertrophiées, congestionnées, pleines de liquide de sécrétion; contractions spontanées caractéristiques. 1 γ, 5 γ Ach augmentent nettement l'amplitude des contrac-

tions spontanées. Après 160 γ pyridoxine (Pyrd.) l'effet P-mim de la dose étalon de 5 γ Ach est nettement favorisé (fig. 2).

Exp. 4. Rate n° 18 (fig. 3), sacrifiée en O. Pendant le traitement, elle n'a plus présenté une phase de D depuis 20 jours. Cornes utérines grandes, dilatées, pleines de liquide de sécrétion. 1 γ, 5 γ, 10 γ et 50 γ Ach élèvent le tonus du myomètre, en rapport avec la dose. 160 γ Pyrd. favorisent légèrement l'effet P-mim de la dose de 5 γ Ach.

Ex. histologique. Ovaires grands, avec des follicules de divers degrés et de nombreux corps jaunes [21] de différentes dimensions. Grosseur inégale de l'utérus, musculature développée, chorion gros, avec de nombreuses glandes ramifiées.

Exp. 5. Rate n° 8, sacrifiée en O. Pendant toute la durée du traitement elle a présenté des périodes avec O prolongés jusqu'à 4 jours et un seul D net pendant 22 jours. Cornes utérines plus développées, congestionnées; contractions spontanées, régulières, amples. 0,1—0,5 γ Ach sont des doses isominétiques. 1 γ et 5 γ Ach augmentent l'amplitude des contractions. 10 γ et 50 γ Ach provoquent une nette hypertonie. 160 γ et 320 γ Pyrd. favorisent l'effet P-mim de la dose de 10 γ Ach.

Ex. histologique. Les ovaires présentent des follicules du Ier, IIe et IIIe degré et de nombreux corps jaunes [21] de diverses dimensions. L'utérus à paroi épaissie, richement vascularisée, chorion gros, laxe, avec de nombreuses glandes.

En conclusion, dans la phase de O, le myomètre présente la RP-mim envers l'Ach augmentée, vu que l'on sait que chez les témoins, dans cette phase, les petites doses de ce médiateur (0,1 γ, 0,5 γ, 1 γ et même 5 γ parfois) produisent un effet sympathomimétique (S-mim),

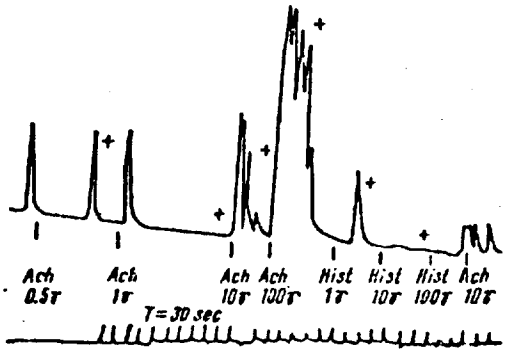

Fig. 4

c'est-à-dire la diminution de l'amplitude des contractions spontanées, parfois jusqu'à la disparition, et que les doses de 5 γ, 10 γ, et même 50 γ rarement, produisent une hypertonie si évidente (fig. 4).

Ces aspects de la R de l'utérus et l'aspect histologique persistent chez les rates traitées par la pyridoxine dans les autres phases de M ou de M—D . Par exemple :

Exp. 6. Rate n° 13 (fig. 5) sacrifiée en M vers D. A présenté des D très rares pendant le traitement. Cornes utérines modérément hyper-

trophiées, congestionnées, présentant des contractions spontanées rythmiques. 1 γ Ach — sans effet évident ; 5 γ, 10 γ et 50 γ Ach élèvent nettement le tonus du myomètre.

Ex. Histologique. Ovaires avec follicules du Ier, IIe et IIIe degrés, corps jaunes assez nombreux [17] de diverses dimensions. La paroi de l'utérus de grosseur moyenne, avec la musculature encore bien dévelop-

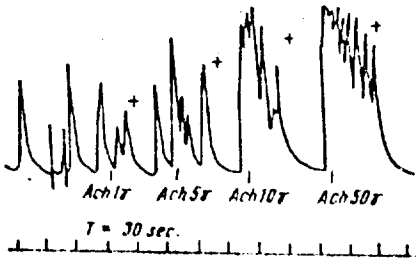

Fig. 5

pée, avec de nombreux vaisseaux, à épithélium cubique nécrosé (2/3), chorion laxe, avec glandes.

En conclusion, bien qu'en phase de M—D, l'aspect histologique de l'utérus présente une couche musculaire encore bien développée, de nombreux vaisseaux et avec la RP-mim envers l'Ach constamment accrue.

On voit donc que la plupart des animaux traités à la longue par la pyridoxine présentent généralement une augmentation de la R du tractus génital, due à la stimulation hypophyso-utéro-ovarienne et par conséquent à la stimulation folliculino-progestéronique. Cette stimulation se traduit par :

— disparition du D (20—24 jours) dans la grande majorité des cas ; parfois prolongement de la phase de O, apparition d'un P transitoire ou d'un P—O, tendance d'écourtement des cycles sexuels ;

— hypertrophie des ovaires et des cornes utérines avec hyperémie accentuée. Histologiquement, les ovaires présentent de nombreux follicules de tous les degrés et surtout de nombreux corps jaunes de diverses dimensions (fig. 6, rate n° 9) ; les cornes utérines ont une musculature plus développée et vascularisée (fig. 7, rate n° 11), le chorion gros avec de nombreuses glandes et avec liquide de sécrétion plus abondant dans la lumière, aspects qui apparaissent aussi en P et persistent en M jusqu'au cycle suivant ;

— la RP-mim envers l'Ach de l'utérus est plus augmentée en O et en P chez les animaux traités, se maintenant élevée aussi en M, comparativement avec les aspects caractéristiques des mêmes phases chez les témoins. Dans la grande majorité des expériences, les diverses doses de pyridoxine ont favorisé l'effet P-mim de l'Ach sur l'utérus isolé de rate, dans la phase de O ; dans les autres phases du cycle leur action n'est pas évidente.

Compte tenu des recherches antérieures des auteurs qui avaient démontré le rôle de co-enzyme de la pyridoxine pour beaucoup des

Fig. 6

Fig. 7

enzymes impliqués dans le métabolisme protéique, parmi lesquels les transaminases, compte tenu aussi de l'effet gonadostimulateur, de l'influence stimulatrice sur le système nerveux et de son action favorisante sur l'effet de l'Ach (rôle dans le mécanisme cholinergique), nous pouvons supposer le rôle activateur de cette vitamine (au point de vue de la pharmacodynamie non spécifique).

A l'encontre des résultats de nos recherches précédentes [7] effectuées par la thiamine en administration chronique et pendant une longue période, dans lesquelles son action anti-cholinergique (de type curarimimétique) a été prédominante et qui a inhibé le tractus génital (tant physiologique, que histologique), la pyridoxine utilisée dans les mêmes conditions, toujours en doses thérapeutiques, a stimulé l'axe hypophyso-utéro-ovarien.

Ces effets sont similaires à ceux décrits dans des publications plus anciennes [6] concernant l'étude de l'influence des substances activantes dans le mécanisme neuro-endocrino-effecteur. En analysant l'action pharmacodynamique non spécifique de l'ésérine, nous avons montré l'effet réactivant de cette substance sur le tractus génital, par la favorisation de l'action de l'Ach et la stimulation hypophyso-utéro-ovarienne. Grâce à sa qualité de substance activante excitatrice au niveau de ces systèmes, l'ésérine a augmenté la R des centres et des glandes endocrines (hypophyse et ovaires), déterminant l'accroissement de la fréquence des cycles œstraux, le prolongement du O et du M, allant jusqu'à faire disparaître le D chez certains animaux, de sorte qu'ils entraient directement en M ou en P, ou même en O sans passer par la phase de repos sexuel (D). Le mécanisme de l'action activante excitatrice de l'ésérine s'explique tant par son action anticholinestérasique (donc favorisante de l'Ach), que par la stimulation de la folliculine, dont l'effet anticholinestérasique, donc cholinergique, est bien connu.

Compte tenu de l'effet cumulatif du traitement chronique par la pyridoxine et de la durée d'administration, on pourrait expliquer les modifications morphophysiologiques au niveau du tractus génital et de l'évolution du cycle sexuel.

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Influence of Pyridoxine on Tyrosine Transaminase Activity in Maternal and Fetal Rat Liver [1]

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ABSTRACT Massive doses of pyridoxine (100 mg/100 g body weight) were administered to nonpregnant and pregnant intact and adrenalectomized rats to determine whether alterations in tyrosine transaminase activity (TTA) and holotyrosine transaminase activity (HTA) could be produced and whether the responses differed in pregnant and nonpregnant states. Observations were made 5 hours after intraperitoneal injections of saline, cortisol or pyridoxine. Hepatic HTA tended to be higher and TTA was higher in saline-treated pregnant than in nonpregnant animals. Similar enzyme responses were observed in cortisol-treated animals compared with saline treated controls. Pyridoxine administration caused apparent stimulation of TTA and HTA in intact animals, but no increases in the enzyme activities were observed in similarly treated adrenalectomized rats. Similarities between the enzyme responses observed following pyridoxine administration and those following tyrosine administration are discussed in relation to age dependency. Increased TTA and HTA were observed only in fetuses from rats treated with pyridoxine, suggesting that the mechanism of stimulation of the enzyme by pyridoxine may be operative in fetal liver.

Massive doses of pyridoxine (100 mg/100 g body weight) have been reported to produce increased tyrosine transaminase (L-tyrosine-2-oxo-glutarate aminotransferase, EC 2.6.1.5) activity in livers of intact or adrenalectomized male rats (1, 2). Induction of the enzyme was suggested to be analogous to the induction produced by cortisol administration, since the increase in tyrosine transaminase activity following pyridoxine administration was inhibited by the simultaneous administration of puromycin or actinomycin (2).

Increased activity of holotyrosine transaminase relative to the total enzyme activity measured in animals treated with massive doses of pyridoxine appeared to reflect the presence of additional amounts of cofactor in the liver (1). Reduced hepatic holoenzyme activity in pyridoxine-deficient rats has also been observed (3).

A relative deficiency of pyridoxine in normal pregnancy has been suggested by the alterations of tryptophan metabolism observed in pregnant women (4). Endocrine factors have been implicated in the alterations since the urinary tryptophan metabolites differed from those found in pyridoxine deficiency per se. Daily administration of 10 mg pyridoxine to pregnant women partially corrects the faulty tryptophan metabolism.

This study was undertaken to determine the effect of pharmacological doses of pyridoxine (100 mg/100 g body weight) on holotyrosine transaminase and total tyrosine transaminase activities of maternal and fetal rat liver, and to determine the influence of the adrenal glands and of cortisol on the responses.

PROCEDURES

Animals and treatments. Female rats of the Sprague-Dawley strain weighing 200 to 250 g were housed individually in suspended, wire-bottomed cages in a room with controlled heat (22°), humidity (40%) and lighting (lights on: 7:30 AM to 7:30 PM). Food [2] and water were provided ad libitum. The day on which mating was confirmed by the presence of sperm in a vaginal smear was designated day 1. A control from the corresponding nonpregnant group was assigned with each pregnant animal. Bilateral adrenalectomies were performed on one-half of the

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[2] Wayne Lab-Blox Meal, Allied Mills, Inc., Chicago, Ill.

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animals on day 15 of the experiment. Following adrenalectomy, animals were maintained with 0.9% saline as drinking water.

Injections were adjusted to a constant volume of 2.0 ml with physiological saline and administered intraperitoneally on day 21. One intact and one adrenalectomized group received either saline injections, a physiological dose of 0.125 mg cortisol[3]/100 g body weight, or a massive dose (100 mg/100 g body weight) of neutralized pyridoxine hydrochloride.

Procedures. Rats were killed between 1:00 and 2:00 PM by electric shock 5 hours after the injections. Livers were quickly removed, blotted and weighed. The uterus was promptly removed from pregnant animals. Livers of decapitated fetuses were pooled by litter and weighed. Tissues were placed on dry ice for enzyme determination within 36 hours.

Tyrosine transaminase activity was measured by the method of Rosen et al. (5). Tissue homogenates were incubated for 3 minutes at 37° with pyridoxal phosphate in the presence of α-ketoglutaric acid, sodium phosphate buffer, diethyldithiocarbamate and sucrose, to permit combination of the cofactor and apoenzyme. Tyrosine was then added to initiate the reaction and incubation was continued for 10 minutes at 37°. Precipitation of the protein with trichloracetic acid terminated the reaction. The *p*-hydroxyphenylpyruvic acid formed was determined colorimetrically by adding a solution containing ammonium molybdate in hydrochloric acid, potassium phosphate and water. Optical density was measured in a spectrophotometer at 850 mμ, following color development at room temperature for 1 hour. Tissue protein concentration was determined by a modification[4] of the method of Lowry et al. (6).

Linear regression equations for protein and *p*-hydroxyphenylpyruvic acid standards were calculated. Enzyme activity was expressed as μmoles *p*-hydroxyphenylpyruvic acid produced per gram protein per hour. Three-way factorial analysis of variance values were computed to distinguish effects between treatments ($P < 0.01$). Student's *t* tests were used to compare the means of individual groups ($P < 0.05$).

RESULTS AND DISCUSSION

Food consumption, body weight gains and reproductive performance (table 1) were not affected by any of the treatments. No effects from cortisol or pyridoxine injections were expected since the compounds were administered only 5 hours prior to termination of the experiment. Adrenalectomies were performed 6 days earlier, but no effect on these measurements was observed. Relative liver sizes (liver weight × 100/body weight) were not affected by the compounds administered nor by adrenalectomy (table 1). A small but significant increase ($P < 0.05$) in relative liver size was observed in the pregnant animals compared with the nonpregnant, reflecting a widely observed phenomenon in pregnant rats. Liver protein concentration in pregnant animals was slightly lower than in nonpregnant animals and concentrations in fetal liver were markedly lower than in adult animals. A small but significant increase ($P < 0.05$) in liver protein concentration was observed in all adrenalectomized rats receiving pyridoxine and in fetuses from those mothers. The data were insufficient for determining the reason(s) for this increase.

Holotyrosine transaminase activity (HTA) tended to be higher in saline-treated pregnant rats than in nonpregnant rats but the increase was not significant (table 2). Removal of the adrenal glands did not significantly alter holoenzyme activity. In view of the "relative pyridoxine deficiency" associated with the pregnant state (4) and the observations of Lin et al. (3) that HTA tended to be depressed during pyridoxine deficiency in nonpregnant rats, the absence of a depression in pregnant animals in this study appeared to indicate that pyridoxine nutritional status was adequate. Based on the average daily food intake and the pyridoxine content of the diet, the daily

[3] Hydrocortone acetate, Merck, Sharp and Dohme Company, West Point, Pa.

[4] Leaver, D. J., and J. Key. Dept. Botany and Plant Pathology, Purdue University, Lafayette, Ind. (unpublished data, personal communication).

TABLE 1

Reproductive performance, relative liver size and liver protein content of intact and adrenalectomized rats administered cortisol or pyridoxine

Treatment [1]	Reproductive performance			Relative liver size		Protein content		
	Live young		Resorptions	Nonpregnant	Pregnant	Nonpregnant	Pregnant	Fetus [2]
	No.	*Body wt, g*	*No.*	*Liver wt x 100/body wt*		*mg/g liver*		
Intact								
Saline	12.1 ± 1.1 [3]	4.06 ± 0.08	0.8 ± 0.5	3.22 ± 0.06	3.54 ± 0.11 [4]	24.0 ± 0.9	22.8 ± 0.7	12.9 ± 0.5
Cortisol	12.7 ± 1.2	3.98 ± 0.15	1.0 ± 0.7	3.14 ± 0.05	3.38 ± 0.06 [4]	23.1 ± 0.8	22.6 ± 0.9	13.0 ± 0.1 [5]
Pyridoxine	13.8 ± 0.9	3.99 ± 0.11	0.5 ± 0.2	3.15 ± 0.09	3.34 ± 0.06 [4]	25.1 ± 1.1	24.0 ± 0.6	13.7 ± 0.3 [5]
Adrenalectomized								
Saline	10.3 ± 0.9	3.97 ± 0.07	1.8 ± 0.9	3.10 ± 0.10	3.46 ± 0.10 [4]	23.8 ± 0.7	23.5 ± 0.5	13.1 ± 0.3
Cortisol	12.7 ± 0.8	3.99 ± 0.21	0.8 ± 0.5	3.22 ± 0.11	3.54 ± 0.09 [4]	24.0 ± 1.1	22.9 ± 0.9	13.8 ± 0.1 [6]
Pyridoxine	11.3 ± 1.3	3.66 ± 0.21	1.6 ± 0.7	3.08 ± 0.06	3.16 ± 0.05	25.8 ± 0.7 [6]	25.1 ± 0.6 [6]	15.6 ± 0.4 [6]

[1] Intraperitoneal injections of 2 ml saline (0.9% NaCl), or 0.125 mg cortisol, or 100 mg pyridoxine per 100 g body weight in 2 ml saline were administered 5 hours before the rats were killed; adrenalectomies were performed on day 15.
[2] Fetuses from mothers receiving treatment.
[3] Mean of 6 rats or litters ± SE, except average body weight of fetus represents mean of 4 litters ± SE.
[4] Differs from similar nonpregnant group ($P < 0.05$).
[5] Differs from similar adrenalectomized group ($P < 0.05$).
[6] Differs from similar saline treated group ($P < 0.05$).

TABLE 2

Effect of cortisol and pyridoxine administration on tyrosine transaminase activity in intact and adrenalectomized pregnant and nonpregnant rats

Treatment [1]	Holoenzyme [2]			Total enzyme [3]			Stimulation [4]		
	Nonpregnant	Pregnant	Fetus [5]	Nonpregnant	Pregnant	Fetus	Nonpregnant	Pregnant	Fetus
	μmoles p-hydroxyphenylpyruvic acid/g protein/hr						%		
Intact									
Saline	296 ± 67 [6]	356 ± 47	40.2 ± 9.7	383 ± 87	572 ± 71	41.3 ± 7.4	30.9 ± 5.5	61.2 ± 3.6 [9]	9.0 ± 6.5
Cortisol	364 ± 26	423 ± 37	30.8 ± 7.3	539 ± 29	839 ± 44 [8*,9*]	34.5 ± 7.6	50.6 ± 9.4	102.2 ± 11.1 [8,9]	13.6 ± 5.9
Pyridoxine	427 ± 95 [7]	474 ± 52 [7*]	41.8 ± 4.4	613 ± 98 [7]	792 ± 53 [7*,8]	49.9 ± 5.2	50.4 ± 8.1	71.1 ± 8.8	20.0 ± 3.7
Adrenalectomized									
Saline	274 ± 42	298 ± 54	27.7 ± 2.9	393 ± 56	523 ± 91	33.4 ± 3.4	65.1 ± 18.3	79.7 ± 10.3	20.7 ± 3.2
Cortisol	463 ± 88 [8]	400 ± 56	30.0 ± 3.2	666 ± 96 [8]	346 ± 91 [8]	35.6 ± 3.5	50.2 ± 6.9	118.7 ± 18.5 [8,9]	19.9 ± 5.6
Pyridoxine	167 ± 23	290 ± 21 [9*]	50.3 ± 7.2 [8*]	274 ± 36	489 ± 34 [9*]	60.9 ± 9.5 [8]	66.7 ± 6.8	69.6 ± 8.1	20.5 ± 6.2

[1] Intraperitoneal injections of 2 ml saline (0.9% NaCl), or 0.125 mg cortisol, or 100 mg pyridoxine per 100 g body weight in 2 ml saline were administered 5 hours before the rats were killed; adrenalectomies were performed on day 15.
[2] Activity measured without pyridoxal phosphate added in vitro.
[3] Activity measured with pyridoxal phosphate added in vitro.
[4] $\frac{\text{Total enzyme} - \text{holoenzyme}}{\text{holoenzyme}} \times 100.$
[5] Fetuses from mothers receiving treatment.
[6] Mean of 6 rats or litters ± SE.
[7] Differs from similar adrenalectomized group ($P < 0.05$).
[8] Differs from similar saline treated group ($P < 0.05$).
[9] Differs from similar nonpregnant group ($P < 0.05$).
* $P < 0.01$.

pyridoxine intake was 124 μg for nonpregnant rats and 168 μg for pregnant. These levels are considerably higher than the suggested requirements for growth and gestation in the rat (7). The data suggested that dietary pyridoxine was adequate to provide for sufficient pyridoxal phosphate for formation of holoenzyme in the livers of pregnant rats.

Pregnant rats showed higher ($P < 0.01$ by analysis of variance) total tyrosine transaminase activity (TTA) than the corresponding nonpregnant animals. Milkovic and Milkovic (8) reported that the level of circulating adrenocortical steroids in maternal blood is elevated which lent support to the findings of Sereni et al. (9) that TTA was partially induced in pregnant rats. In the present study, adrenalectomy of saline-treated pregnant rats did not cause a significant decrease of TTA, in agreement with observations by Lin and Knox (10) and Rosen et al. (5), indicating that elevated enzyme activity during pregnancy is influenced by factors other than the level of adrenocortical steroids.

HTA appeared to be increased in all animals treated with cortisol compared with saline-treated controls, although the increase was significant ($P < 0.05$) only in the adrenalectomized nonpregnant group. HTA showed a 23% increase in nonpregnant intact animals treated with cortisol and a 19% increase in similarly treated pregnant animals when compared with corresponding values of saline-treated rats. Cortisol-treated adrenalectomized animals demonstrated increases of 87 and 34% in nonpregnant and pregnant rats, respectively. No reports of HTA in cortisol-treated rats have been found, but these data indicated that both nonpregnant and pregnant rats obtained sufficient dietary pyridoxine to provide pyridoxal phosphate for formation of additional holoenzyme in the liver.

TTA was increased in all animals receiving cortisol compared with those receiving saline, but the increase was not significant ($P < 0.05$) in intact nonpregnant animals. Intact nonpregnant and pregnant rats treated with cortisol demonstrated enzyme activity increases of 41 and 47%, respectively. Cortisol treatment of adrenalectomized rats produced enzyme activity increases of 69% in nonpregnant animals and 62% in pregnant animals. The increased sensitivity of adrenalectomized rats to cortisol administration originally reported by Lin and Knox (10) may be due to a limited ability of the animals to maintain enzyme repressor levels. Response of enzyme activity to cortisol treatment in pregnant rats, also observed by Rosen and Nichol (11) and Wicks (12), suggested that the amino acid pools in the liver are sufficient to permit tyrosine transaminase synthesis even during amino acid withdrawal for rapid growth of fetal tissues.

Administration of pyridoxine to intact nonpregnant and pregnant animals tended to elevate HTA compared to saline-treated animals. Increased HTA would be expected in the presence of large amounts of pyridoxine in vivo. However, HTA in adrenalectomized animals was not increased by pyridoxine administration. Greengard and Gordon (1) observed increased HTA in adrenalectomized young male rats receiving similar pyridoxine doses.

TTA increased following administration of pyridoxine to intact rats, and the increase was significant ($P < 0.01$) in intact pregnant animals when compared to saline-treated controls. Apparent stimulation of TTA in intact animals by pyridoxine was not observed in similarly treated adrenalectomized animals, in fact, a slight depression was observed.

The enzyme responses in pyridoxine-treated rats closely resembled TTA patterns following tyrosine administration, as reported by Lin and Knox (10). They observed increased TTA in intact adult rats receiving tyrosine; however, administration of tyrosine to adrenalectomized animals caused a slight depression in the liver enzyme activity. Administration of tyrosine to intact animals may have promoted release of adrenocortical hormones resulting in increased tyrosine transaminase activity. Kenney and Flora (13) found that elevated tyrosine transaminase activity in intact rats and slightly depressed enzyme activity in adrenalectomized rats were also produced by injections of a stressing agent (diatomaceous

earth). Kenney and Albritton (14) demonstrated that the depressed enzyme activity occurred at a rate equal to the known rate of tyrosine transaminase degradation, indicating nearly complete cessation of enzyme synthesis. They proposed that pituitary hormone(s) secreted or synthesized in response to stress may function as repressors or stimulate repressor synthesis in the liver. Data obtained in the present study indicated that pyridoxine, in the dosage administered, produced TTA patterns similar to those produced by a stressing agent.

Greengard and Gordon (1), who reported increased TTA in pyridoxine-treated (100 mg/100 g body weight) adrenalectomized rats, used male rats 30 to 50 days of age, whereas female rats 103 to 135 days of age were used in the present study. Administration of tyrosine to adrenalectomized rats less than 75 days of age by Knox et al. (15) produced elevated tyrosine transaminase activity, whereas similar treatment of rats more than 14 weeks old caused no increase in enzyme activity. Since TTA responses to pyridoxine administration in the present study were similar to those produced in adult animals by tyrosine (10), the contrast between the observations of Greengard and Gordon (1) and the present data may reflect the difference in ages of the experimental animals.

There were no significant differences in percentage stimulation (percentage increase in enzyme activity produced by the addition of pyridoxal phosphate in vitro) between treatments of any nonpregnant animals. Statistically significant differences ($P < 0.05$) in percentage stimulation were noted between pregnant and nonpregnant intact rats receiving saline or cortisol and in adrenalectomized rats receiving cortisol. Interpretation of these findings is difficult. Since cortisol-treated and pregnant animals were capable of forming additional HTA, a change in the balance of HTA and TTA may be a normal effect of pregnancy. This suggestion is supported by the fact that even in the pyridoxine-treated rats with depressed activity percentage stimulation was not decreased.

There were no significant effects ($P < 0.05$) of maternal treatment on tyrosine transaminase activity in fetuses, except in those from adrenalectomized rats receiving pyridoxine. A statistically nonsignificant ($P > 0.05$) increase of tyrosine transaminase activity was noted in fetuses from pyridoxine-treated intact rats. Studies of tyrosine transaminase activity in fetuses following pyridoxine administration have not appeared in the literature, but stimulation of fetal liver tyrosine transaminase activity following administration of glucagon in utero was reported by Greengard and Dewey (16). Wicks (12) suggested that the mechanism of tyrosine transaminase stimulation by cortisol may be repressed in fetal liver by pituitary and placental secretion while the mechanism of stimulation by glucagon is not repressed. Data obtained in the present study suggest that the mechanism of stimulation of tyrosine transaminase by pyridoxine is operative in fetal liver. No significant influences of maternal treatment on percentage stimulation of enzyme activity in fetuses were observed.

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ANTAGONISM BETWEEN PYRIDOXINE, PYRIDOXINE DISULPHIDE AND ETHYL ALCOHOL

Antagonismo entre piridoxina y disulfuro de piridoxina y alcohol etílico

J. Teijeira and J.M. Martinez-Lage

It is conceded that pyridoxine acts as an antagonist to the toxic effects of alcohol, even though the results obtained for acute alcoholic intoxication are, in general, debatable. For some the favorable effects are patent (Duchesnay (4) and Wordsworth (22)). Other observations are very dubious (Abele (1), Berg (2), Gould (5) and Gruber(6)), and it has even been rejected as useless (Newman and Edmonds (13)). From the biochemical aspect, even though the degradation of the ethyl alcohol is related to numerous metabolic reactions, and among these some that depend on enzymes into which pyridoxine falls (Hakkinen and Kulonem (7) and Rosenfeld (14)), the data that

are found in the bibliography are insufficient to establish a possible antagonistic influence of pyridoxine on the toxic effects of alcohol. Due to the uncertainty of these studies, and considering that the percentage of positive results and current clinical experience are beginning to justify its use in the treatment of acute alcoholism, we have performed experiments related to this problem. We have also tested pyridoxine disulphide, the effects of which have not been experimentally tested in relation to alcoholic intoxication. This new compound can, according to our results in other types of expeiments, be substituted for pyridoxine successfully (Teijeira and cols. (17, 20). Pharmacologically it presents clear differences with pyridoxine, according to Hotovy (personal communication); but since it also has indubitable similarities, it seemed logical to perform comparative tests. In initial tests we found that the mortality of the rats in cases of acute alcoholic intoxication was reduced by a considerable percentage using pyridoxine, and even more so using pyridoxine disulphide (Teijeira (19)). We have tried to expand on our first results with the experiments that we are about to describe.

With the objective of appraising the neurological effects, we studied electroencephalograms; and to objectify the general state of the animal approximately, we also registered the ECG. The EEG and the cardiac rhythm change during acute alcoholic intoxication, and the improvement under the effects of pyridoxine and its disulphide can be taken as a criterion of its anti-alcoholic effectiveness.

Materials and Methods

All experiments on animals without anaesthesia: guinea pigs and rats mainly, a series of tests on a group of cats and some dogs. After the anaesthesia, indispensable for the operations, we waited long enough to be sure of the animal's recuperation from the narcosis, verifying it with the electroencephalographic control prior to administration of the alcohol. We maintained the animals under adequate conditions that ensured the normality of the temperature in the control conditions.

For one group of guinea pigs we used a dose of urethane of 10 cc./kg. of weight; but since recuperation was so slow, it did not seem practical. In the majority of cases we used a thiobarbitol dose of 10 mg./kg.

We administered the alcohol in one dose intraperitoneally in most cases. The dose was determined according to our previous results. For guinea pigs the dose was 20 cc. of 20 % per kilogram of weight to one group and 30 cc. per kilogram to an another group. We gave the latter dose to rats. We gave the cats 40, 20 and 10 cc. per kilogram of weight with the same concentration. In some experiments we administered the alcohol by slow intravenous injection.

Results

Effects of Pyridoxine and Pyridoxine Disulphide on Alcoholic Arhythmia.

Experiences with urethane.- Alcohol was administered to 15 animals previously anaesthetized with urethane when the activity of normal amplitude and acceptable regularity on the EEG predominates over the slow peaks; normally 3 hours after the anaesthesia. Anaesthesia with urethane produces hypersynchronic slow arhythmia that alternates with segments having characteristics close to those of wakefulness, during 3 or more hours (Fig. 1). With the passage of time after the anaesthesia, the low voltage activity dominates the graph, but still maintaining a slow and relatively isorhythmic quality in most cases.

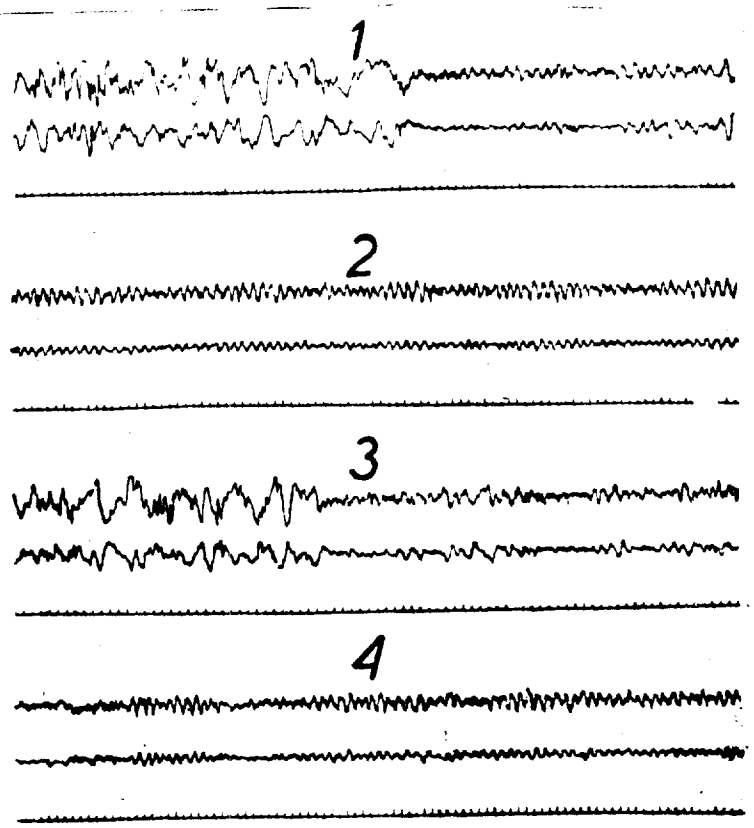

Figure 1.- Guinea pig anaesthetized with urethane: 1. intermitent paroxysmal activity, 2 hours and 30 minutes after anaesthesi. 2. monorhythmia 3 hours after anaesthesia. 3. intermitent arhythmia due to 20 % alcohol in 0.3 cc./min. continuous intravenous injection. 4. effect of intravenous pyridoxine.

With the slow continuous intravenous injection of alcohol (less than 0.3 cc. per minute), slow intermittent activity having a morphology and rhythm similar to that caused by the urethane reappears on the EEG (Fig. 1.3). Larger doses cause continuous hypersynchronic slow arhythmia. If the injection of alcohol is continued, there is a progressive decrease in the amplitude and frequency until extinction of electric cerebral activity which coincides with progressive reduction of the cardiac frequency and signs of anoxia on the EEG. On occasion paoxysmal activity of an irritative type is registered. When a dose of 20 cc. of alcohol per kilogram of weight is administered intraperitoneally, continuous hypersynchronic slow arhythmia quickly arises.

The changes produced by the injection of a dose of 5 mg/kg of pyridoxine are not significant because the immediate desynchronization of the graph due to the injection (Fig. 1.4) does not differ substantially from that caused by other stimuli. For that reason the data obtained from these experiments are not very useful, and we based our results on the charts obtained using another type of anaesthesia.

Effects of alcohol.- We selected the sample from guinea pigs previously anaesthetized with sodium thiopental, taking into consideration the age, weight, etc., the characteristics of the EEG and the cardiac rhythm. We found a linear correlation between the degree of regularity of the EEG and cardiac frequency. With cardiac frequencies less than 225 per minute, the EEG graph becomes progressively arhythmic. Changes permit the determination of the degree of deterioration suffered by

the animal, and whether to reject or use it for the test. Below this cardiac frequency, in effect, we observed greater sensitivity to alcohol which is manifested by the precocity of the slowness which appears inside of 2 minutes after the injection of alcohol and by the reduction in the recuperative capacity of the EEG. At cardiac frequencies above 225, the distribution of the phenomenon normalizes sensibly, and starting at 275 influences the morphology of the distribution curve equally, registering a mean value of 3 minutes with a dispersion of 1 to 4 minutes. For the comparative study all animals with cardiac frequencies below 225 were eliminated. Therefore, a total of 49 animals were used.

With these guinea pigs we started the experiments 2 hours after anaesthetization with thiobarbital, when the EEG and ECG recordings were normal and could be used as controls. All the animals received 20 cc./kg. of weight of 20 % alcohol intraperitoneally. They were divided into the following groups: 10 as controls; 10 receiving a dose of 50 mg/kg. of pyridoxine intravenously 5 minutes before the alcohol; 9 receiving a dose of 5 mg./kg. of pyridoxine intravenoulsy 5 minutes before the alcohol; 6 receiving a dose of 5 mg/kg of pyridoxine intravenously 5 minutes after the alcohol; 14 receiving a dose of 5 mg/kg of pyridoxine disulphide 5 minutes before the alcohol.

To describe the effects of the alcohol on the EEG we started from the graphs for the control group. We observed that slowness started most often 3 minutes after injection of the alcohol, with the slowness times varying between 1 and 4 minutes.

Besides the slowness, irritative paroxysmal activity consisting of hypersynchronic point discharges that dominate over 50 % of the charts, and to a lesser degree all of it. In no case was the recuperation of the electrical activity charted through to normality. In 30 % of the cases, after the slowness and paroxysmal activity have reached a maximum, the paroxysmal activity decreases at a more normal rate. In cases where a rapid evolution toward death is registered, it is preceded by progressive depression and even desynchronization of the electroencephalographic activity, appearing like a chart for anoxia.

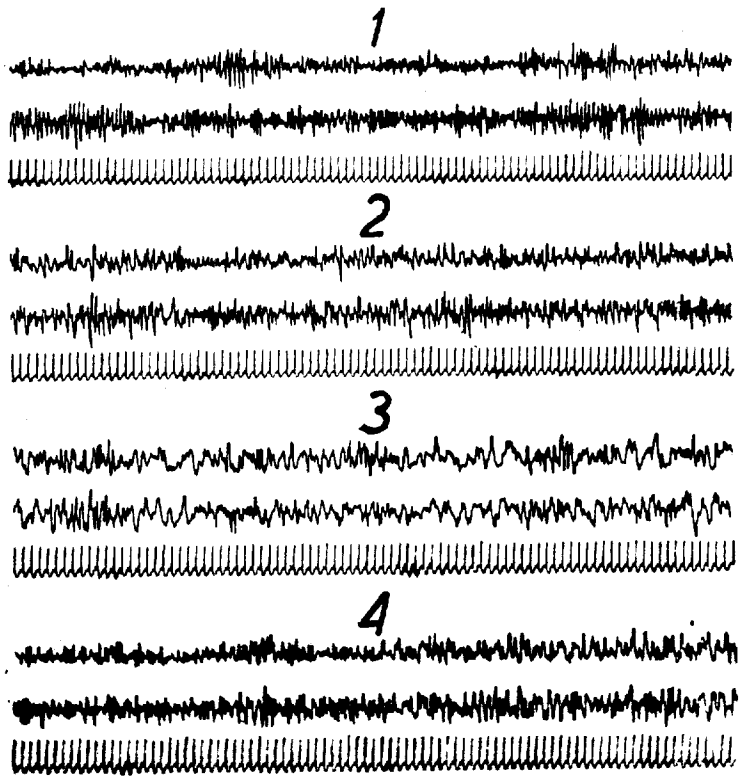

Figure 2.- Guinea pig anaesthetized with thiobarbital and treated with 5 mg/kg of pyridoxine disulphide. 1, basic chart: EEG normal; cardiac frequency 276/min. 2, 5 minutes after the alcohol: moderate EEG irregularity with reduction of the peaks of 20%, cardiac frequency 273/min. 3, 10 minutes after the alcohol, EEG with slow intense arhythmia and average paroxysmal activity; cardiac frequency 267/min. 4, 40 minutes after the alcohol: EEG reasonably recovered (grade 2); cardiac frequency 267/min.

To study the effect of pyridoxine and its disulphide on the EEG (Fig.2), we payed particular attention to their influence on the slowing of the chart, the paroxysmal irritative activity and the degree of recuperation.

We determined the slowing by measuring the time transpired between the administration of the alcohol and the loss of 20% of the peaks per minute compared to the base chart without analyzing the individual characteristics of the peaks. In the group receiving the strong dose of pyridoxine, the slowing takes longer to appear compared to the control group. The weak doses do not exert a noticeable effect with respect to the distribution of the phenomenon in the contol group.

We determined the irritative paroxysmal activity as a function of the percentage of hypersynchronic points that interfered with the chart slowed down by the alcohol. The value 4 corresponds to charts totally dominated by hypersynchronic point discharges. The value 0 corresponds to the absence of these. We did not register irritative activity in 52 % of the animals treated with pyridoxine disulphide or in 20 % of those treated with the strong dose of pyridoxine. In contrast, all of the animals in the control group showed paroxysmal activity.

We valued the electroencephalic recovery by the percentage of maximum frequency and amplitude gain referenced to the greatest arhythmia not taking time into consideration. The degree of recovery is greater for the groups treated with pyridoxine and its disulphide. Total recovery was registered in 6 % of the group receiving the strong dose of pyridoxine.

All of these results are shown in Figure 3 and in Tables I, II and III.

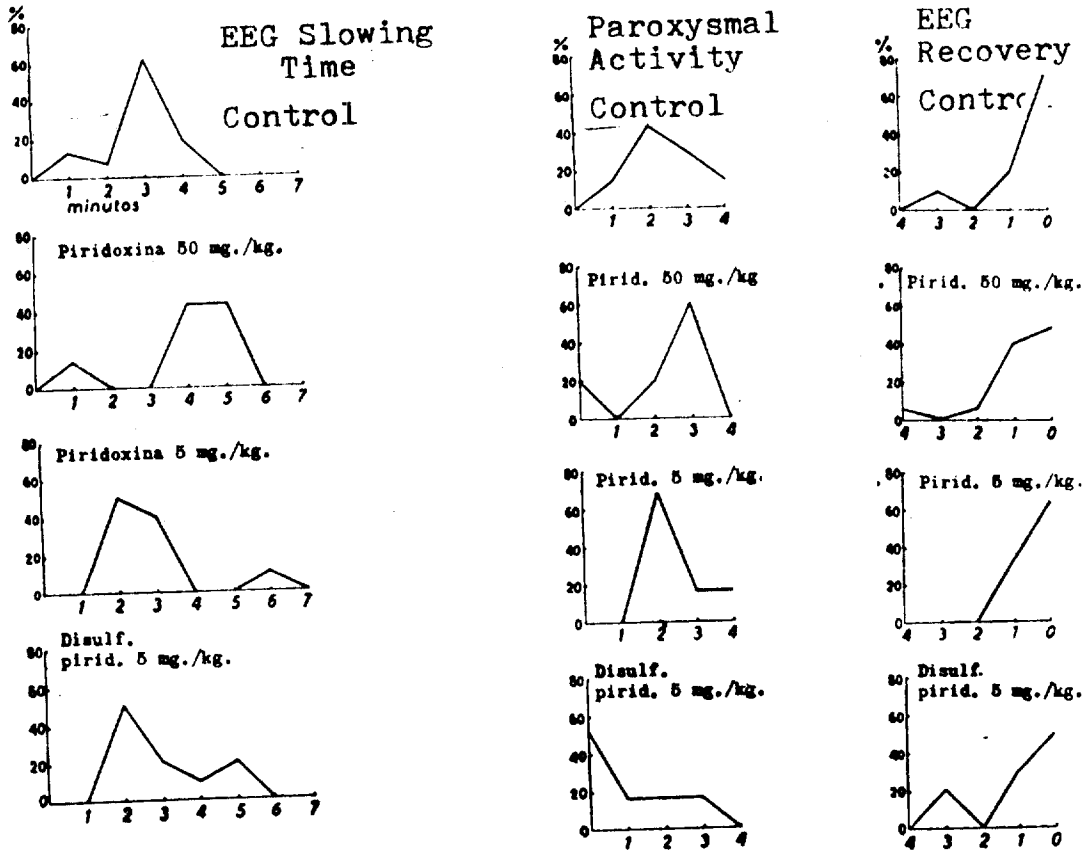

Figure 3.- Effects of alcohol on guinea pigs and changes arising from the previous treatment with pyridoxine and pyridoxine disulphide. EEG slowing appears later in the animals treated with strong dose of pyridoxine and pyridoxine disulphide.

Paroxysmal activity caused by the alcohol was not registered in 52 % of the cases treated with pyridoxine disulphide and in 20 % of those treated with the strong dose of pyridoxine. It was registered in all of the control animals.

The protective effect is also clear with regard to the degree of recovery from the cerebral arhythmia caused by the alcohol.

No electrical recovery is registered in 70 % of the control animals, while only 48 % of those treated with the strong dose of pyridoxine and 50 % of those treated with pyridoxine disulphide failed to recover.

The results obtained for cats are more debatable. With doses of alcohol which produce only moderate slowing - 20 cc/kg

of the same concentration as in the other experiments - we observed spontaneous recovery; and for this reason the difference between the treated and the control animals is dubious. Furthermore, we did not notice an appreciable effect of the pyridoxine on animals injected with progressively more toxic doses of alcohol.

TABLE I

Slowing of the EEG

Group	No.	Minutes 1	2	3	4	5	6
Control	16	13	7	61	19		
Pyridoxine 50 mg/kg	10	14		43	43		
Pyridoxine 5 mg/kg	9		50	40			10
Pyridoxine Disulphide 5 mg/kg	14		50	20	10	20	

For the purposes of this calculation, the 6 guinea pigs injected with pyridoxine 5 minutes after the alcohol have been added to the 10 guinea pigs in the control group. The values contained in the Table express the percentage of animals that slow at the times indicated.

TABLE II

Degree of Paroxysmal Activity

Group	N.	0	1	2	3	4
Control	10		14	43	29	14
Pyridoxine 50 mg/kg	10	20		20	60	
Pyridoxine 5 mg/kg	9			68	16	16
Pyridoxine Disulphide 5 mg/kg	14	52	16	16	16	

0: not measurable
1-2-3: intermediate paroxysmal arhythmia
4: totally dominates the chart

TABLE III

Degree of Recovery of the EEG

Group	No	4	3	2	1	0
Control	10		10		20	70
Pyridoxine 50 mg/kg	10	6		6	40	48
Pyridoxine 5 mg/kg	9				33	67
Pyridoxine Disulphide 5 mg/kg	14		20		30	50

Maximum observation time: 6 hours
4: Close to total recovery
3-2-1: Intermediate degrees
0: No recovery was registered

The Effects of Pyridoxine and Pyridoxine Disulphide on the Circulatory Changes Caused by the Alcohol

The electrocardiogram shows that the reduction of the cardiac frequency is a constant manifestation of alcoholic intoxication, although varying from animal to animal. It is greater in those animals which had low cardiac frequencies initially, indicative of ill state of health. The data shown in Table IV and Figure 4 corresponds to the same groups of guinea pigs used for the EEG studies. It was noticed that the cardiac frequency of the animals treated with pyridoxine and pyridoxine disulphide is more stable than that of the control animals, that is, they resist the toxic action of the alcohol better. The results are more favorable for larger doses of pyridoxine, in spite of the use of the animals with the lowest base cardiac frequency in this test.

In Figure 5 we show the curve of cardiac frequency versus time for an experiment using four guinea pigs, two as controls

and the other two receiving doses of 5 and 10 mg/kg of pyridoxine intramuscularly. All were injected with 30 cc/kg of 20 % alcohol intraperitoneally. The cardiac frequency curves for the animals treated with pyridoxine start to diverge from those for the control animals 10 minutes after injection of the alcohol, showing higher cardiac frequencies with differences greater than the dispersion of the curves for the control animals. After one hour the cardiac frequency of the control animals decreased by more than 120 beats, while that of the animals receiving moderate and strong doses of B_6 decreased by 84 and 69 beats, respectively.

TABLE IV

Average Cardiac Frequency

Group	N.°	b	d(5-b)	5m	d(10-5)	10m	d(10-b)
Control	10	316	28	288	36	252	64
Pyridoxine 50 mg/kg	10	255	1	254	6	248	7
Pyridoxine 5 mg/kg	9	301	26	275	8	267	34
Pyridoxine Disulphide 5 mg/kg	14	273	2	271	22	249	24

b: Base

5^m and 10^m: 5 and 10 minutes after administration of the alcohol

The cardiac frequencies for 10 rats for which ECGs were taken simultaneously are shown in Figure 6. Five rats were used as controls and the other five received doses of 75 mg/kg of pyridoxine. All the rats were injected with 30 cc/kg of weight of 30 % alcohol intraperitoneally. The curves show considerable

dispersion. The curves for the animals treated with pyridoxine were higher than those for the control rats, some of them returning to normal.

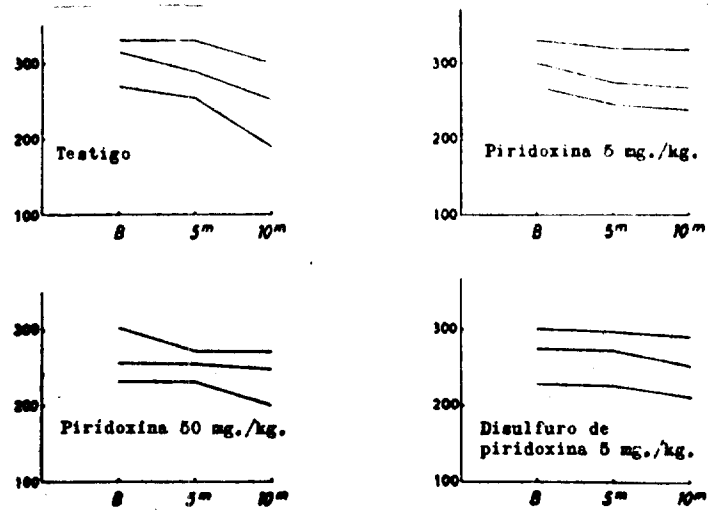

Figure 4.- Curves showing the average and the extreme cardiac frequencies. Ordinates: cardiac frequencies. Abscissas: B, base, 5^m and 10^m, 5 and 10 minutes after administration of the alcohol
The slope of the cardiac frequency curves is much smaller for the animals protected with pyridoxine and pyridoxine disulphide.

In cats (Fig. 7) the alcohol also causes progressive reduction of the cardiac frequency, approximately 15 to 20 % per hour compared to the base. The slope is mostly constant with occasional undulations. A slight recovery of the cardiac frequency, which in one case lasted for half an hour, was observed in the animals treated with pyridoxine. The pyridoxine, however, was not sufficient under these conditions to raise the cardiac frequency to normal levels; not being able to stop the drop in frequency and the heart stoppage which, in general, occurred 4 or 5 hours after the start of the experiment.

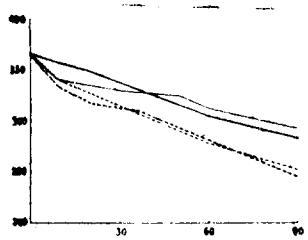

Figure 5.- Guinea pigs without surgical trauma under the effects of 30 cc. of 20 % alcohol injected intraperitoneally. Ordinate: cardiac frequency. Abscissa: time in minutes. Dotted lines: control group. Solid lines: animals treated with pyridoxine. The decrease in frequency is much more pronounced for the control group.

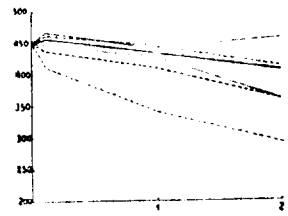

Figure 6.- Group of rats under the effects of 30 cc. of 20 % alcohol injected intraperitoneally. Intact animals. Ordinates: cardiac frequency. Abscissa: time in minutes. Dotted lines: control group. Solid lines: animals treated with pyridoxine. The decrease in cardiac frequency was much more pronounced for the control group.

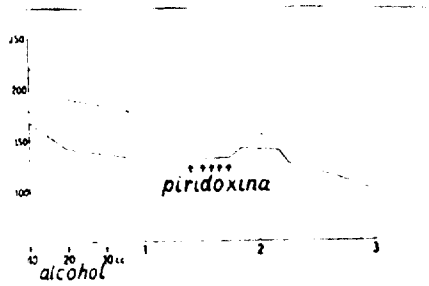

Figure 7.- Cats under the effects of 40, 20 and 10 cc/kg of 20 % alcohol at 20 minute intervals. Ordinate: cardiac frequency. Abscissa: time in hours. Dotted line: control group. Solid line: cat receiving intravenous injection of 200, 50, 50, 50 and 300 mg/kg of pyridoxine at the times shown by arrows on the cardiac frequency curve. There is a noticeable recovery in the curve starting from the point of injection of pyridoxine. This effect persists for more than 30 minutes after the last dose.

DISCUSSION

The results shown, although in some cases debatable and not conclusive, if considered as a whole reveal a protective effect of pyridoxine and pyridoxine disulphide against the

effects of acute alcoholic intoxication. Another point supporting this conclusion is that the animals used in the control groups had, for the parameters tested, the best base results. In spite of using the worst animals for the pyridoxine and pyridoxine treatments, they showed better results than the control animals.

The changes in the EEG during alcoholic intoxication has been studied by various authors. It has been found that small doses in dogs has resulted in increases in the base frequency (Malmejac and cols. (11)). For cats without anaesthesia a biphasic action of the alcohol has been described, starting with an increase in frequency and amplitude with progressive slowing as the concentration of alcohol in the blood is increased (Horsey (9), Horsey and Akert (10)). In the human EEG mainly slowing is also observed (Holmberg and Martens (8), Newman (12), Sutherland (18) and Ungerleider (2)). The results we have described do not differ essentially from those published by these authors, in the sense of considering the slowing of the EEG as the best indication of the toxic effect, and constitute a verification of the usefulness of the EEG in the study of pharmacological action in accordance with recent studies (Bickford (3)). With regard to the electroencephalographic effect of pyridoxine and pyridoxine disulphide, it is comparable to that which follows from its use in other pathological situations studied by us (Soria and Teijeira (17), Teijeira, Martinez-Lage, Anaya and de la Herran (20)) but has no specific character.

In some of our previous observations, the indicated compounds, above all pyridoxine disulphide, could be considered general stimulants; and in this case their antialcoholic effects could be interpreted in the same way as those of other stimulants (Rutenfranz and Jansen (15)). However, if this effect exists it seems to us to be secondary; and we believe that it is more a case of an influence on cerebral metabolism that speeds recovery from the toxic effect of alcohol. Sufficient data to support this possible metabolic mechanism cannot be found in the bibliography, but many hypothetical explanations could be formulated because the relations of structure and function of pyridoxine and its derivatives are greater than in any other vitamin.(Snell (16)).

Summary

Pyridoxine and pyridoxine disulphide antagonism against ethyl alcohol

An experimental investigation was planned in order to base a possible antagonistic influence of pyridoxine and pyridoxine disulphide upon the toxic effect of alcohol.

Pyridoxine disulphide is a new compound that can substitute the pyridoxine advantegeously. Initially we have found that pyridoxine and pyridoxine disulphide considerably reduces the percentage in mortality rate in rats with acute alcoholic intoxication. The present work was carry out using guinea-pigs principally and rats, and a series of cats and some dogs, narcotized previously with thiopental and some cases with uretane, even though we waited sufficient time for the recuperation from narcosis, does proving with the EEG control previous to the administration of alcohol. It was administered in most cases by intraperitoneal injection (one dose only), and some cases by slow intravenous injection. Simultaneously EEG and ECG was registered, studying its alterations during the alcoholic intoxication and the favourable modification of the same below the effect of the pyridoxine and the pyridoxine disulphide which can be taken as criteria of the antialcoholic effectiveness.

A lineal correlation was found between the grade of regularity of the EEG and the cardiac frequency; at the same time that this diminished the EEG became progressively disrhytmic. Both characteristics were considered to discard the animals not apt for the experiment.

In guinea-pigs, the intraperitoneal injection of the 20 cc/kg of alcohol to the 20 % arise lentification of the EEG on the average within 3 minutes after injection. There also appear in the EEG, after the injection of alcohol an irritating paroxistic activity with hypersynchronic spikes.

In no case were these alterations spontaneously reversible towards normality, being observed in some cases a rapid evolution towards death, preceded by a progressive depression in the electroencephalographic activity stimulating a record of anoxia.

The administration of pyridoxine in high level doses and of pyridoxine disulphide slows down the appearance of the EEG lentification following the injection of alcohol, prevents the irritative paroxistic activity caused by it, and improves the degree of recuperation in relation to the group control.

The acute alcoholic intoxication causes a progressive diminishing of the cardiac frequency, an effect which neither progressive nor is stablished in animals treated with pyridoxine and pyridoxine disulphide.

These exposed results, considered as a whole, reveal a so called protector effect of pyridoxine and pyridoxine disulphide against the effects of acute alcoholic intoxication.

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Antagonismo entre piridoxina y disulfuro de piridoxina y alcohol etílico

por
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(Recibido para publicar el 17 de febrero de 1961)

La piridoxina se admite que actúa como antagonista de los efectos tóxicos del alcohol, aunque los resultados obtenidos en la intoxicación alcohólica aguda en general son discutibles. Para unos el efecto favorable es patente [DUCHESNAY (4) y WORDSWORTH (22)]. Otras observaciones son muy dudosas [ABELE (1), BERG (2), GOULD (5) y GRÜBER (6)]. Y hasta se ha rechazado como inútil [NEWMAN y EDMONDS (13)]. En el aspecto bioquímico, aunque la degradación del alcohol etílico se relaciona con numerosas reacciones metabólicas, y entre éstas algunas que dependen de enzimas en los que entra la piridoxina [HAKKINEN y KULONEM (7) y ROSENFELD (14)], los datos que se encuentran en la bibliografía son insuficientes para fundamentar una posible influencia antagonista de la piridoxina sobre el efecto tóxico del alcohol. Ante la inseguridad de estos estudios, y teniendo en cuenta que el porcentaje de datos positivos publicados junto con la experiencia clínica corriente viene justificando su empleo en el tratamiento del alcoholismo agudo, nos hemos planteado investigaciones experimentales sobre el problema. Además ensayamos el disulfuro de piridoxina cuyos efectos no han sido investigados experimentalmente en relación con la intoxicación alcohólica. Este compuesto nuevo, según resultados nuestros en investigaciones de otro tipo, puede sustituir con ventaja a la piridoxina [TEIJEIRA y col. (17, 20)]. Farmacológicamente presenta claras diferencias con la

piridoxina, según HOTOVY (comunicación personal). Pero como también tiene indudables analogías, era lógico realizar un estudio comparativo. En ensayos iniciales encontramos que la mortalidad de los ratones en la intoxicación alcohólica aguda se reduce en un porcentaje considerable por la piridoxina, y más aún por el disulfuro de piridoxina [TEIJEIRA (19)]. En las investigaciones que exponemos en este trabajo tratamos de ampliar nuestros primeros resultados.

Con el objeto de valorar los efectos neurológicos estudiamos el registro electroencefalográfico, y para objetivar aproximadamente el estado general del animal registramos también el ECG. El EEG y el ritmo cardíaco se alteran en la intoxicación alcohólica aguda, y la modificación favorable bajo el efecto de la piridoxina y disulfuro de piridoxina puede tomarse como criterio de su eficacia antialcohólica.

Material y métodos

Todas las experiencias en animales sin anestesia: cobayas y ratas principalmente, una serie de ensayos en un grupo de gatos, y algunos perros. Después de la anestesia imprescindible para las manipulaciones operatorias, esperamos lo suficiente para tener seguridad de que el animal está recuperado de la narcosis, comprobándolo así con el control electroencefalográfico previo a la administración de alcohol. Y mantenemos los animales en las condiciones adecuadas que aseguran la normalidad de la temperatura en las condiciones de control.

En un grupo de cobayas hemos empleado uretano a la dosis de 10 cc./kg. de peso, pero como la recuperación es muy lenta no nos ha parecido práctico y en la mayoría de los casos utilizamos tiobarbital a la dosis de 10 mg./kg.

Administramos el alcohol en la mayoría de los casos por vía intraperitoneal en dosis única, determinada de acuerdo con nuestros anteriores resultados. En cobayas 20 cc. al 20 % por kilo de peso en un lote de animales, y 30 cc. por kilo en otro grupo. En ratas esta última dosis. En gatos 40, 20 y 10 cc. a la misma concentración por kilo de peso. En algunas experiencias administramos el alcohol en inyección intravenosa lenta.

Resultados

EFECTOS DE LA PIRIDOXINA Y DISULFURO DE PIRIDOXINA SOBRE LA DISRITMIA ALCOHÓLICA.

Experiencias con uretano. — En 15 animales, previamente anestesiados con uretano, se administra el alcohol cuando en

el EEG la actividad de amplitud normal y regularidad aceptable predomina sobre los brotes lentos, ordinariamente a las tres horas de la anestesia. La anestesia con uretano origina disritmia lenta hipersincrónica que alterna con segmentos de características próximas a las de vigilia, durante tres horas o más (fig. 1). A medida que transcurre el tiempo después de la anestesia, la actividad de bajo voltaje domina el trazado, pero conservando un carácter lento y relativamente isorrítmico en la mayoría de los casos.

Con el alcohol en inyección intravenosa continua lenta — menos de 0'3 cc. por minuto — reaparece la actividad lenta

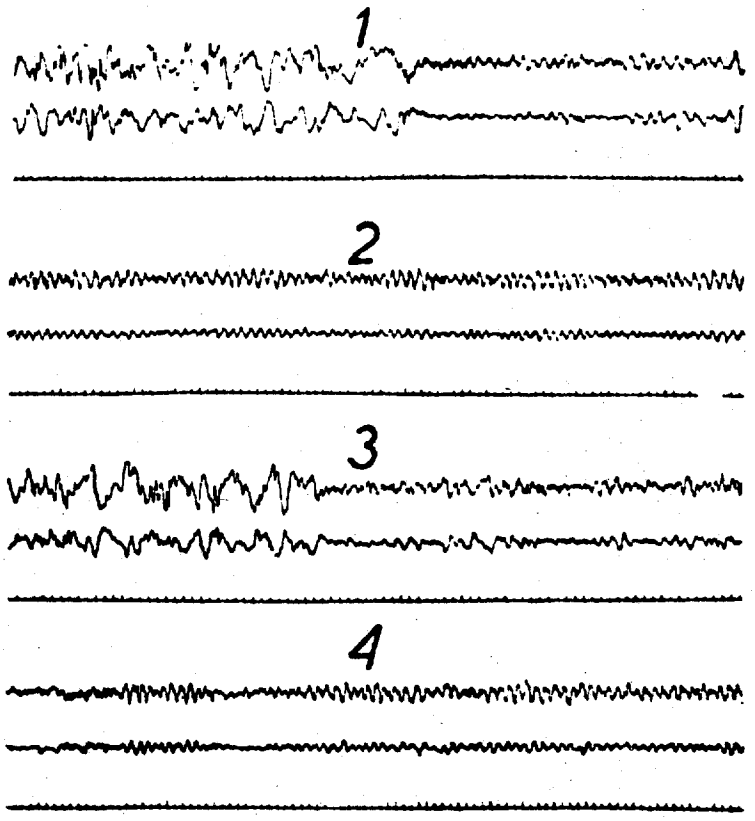

Figura 1. — Cobayo anestesiado con uretano. 1. actividad paroxística intermitente, a las 2 horas 30 minutos de la anestesia. 2, monoritmia a las 3 horas de la anestesia. 3. disritmia intermitente por el alcohol al 20 %, en inyección intravenosa continua, 0,3 cc/min. 4, efecto de la piridoxina intravenosa.

intermitente en el EEG, de morfología y cadencia similar a la desencadenada por el uretano (fig. 1, 3). Dosis mayores originan disritmia lenta hipersincrónica continua. Si se sostiene la inyección de alcohol llega a producirse progresiva disminución de la amplitud y frecuencia hasta la extinción de la actividad eléctrica cerebral, coincidiendo con retardo progresivo de la frecuencia cardíaca y signos EEG de anoxia. En ocasiones se registra actividad paroxística de tipo irritativo. Cuando se administra el alcohol por vía intraperitoneal a la dosis de 20 cc. por kilo de peso se instaura rápidamente disritmia lenta hipersincrónica continua.

Las modificaciones que produce la inyección de piridoxina a la dosis de 5 mg./kg no son significativas, porque la desincronización inmediata del trazado por la inyección (fig. 1, 4) no difiere esencialmente de la ocasionada por otros estímulos. Por eso los datos obtenidos en estas experiencias son poco aprovechables y valoramos nuestros resultados por los registros obtenidos con otro tipo de anestesia.

Efectos del alcohol. — En cobayas previamente anestesiados con tiopental sódico seleccionamos la muestra, teniendo en cuenta además de la edad, peso, etc., las características del EEG y el ritmo cardíaco. Encontramos una correlación lineal entre el grado de regularidad del EEG y la frecuencia cardíaca. Con frecuencia cardíaca inferior a 225 por minuto, el trazado EEG se hace progresivamente disrítmico. Una y otra alteración permiten fijar el grado de deterioro sufrido por el animal, y rechazarlo o utilizarlo para el ensayo. Por debajo de esta frecuencia cardíaca, en efecto, observamos mayor sensibilidad al alcohol que se pone de manifiesto, con carácter constante, por la precocidad en la lentificación que aparece antes de dos minutos de la inyección de alcohol y por la disminución de la capacidad de recuperación del EEG. Con frecuencia cardíaca superior a 225, la distribución del fenómeno se normaliza sensiblemente, y a partir de 275 influyen por igual en la morfología de la curva de distribución registrando un valor medio de 3 minutos con dispersión de 1 a 4 minutos. Se han eliminado para el estudio comparativo todos los animales de frecuencia cardíaca inferior a 225, utilizando un total de 49 animales.

En estos cobayas iniciamos las experiencias a las 2 horas de la anestesia con tiobarbital, cuando los registros de EEG y ECG son normales y podemos tomarlos como controles. A todos estos animales se les administró alcohol intraperitoneal al 20 %, 20 cc./kg. de peso. Fueron distribuidos en los siguientes grupos: 10, como testigo; 10, con piridoxina intravenosa, cinco minutos antes del alcohol a la dosis de 50 mg./kg.; 9, con

piridoxina intravenosa a la dosis de 5 mg./kg. cinco minutos antes del alcohol; 6, con piridoxina intravenosa cinco minutos después del alcohol a la dosis de 5 mg./kg.; 14, con disulfuro de piridoxina a la dosis de 5 mg./kg., cinco minutos antes del alcohol.

Para describir los efectos del alcohol sobre el EEG tomamos los trazados del grupo testigo. Observamos que la lentificación se origina con mayor frecuencia a los tres minutos de

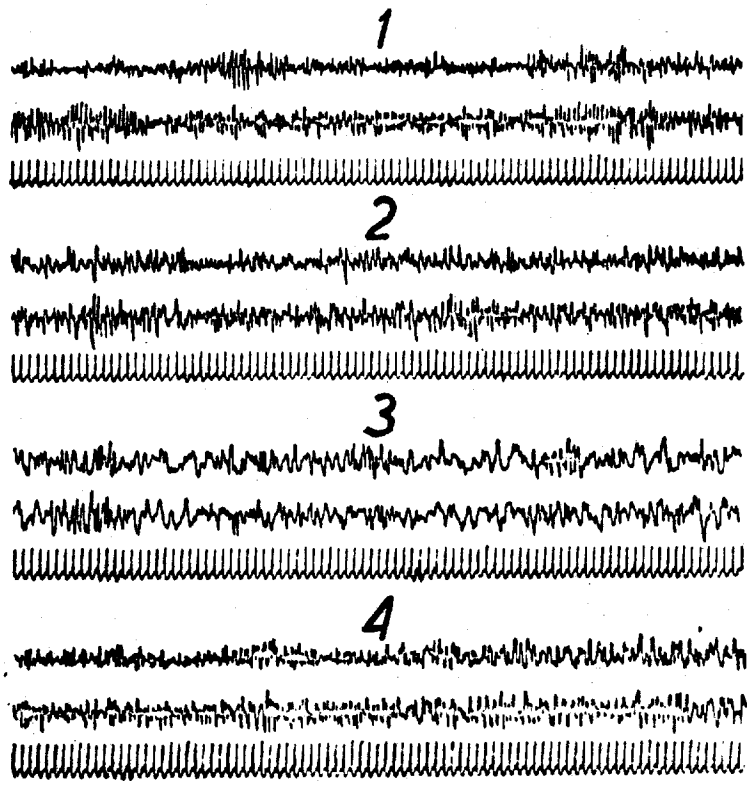

Figura 2. — Cobayo anestesiado con tiobarbital, y tratado con 5 mg/kg de disulfuro de piridoxina. 1, registro basal: EEG normal; frecuencia cardíaca 276/min. 2, cinco minutos después del alcohol: moderada irregularidad EEG, con reducción de grafoelementos en un 20 %, frecuencia cardíaca 273/min. 3, diez minutos después del alcohol: EEG con disritmia lenta intensa y actividad paroxística media; frecuencia cardíaca 267/min. 4, cuarenta minutos después del alcohol: EEG sensiblemente recuperado (grado 2); frecuencia cardíaca 267/min.

inyectado el alcohol, oscilando los tiempos de lentificación entre uno y cuatro minutos. En todos los animales se ha registrado, además de lentificación, actividad paroxística irritativa consistente en descargas de puntas hipersincrónicas, que dominan en general más del 50 % del trazado, y en moderado porcentaje la totalidad del mismo. No se ha registrado en ningún caso recuperación de la actividad eléctrica hasta la normalidad. En el 30 % de los casos, después que la lentificación y actividad paroxística alcanzan un máximo, la actividad paroxística disminuye de modo más sensible. En los casos en que se registra una evolución rápida hacia la muerte, ésta va precedida de depresión progresiva y aun desincronización de la actividad electroencefalográfica, simulando un trazado de anoxia.

Para estudiar el efecto de la piridoxina y del disulfuro de piridoxina sobre el EEG (fig. 2) atendemos a su influencia en la lentificación del trazado, la actividad irritativa paroxística y el grado de recuperación.

Determinamos la lentificación midiendo el tiempo transcurrido entre la administración de alcohol y la pérdida de un 20 % de grafoelementos por minuto con respecto al trazado de base, sin analizar las características individuales de los grafoelementos. En el grupo de la piridoxina a dosis fuerte la lentificación tarda en presentarse comparativamente al grupo control. Las dosis débiles no ejercen efecto sensible con respecto a la distribución del fenómeno en el grupo control.

Calificamos la actividad paroxística irritativa en función del porcentaje de puntas hipersincrónicas que interfieren el trazado lentificado por el alcohol. El valor 4 corresponde a trazados totalmente dominados por las descargas de puntas hipersincrónicas. El 0 a la ausencia de los mismas. No registramos actividad irritativa en el 52 % de los animales tratados con disulfuro de piridoxina y en el 20 % de los tratados con piridoxina a dosis fuerte. En cambio todos los componentes del grupo control presentan actividad paroxística.

La recuperación electroencefalográfica la valoramos por el porcentaje de ganancia máxima en frecuencia y en amplitud a partir de la máxima disritmia sin tener en cuenta el tiempo. El grado de recuperación se hace más sensible en el grupo tratado con piridoxina fuerte y con disulfuro de piridoxina. Se registra recuperación total en el 6 % del grupo de piridoxina fuerte.

El conjunto de estos resultados se expone en los cuadros I, II y III y en la figura 3.

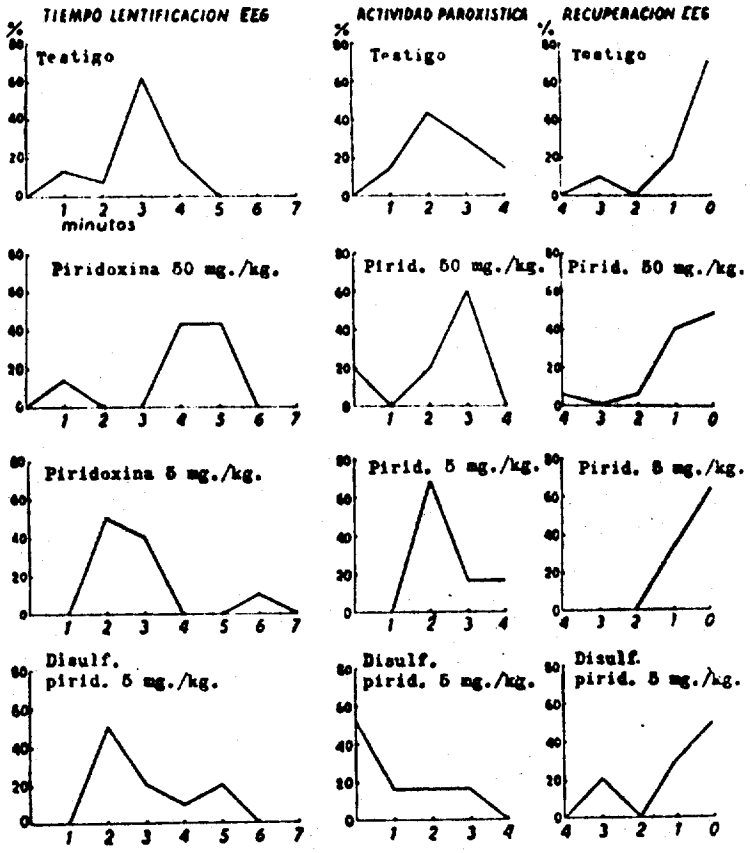

Figura 3. — Efectos del alcohol en cobayas y modificaciones que origina el tratamiento previo con piridoxina y disulfuro de piridoxina. La lentificación EEG, se presenta más tarde en los animales tratados con piridoxina fuerte y disulfuro de piridoxina.

La actividad paroxística ocasionada por el alcohol no se registra en el 52 % de los casos tratados con disulfuro de piridoxina, y en el 20 % de los tratados con piridoxina a dosis fuerte. Se registra en todos los animales testigos.

El efecto protector también es claro en cuanto al grado de recuperación de la disritmia cerebral ocasionada por el alcohol.

No se registra recuperación eléctrica en el 70 % de los animales testigos, mientras que la falta de recuperación afecta solamente a 48 % de los tratados con piridoxina a dosis fuertes y al 50 % de los tratados con disulfuro de piridoxina.

CUADRO I

Lentificación del EEG

Grupo	N.º	Minutos					
		1	2	3	4	5	6
Testigo	16	13	7	61	19		
Piridoxina 50 mg/kg.	10	14		43	43		
Piridoxina 5 mg/kg.	9		50	40			10
Disulfuro de piridoxina 5 mg/kg.	14		50	20	10	20	

Para este cálculo se han sumado a los 10 cobayas del grupo testigo los 6 cobayas inyectados con piridoxina, cinco minutos después del alcohol. Los valores contenidos en el cuadro expresan el porcentaje de animales que lentifican a los minutos señalados.

CUADRO II

Grado de actividad paroxística

Grupo	N.º	0	1	2	3	4
Testigo	10		14	43	29	14
Piridoxina 50 mg/kg.	10	20		20	60	
Piridoxina 5 mg/kg.	9			68	16	16
Disulfuro de piridoxina 5 mg/kg.	14	52	16	16	16	

0: no se registra.
1-2-3: disritmia paroxística intermedia.
4: domina totalmente el trazado.

Los resultados en gatos son más discutibles. Con dosis de alcohol que sólo producen moderada lentificación — 20 cc./kg. a la misma concentración que en las experiencias anteriores — observamos recuperación espontánea, y por este motivo es du-

dosa la diferencia entre los controles y los animales tratados. Pero tampoco apreciamos efecto claro de la piridoxina en animales inyectados con dosis de alcohol progresivamente más tóxicas.

CUADRO III

Grado de recuperación del EEG

Grupo	N.º	4	3	2	1	0
Testigo	10		10		20	70
Piridoxina 50 mg/kg.	10	6		6	40	48
Piridoxina 5 mg/kg.	9				33	67
Disulfuro de piridoxina 5 mg/kg.	14		20		30	50

Tiempo de observación máxima: 6 horas.
4. — Recuperación próxima a la total.
3-2-1. — Grados intermedios.
0. — No recuperan en ningún momento del registro.

EFECTO DE LA PIRIDOXINA Y DISULFURO DE PIRIDOXINA SOBRE LAS ALTERACIONES CIRCULATORIAS OCASIONADAS POR EL ALCOHOL.

El registro electrocardiográfico demuestra que la caída progresiva de frecuencia cardíaca es una manifestación constante de la intoxicación alcohólica, aunque de curso muy variable de unos a otros animales. Más fuerte en aquéllos que inicialmente tienen frecuencia cardíaca baja, sintomática de su mal estado. El conjunto de los datos que exponemos en el cuadro IV y figura 4 corresponde a los mismos grupos de cobayas utilizados para el estudio del EEG. Se aprecia que la frecuencia cardíaca de los tratados con piridoxina y disulfuro de piridoxina es más estable que la de los controles, es decir, resisten mejor la acción tóxica del alcohol, y el resultado es más favorable con dosis fuertes de piridoxina, a pesar de haber utilizado para esta prueba los de frecuencia cardíaca basal más baja.

En la figura 5 representamos la curva de frecuencia cardíaca en una experiencia con cuatro cobayas en los que efectuamos el registro simultáneo: dos como control, dos con piridoxina

CUADRO IV

Frecuencia cardiaca media

Grupo	N.º	b	d(5-b)	5m	d(10-5)	10m	d(10-b)
Testigo	10	316	28	288	36	252	64
Piridoxina 50 mg/kg.	10	255	1	254	6	248	7
Piridoxina 5 mg/kg.	9	301	26	275	8	267	34
Disulfuro de piridoxina 5 mg/kg.	14	273	2	271	22	249	24

b: basal.
5^m y 10^m: a los 5 y 10 minutos de administrar el alcohol.

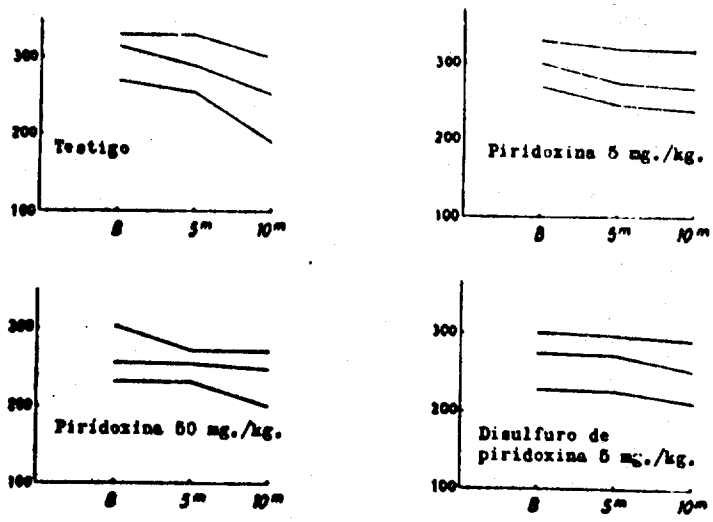

Figura 4. — Curvas medias y extremas de la frecuencia cardíaca. Ordenadas, frecuencia cardíaca. Abscisas: B, basal; 5^m y 10^m a los cinco y diez minutos de la administración de alcohol.
La pendiente de la curva de la frecuencia cardíaca disminuye considerablemente en los animales protegidos con piridoxina y disulfuro de piridoxina.

intramuscular a la dosis de 5 y 10 mg./kg., respectivamente. Todos inyectados con alcohol, 30 cc./kg. al 20 %, intraperitoneal. En los animales tratados con piridoxina la curva de retardo de frecuencia cardíaca se disocia a partir de los 10 minutos de la inyección de alcohol destacándose por encima de la banda de pendiente de los testigos, con un margen superior a la dispersión de ésta. Al cabo de una hora la frecuencia cardíaca de los animales testigos ha perdido más de 120 pulsaciones, mientras que la de los animales sometidos a dosis moderadas y fuertes de B_6 ha perdido sólo 84 y 69 respectivamente.

En la figura 6 se representan las curvas de frecuencia cardíaca en 10 ratas en las que se efectuó el registro simultáneo del ECG: 5 como testigo y 5 con piridoxina a la dosis de 75 mg./kg. Todos los animales inyectados con alcohol, 30 c.c. al 20 % por kg. de peso, por vía intraperitoneal. Las curvas muestran un grado considerable de dispersión. Las correspondientes a los animales tratados con piridoxina se mantienen por encima de los controles incluso recuperando la normalidad alguno de ellos.

En gatos (fig. 7) el alcohol produce también retardo progresivo de la frecuencia cardíaca, que disminuye aproximadamente un 15 a 20 % por hora en relación a la basal en pendiente constante, y ondulante en ocasiones. En los animales tratados con piridoxina se observa, con carácter variable pero constante, una ligera recuperación de la frecuencia cardíaca que en uno de los casos se mantiene durante más de media hora. La piri-

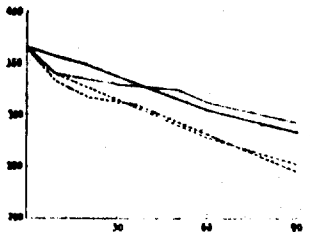

Figura 5. — Cobayas sin trauma quirúrgico, bajo el efecto del alcohol por vía intraperitoneal, 30 cc. al 20 %. Ordenadas, frecuencia cardíaca. Abscisas, tiempo en minutos. Línea de trazos, grupo testigo. Línea continua, animales tratados con piridoxina. La pérdida de frecuencia es mucho más acusada en los animales testigo.

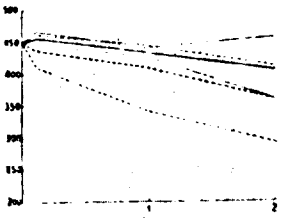

Figura 6. — Grupo de ratas bajo el efecto del alcohol por vía intraperitoneal, 30 cc. al 20 %. Animal intacto. Ordenadas, frecuencia cardíaca. Abscisas, tiempo en minutos Línea de trazos, grupo testigo. Línea continua, animales tratados con piridoxina. La pérdida de frecuencia es mucho más acusada en los animales testigo.

doxina, sin embargo, es insuficiente en estas condiciones para recuperar la frecuencia cardíaca normal, no pudiendo impedir la caída y el paro cardíaco que en general se produce hacia las 4 ó 5 horas de iniciar la experiencia.

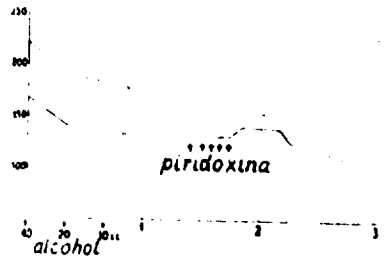

Figura 7. — Gatos bajo el efecto del alcohol, 40, 20 y 10 cc./kg. al 20 %, con intervalo de 20 minutos. Ordenadas, frecuencia cardíaca. Abscisas, tiempo en horas. Línea de trazos, grupo testigo. Línea continua, gato al que se le inyectan por vía i.v.: 200, 50, 50, 50 y 300 mg./kg. de piridoxina en los momentos señalados con una flecha sobre la curva de frecuencia cardíaca. Se registra una evidente modificación de la curva hacia la recuperación a partir de la inyección de piridoxina, efecto que persiste durante más de 30 minutos a partir de la última dosis.

Discusión

Los resultados expuestos, aunque en algunos casos son discutibles y poco demostrativos, si se consideran en conjunto revelan un efecto protector de la piridoxina y el disulfuro de piridoxina frente a los efectos de la intoxicación alcohólica aguda. Y en apoyo de esta conclusión se ha de tener en cuenta que los datos positivos son tanto más valorables cuanto que se han utilizado como testigos los que ofrecían mejores condiciones basales en los parámetros a estudiar. Y a pesar de utilizar los peores para el tratamiento con piridoxina y disulfuro de piridoxina, éstos evolucionan mejor que los controles.

La alteración del EEG en la intoxicación alcohólica ha sido estudiada por varios autores. En perros con dosis bajas se ha encontrado aumento de la frecuencia de base [MALMEJAC y *col.* (11)]. En gatos sin anestesia se ha descrito una acción bifásica del alcohol, comenzando por aumento de frecuencia y amplitud con lentificación progresiva al aumentar la concentración de alcohol en sangre [HORSEY (9), HORSEY y AKERT (10)]. En el EEG humano también se observa principalmente lentificación [HOLMBERG y MARTENS (8), NEWMAN (12), SUTHERLAND

(18) y UNGERLEIDER (21)]. Los resultados que hemos descrito no difieren esencialmente de los publicados por estos autores, en el sentido de considerar la lentificación del EEG como el dato más expresivo del efecto tóxico, y constituyen una comprobación de la utilidad del registro EEG en el estudio de acciones farmacológicas, de acuerdo con estudios recientes [BICKFORD (3)]. En cuanto al efecto electroencefalográfico de la piridoxina y el disulfuro de piridoxina es comparable al que acompaña a su influencia en otras situaciones patológicas estudiadas por nosotros [SORIA Y TEIJEIRA (17), TEIJEIRA, MARTÍNEZ-LAGE, ANAYA y DE LA HERRÁN (20)], pero no tiene un carácter específico. En algunas de nuestras observaciones anteriores los indicados compuestos, sobre todo el disulfuro de piridoxina pudieron considerarse en cierto modo como estimulantes centrales, y en este caso cabría interpretar su efecto antialcohólico como el de otros estimulantes [RUTENFRANZ y JANSEN (15)]. Sin embargo, si esta acción existe nos parece secundaria, y creemos que se trata más bien de una influencia sobre el metabolismo cerebral que favorece la recuperación del efecto tóxico del alcohol. En la bibliografía no se encuentran suficientes datos para interpretar este posible mecanismo metabólico, pero se podrían formular muchas explicaciones hipotéticas porque las relaciones de estructura y función de la piridoxina y derivados son más amplias que en ninguna otra vitamina [SNELL (16)].

Summary

Pyridoxine and pyridoxine disulphide antagonism against ethyl alcohol

An experimental investigation was planned in order to base a possible antagonistic influence of pyridoxine and pyridoxine disulphide upon the toxic effect of alcohol.

Pyridoxine disulphide is a new compound that can substitute the pyridoxine advantegeously. Initially we have found that pyridoxine and pyridoxine disulphide considerably reduces the percentage in mortality rate in rats with acute alcoholic intoxication. The present work was carry out using guinea-pigs principally and rats, and a series of cats and some dogs, narcotized previously with thiopental and some cases with uretane, even though we waited sufficient time for the recuperation from narcosis, does proving with the EEG control previous to the administration of alcohol. It was administered in most cases by intraperitoneal injection (one dose only), and some cases by slow intravenous injection. Simultaneously EEG and ECG was registered, studying its alterations during the

alcoholic intoxication and the favourable modification of the same below the effect of the pyridoxine and the pyridoxine disulphide which can be taken as criteria of the antialcoholic effectiveness.

A lineal correlation was found between the grade of regularity of the EEG and the cardiac frequency; at the same time that this diminished the EEG became progressively disrhytmic. Both characteristics were considered to discard the animals not apt for the experiment.

In guinea-pigs, the intraperitoneal injection of the 20 cc/kg of alcohol to the 20 % arise lentification of the EEG on the average within 3 minutes after injection. There also appear in the EEG, after the injection of alcohol an irritating paroxistic activity with hypersynchronic spikes.

In no case were these alterations spontaneously reversible towards normality, being observed in some cases a rapid evolution towards death, preceded by a progressive depression in the electroencephalographic activity stimulating a record of anoxia.

The administration of pyridoxine in high level doses and of pyridoxine disulphide slows down the appearance of the EEG lentification following the injection of alcohol, prevents the irritative paroxistic activity caused by it, and improves the degree of recuperation in relation to the group control.

The acute alcoholic intoxication causes a progressive diminishing of the cardiac frequency, an effect which neither progressive nor is stablished in animals treated with pyridoxine and pyridoxine disulphide.

These exposed results, considered as a whole, reveal a so called protector effect of pyridoxine and pyridoxine disulphide against the effects of acute alcoholic intoxication.

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METABOLISM OF TRITIUM-LABELLED PYRIDOXINE AND PYRIDOXINE 5'-PHOSPHATE[1] IN THE CENTRAL NERVOUS SYSTEM

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Abstract—[^{3}H]Pyridoxine and [^{3}H]pyridoxine 5'-phosphate have been injected into rats and mice. The uptake in brain tissue has been studied by comparing the concentrations of labelled compounds in serum, cerebrospinal fluid and brain tissue. Labelled pyridoxine passes rapidly into brain tissue, whereas the uptake of pyridoxine 5'-phosphate occurs at a much slower rate.

Perchloric acid extracts of brain have been fractionated by ion-exchange chromatography and the distribution of isotope between the different forms of the vitamin has been determined at different times after the administration. The time sequence of the metabolic transformation is: pyridoxine → pyridoxine 5'-phosphate → pyridoxal 5'-phosphate → pyridoxamine 5'-phosphate. After the initial transformation period about 40 per cent of the isotope is recovered in each of the pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate fractions.

MUCH INFORMATION has accumulated on vitamin B_6 and on the important role of its metabolism in the central nervous system. TOWER (1956) has reviewed the neurochemical aspects of pyridoxine metabolism and COURSIN (1956; 1969) has investigated the physiological effects of the vitamin on the brain. Several authors have reported on neurological disturbances due to B_6 deficiency (STREET, COWGILL and ZIMMERMAN, 1941; SNYDERMAN, CARRETERO and HOLT, 1950; COURSIN, 1954, 1960). The neuropathology in B_6-deficient monkeys has been studied by VICTOR and ADAMS (1956), and studies on the biochemical and clinical effects of pyridoxine in children with brain disorders have been reported by HEELEY, PIESOWICZ and MCCUBBING (1968). Special attention has been paid to convulsive disorders following deprivation of vitamin B_6 from the food (COURSIN, 1954; SNYDERMAN, HOLT, CARRETERO and JACOBS, 1953; MOLONY and PARMELEE, 1954; SCRIVER and HUTCHINSON, 1963; CHICK, ELSADR and WORDEN, 1940) and to B_6-dependent seizures appearing in childhood (HUNT, STOKES, MCCRORY and STROUD, 1954; SCRIVER, 1966; GENTZ *et al.*, 1967). HAGBERG, HAMFELT and HANSSON (1966) found an abnormal tryptophan metabolism in children with cryptogenic epilepsy, and this metabolic abnormality was normalized by pyridoxine. Some vitamin B_6-dependent convulsive disorders cannot be explained by an insufficient intake of the vitamin, but might be caused by irregularities in the normal metabolism of the vitamin. Neurological disorders following medical treatment with hydrazides (such as INH) are well known, and have been explained by an interaction between pyridoxal-P and the inhibiting hydrazide (BIEHL and VILTER, 1954; WIEGAND, 1956; DAVISON, 1956 and KILLAM, DASGUPTA and KILLAM, 1960).

The present study was undertaken in order to obtain information on the interconversion between the different forms of vitamin B_6 in the brain, following the

[1] Nomenclature of vitamin B_6 is that recommended by the IUPAC–IUB Commission on Biochemical Nomenclature.

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administration of physiological amounts of [^{3}H]pyridoxine and [^{3}H]pyridoxine-P and also to study the passage of the vitamin across the blood–CSF and the blood–brain barriers. One unphosphorylated vitamin B_6-form, [^{3}H]pyridoxine and one phosphorylated form, [^{3}H]pyridoxine-P was used in the experiments in order to establish if there was any fundamental difference between phosphorylated and unphosphorylated vitamin forms. Another point of interest was to establish if there existed a difference in the uptake of vitamin in the cerebrum and in the cerebellum.

MATERIALS AND METHODS

Compounds. Pyridoxal 5′-phosphate and pyridoxine hydrochloride were purchased from E. Merck A.G. (Darmstadt, Germany) and pyridoxine 5′-phosphate from Calbiochem (Lucerne, Switzerland). Pyridoxamine 5′-phosphate, pyridoxamine hydrochloride and pyridoxal hydrochloride were gifts from Merck Sharp & Dohme (Rahway, N.J., U.S.A.).

[G-$^3H_3$]Pyridoxine hydrochloride was purchased from the Radiochemical Centre (Amersham, Bucks, England). The purity was controlled by paper chromatography in *n*-butanol–dioxan (100:25, v/v; saturated with 2 M-ammonia), by thin-layer chromatography in *t*-amyl alcohol–acetone–water–ammonia (8:7:4:1 by vol.)[2] and finally by ion-exchange chromatography on a column of Dowex AG 50W-X8. The substance had a specific radioactivity of about 1 Ci/g. The solution used for injection contained about 100 mg/l.

Tritium-labelled pyridoxine 5′-phosphate was synthesized using a method first described by STOCK, ORTANDERL and PFELEIDERER (1966). (Tiselius, to be published). The labelled substance was purified several times before injection and the purity was controlled by ion-exchange chromatography (TISELIUS, 1972), by paper chromatography (SNYDER and WENDER, 1953), by high voltage electrophoresis (CLOTTEN and CLOTTEN, 1962) and by ion-exchange chromatography following hydrolysis (JOHANSSON, LINDSTEDT and TISELIUS, 1968). The specific radioactivity was calculated to be about 3·6 Ci/g. The solution for injection contained about 50 mg/l.

All labelled vitamin forms were stored in darkness at a temperature of +3°C.

Collection of cerebrospinal fluid, blood serum and brain tissue. Rats with a weight of 200–250 g were injected in a tail vein with either 20 μg of [^{3}H]pyridoxine or with 10 μg of [^{3}H]pyridoxine-P. The rats were killed with ether at different times after the administration of tritium-labelled vitamin. Blood was obtained from the animals by cutting off their aortae immediately after death. The blood samples were then centrifuged and the serum collected. CSF was obtained in the following manner: an incision was made in the neck between the superior cervical vertebra and the occipital bone. A very careful dissection was made down to the posterior atlanto-occipital membrane, which was completely uncovered. A glass capillary with an approx. vol. of 100 μl and with a very thin tip was put on a piece of plastic tubing. The glass capillary was then carefully forced through the fluctuating membrane and as much CSF as possible was sucked up. Usually it was possible to obtain about 50–100 μl of CSF. Samples which were not quite clear could not be used because of contamination with trace amounts of blood.

After the collection of CSF and blood, the brain was taken out and divided into three parts: the two hemispheres of the cerebrum and the cerebellum. Each part of the brain was carefully rinsed from blood by repeated washings in physiological saline, and then stored in a desiccator over P_2O_5 *in vacuo* for 24 h. Each brain piece was weighed and the isotope content in the brain tissue was determined by complete combustion in oxygen atmosphere and collection of the water formed (KELLY, PEETS, GORDON and BUYSKE, 1961; DAHLKVIST, LINDSTEDT and TISELIUS, 1969).

Extraction of brain tissue. In the conversion studies male mice of the N.M.R.I. strain were used. They had a weight of about 20–25 g and had been kept on a commercial mouse diet (Harald Forss Co., Stockholm, Sweden). The animals were injected in a tail vein with 20 μg of [^{3}H]pyridoxine or with 10 μg of [^{3}H]pyridoxine-P. The animals were killed at different times after the administration of isotope, their heads were cut off and as much blood as possible was allowed to escape. The brain of each animal was taken out and carefully rinsed from blood in physiological saline. In the short-term experiments (i.e. under 60 min) the brain from one mouse was used whereas in the long-term experiments groups of 4–5 mice were used to obtain a higher accuracy.

The brains were homogenized in a Vir Tis homogenizer (The Vir Tis Co., Inc., Gardiner, New York, N.Y.) together with an equal volume of distilled water. The homogenate was added to an equal vol. of 2 M-perchloric acid, and centrifuged. The pH was adjusted to 4·2 with 3 M-potassium hydroxide, and the extract was kept cold (+3°C) for a few hours. The potassium perchlorate was filtered off

[2] Radiochemical Centre Data Sheet 8322.

and the remaining solution evaporated *in vacuo* at a temperature of less than 40°C to a suitable volume.

Chromatographic procedure. The tissue extracts from the short-term experiments were directly applied onto micro-columns of Dowex AG 50W-X8 (height 200 mm; diam. 4 mm; 200-400 mesh) which had been equilibrated with 0·01 M-ammonium formate at pH 3·2. Elution was first performed with 20 ml of this buffer and then with a gradient system with 20 ml 0·05 M-ammonium formate at pH 4·25 in a mixing bottle, to which was added 0·5 M-ammonium formate at pH 7·5. Fractions with a volume of 0·4 ml were collected and the isotope content in each fraction was determined (TISELIUS, 1972). The different vitamin forms were eluted with the following volumes of effluent: pyridoxal-P, 3–5 ml; pyridoxine-P, 6–8 ml; pyridoxamine-P, 16–18 ml; pyridoxal, 35–36 ml; pyridoxine, 44–46 ml and pyridoxamine, 50–55 ml. Slight variations in elution volumes were observed and the above figures are only approximate.

A solution containing 1 mg of each of the different forms of vitamin B_6 was added to the tissue extracts from the long-term experiments and they were then put onto columns (height 400 mm; diameter 9 mm) of Dowex AG 50W-X8 (200-400 mesh) which had been equilibrated with 0·01 M-ammonium formate buffer at pH 3·2. Elution was performed with 200 ml of the same buffer, and after that with a gradient system composed of 100 ml 0·05 M-ammonium formate (pH 4·25) in a mixing bottle, to which was added 0·5 M-ammonium formate (pH 7·5) (TISELIUS, 1972). Fractions (5 ml) were collected at a flow rate of about 0·5 ml/min. Extinction was determined at 295 nm in each fraction to localize the different B_6-forms. In this chromatographic system the different vitamin forms appeared at the following volumes of effluent: pyridoxal-P, 70–90 ml; pyridoxine-P, 100–130 ml; pyridoxamine-P, 265–275 ml; pyridoxal 325–345 ml; pyridoxine, 375–400 ml and pyridoxamine, 450–500 ml. The fractions containing one vitamin form were combined and the volume was measured.

Isotope determination. From blood serum and CSF volumes of 50 μl were added to 15 ml of a scintillation fluid of the following composition: 2,5-diphenyloxazole (10 g), 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (0·3 g), toluene (1000 ml) and methyl cellosolve (600 ml).

From the chromatographic fractions 500 μl was added to 15 ml of the scintillation fluid. Isotope determinations were performed in a liquid scintillation spectrometer (Tri Carb, 3375, Packard Co., La Grange, Ill., U.S.A.). Correction for quenching was made by using an internal standard.

Fifty per cent of the tritium atoms are lost in the conversion of synthetic pyridoxine-P to pyridoxal-P and to pyridoxamine-P. This was corrected for when calculating the distribution of different vitamin B_6-forms in the metabolic experiments.

RESULTS

Passage of [^{3}H]pyridoxine and [^{3}H]pyridoxine-P into the central nervous system in rats. In order to study the passage of vitamin B_6 across the blood-CSF and the blood–brain barriers, tritium-labelled pyridoxine and pyridoxine-P were administered intravenously to rats, which were killed at different times after the injection. The isotope content of the serum, the CSF and the brain tissue was determined by the methods described above.

Figure 1A shows the ratio between the isotope content of 50 μl of serum and that of 50 μl of CSF during the first 3 h following the intravenous administration of [^{3}H] pyridoxine. Initially, a relatively higher concentration of isotope was obtained in the blood, but during the first 20 min the ratio decreased towards the value 1·0, where an equilibrium between blood and CSF is established.

The concentration ratio of isotope in serum and CSF during the first 3 h after the injection of [^{3}H]pyridoxine-P is shown in Fig. 1B. Initially high values were again found, but a decrease during the first hour to a ratio of 0·94 was observed.

The passage across the blood–brain barrier in the rat was studied by determining the isotope content per mg dried brain tissue and calculating the ratio between the isotope content per mg of brain tissue and in 1 μl of serum.

Figure 2A illustrates the passage of isotope into the brain during the first 3 h after the injection of [^{3}H]pyridoxine and Fig. 2B the passage after [^{3}H]pyridoxine-P injection. It is evident from comparison of Figs. 2A and 2B that the uptake of pyridoxine in the brain occurred at a much more rapid rate than that of pyridoxine-P.

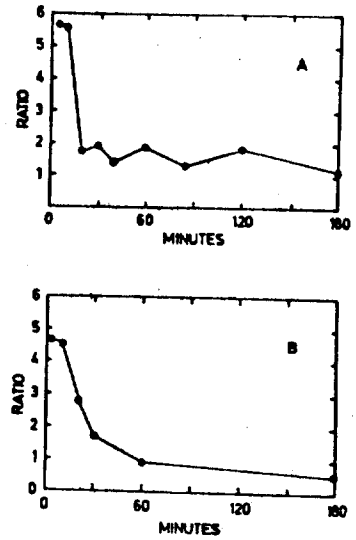

FIG. 1.—Ratio of isotope content of 50 μl of serum to that of 50 μl of cerebrospinal fluid in the rat at different times after the intravenous administration of [^{3}H]pyridoxine (A) and [^{3}H]pyridoxine 5′-phosphate (B). The animals had been injected in a tail vein with [^{3}H]pyridoxine (20 μg, 20 μCi) and [^{3}H]pyridoxine 5′-phosphate (10 μg, 36 μCi).

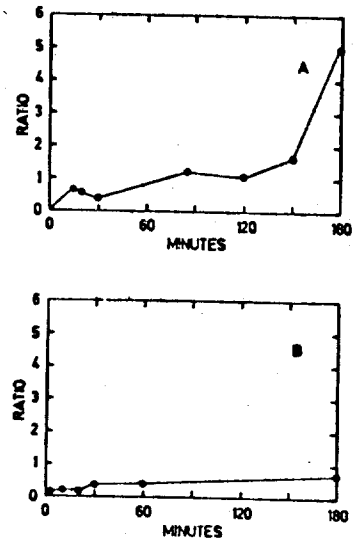

FIG. 2.—Ratio of isotope content of 1 mg of brain tissue (dry weight) to that of 1 μl of serum in the rat at different times after the intravenous administration of [^{3}H] pyridoxine (A) and [^{3}H]pyridoxine 5′-phosphate (B). Dosage and route as in Fig. 1.

This is also evident from the figures given in Table 1, which show the ratio of the isotope concentrations of brain tissue and CSF after administration of pyridoxine and pyridoxine-P.

TABLE 1.—RATIO BETWEEN ISOTOPE CONTENT/MG BRAIN TISSUE AND ISOTOPE CONTENT/μl CEREBROSPINAL FLUID

Time after administration (min)	Ratio following administration of: Pyridoxine	Pyridoxine-P
20	0·99	0·46
30	0·87	0·55
60	—	0·33
85	1·62	—
120	2·06	—
180	6·00	0·41

The cerebrum and the cerebellum were compared with reference to their isotope content per mg of tissue. The result in Fig. 3 shows that the vitamin was equally distributed between the cerebrum and the cerebellum.

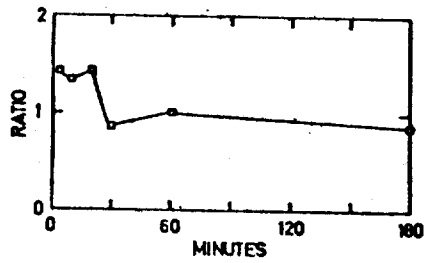

FIG. 3.—Ratio between radioactivity in 1 mg of the cerebrum and in 1 mg of the cerebellum at different times after the intravenous administration of [^{3}H]pyridoxine 5′-phosphate as in Fig. 1.

Metabolism of [^{3}H]pyridoxine in mouse brain. The distribution of tritium-labelled vitamin forms in the brain during the first 50 min after the intravenous injection of [^{3}H]pyridoxine is shown in Fig. 4. There was a rapid decrease in the relative amount of labelled pyridoxine; 10 min after the injection only 26 per cent of isotope was present in the pyridoxine fraction. Pyridoxine was phosphorylated to pyridoxine-P, which reached a maximum 20 min after the injection and then declined. Labelled pyridoxal-P appeared 10 min after the injection, when 17 per cent of isotope was detected in the pyridoxal-P fraction. After 40 min pyridoxal-P was the principal vitamin form present (61 per cent). Pyridoxamine-P seemed to increase rather slowly and during the first 30 min only minute amounts of tritium-labelled pyridoxamine-P could be detected.

In Fig. 5 the distribution of isotope between the different B_6 forms during the first 13 days after the injection of [^{3}H]pyridoxine is shown. The relative amount of labelled pyridoxal-P was rather constant around 50 per cent. An increase of isotope in the

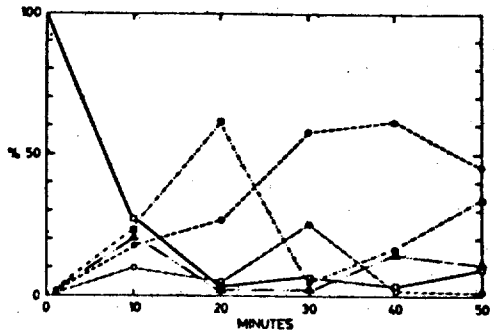

FIG. 4.—Distribution of isotope in mouse brain between the different forms of vitamin B_6 during the first 50 min after the intravenous administration of [^{3}H]pyridoxine (20 μg, 20 μCi). Pyridoxine (□), pyridoxal (○), pyridoxine 5'-phosphate (■), pyridocal 5'-phosphate (●), and pyridoxamine 5'-phosphate (▲).

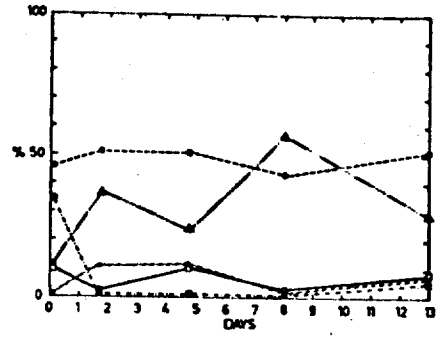

FIG. 5.—Distribution of isotope in mouse brain between the different forms of vitamin B_6 during the first 13 days after the intravenous administration of [^{3}H]pyridoxine (20 μg, 20 μCi). Key to symbols as in Fig. 4.

pyridoxamine-P fraction was observed during the first 2 days. Forty hours after the injection, 35 per cent of isotope appeared as pyridoxamine-P. However, the relative amount varied between 28 and 56 per cent from 2 to 13 days after the isotope administration. Pyridoxal could be found in most animals in small quantities, generally less than 10 per cent.

Metabolism of [^{3}H]pyridoxine-P in mouse brain. The distribution of isotope between the different vitamin forms during the first 4 h after the intravenous injection of [^{3}H] pyridoxine-P is presented in Fig. 6. The relative amount of tritium-labelled pyridoxine-P rapidly decreased during the first hour and no pyridoxine-P could be detected 4 h after the injection. Pyridoxal-P was the first vitamin form to increase and 20 min after the vitamin administration about 50 per cent of isotope was present in the pyridoxal-P fraction. Labelled pyridoxamine-P started to increase after about 40 min and 19 per cent of isotope was present in this fraction 4 h after the injection.

The conversion between different vitamin forms following the injection of [^{3}H] pyridoxine-P was followed during 7 days (Fig. 7). The two principal B_6-forms appeared

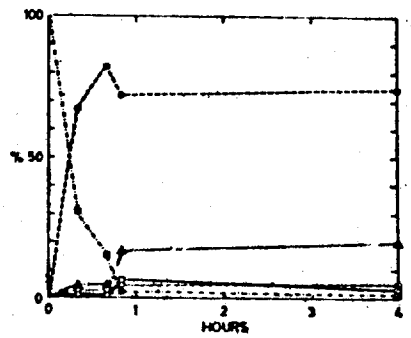

FIG. 6.—Distribution of isotope in mouse brain between the different forms of vitamin B_6 during the first 4 h after intravenous administration of [^{3}H]pyridoxine 5′-phosphate (10 μg, 36 μCi). Key to symbols as in Fig. 4.

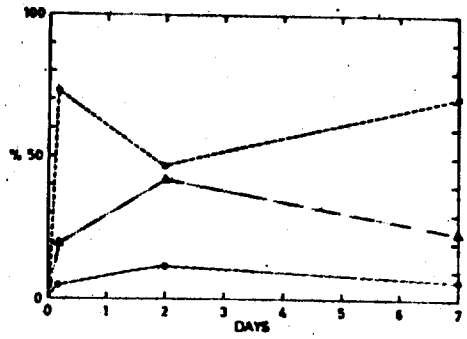

FIG. 7.—Distribution of isotope in mouse brain between the different forms of vitamin B_6 during the first 7 days after the intravenous administration of [^{3}H]pyridoxine 5′-phosphate (10 μg, 36 μCi). Key to symbols as in Fig. 4.

to be pyridoxal-P and pyridoxamine-P. On the seventh day after the administration 70 per cent of isotope could be accounted for as pyridoxal-P and 23 per cent as pyridoxamine-P. Not more than about 10 per cent of isotope was detected in the pyridoxal fraction.

DISCUSSION

In a recent work the distribution of vitamin B_6 between the different organs in the mouse was studied after the injection of tritium-labelled pyridoxine (DAHLQVIST *et al.*, 1969). Most of the isotope was recovered in liver and carcass, and only about 1 per cent could be detected in the brain 7 days after the administration. The high initial concentration found in most organs was not observed in the brain, where the isotope content increased during the first day to a level which seemed to remain unchanged during the first 7 days. COLOMBINI and MCCOY (1970) and CONTRACTOR and SHANE (1971) found a slower build-up of labelled vitamin in the brain than in other organs and related this to the existence of a moderate blood brain barrier.

These facts made it of interest to study in more detail the passage of the vitamin into the nervous system. The passage across the blood–CSF and the blood–brain barriers was studied by calculating the ratios between the isotope concentration in the different pools, thus avoiding inaccurate results due to differences in blood and CSF volumes, amounts of isotope injected and excretion rates of the vitamin in different animals. Rats were used in these experiments because it was almost impossible to obtain clear cerebrospinal fluid from mice.

The ratios of isotope concentration in serum to that of CSF were initially high, but decreased during the first hour to values near 1·0, after pyridoxine as well as pyridoxine-P injection. The passage of pyridoxine-P into the CSF appeared to be somewhat slower than for pyridoxine. The difference between the nonphosphorylated and the phosphorylated forms was significant when the uptake in the brain tissue was compared. It is known that one factor which influences the passage of a compound across the blood–brain barrier is its degree of ionization at physiological pH, i.e. the passage of an unionized compound is favoured over that of an ionized (RALL, STABENAU and ZUBROD, 1959; DOBBIN, 1961). Pyridoxine-P is a much stronger acid than pyridoxine and the observed difference may be due in part to this fact.

LYON, BAIN and WILLIAMS (1962) have found pyridoxal-P and pyridoxamine-P to be the dominating B_6-forms in mouse brain; the ratio between the two forms was about 1:2. They also found that pyridoxal-P decreased on a vitamin B_6-deficient diet more rapidly than pyridoxamine-P. Using [^{14}C]-labelled pyridoxine, COLOMBINI and MCCOY (1970) and CONTRACTOR and SHANE (1971) also reported pyridoxal-P and pyridoxamine-P to be the main vitamin forms in brain tissue. LOO and MACK (1971) investigated the subcellular distribution of vitamin forms in cerebral cortex of rats and found pyridoxamine-P to be the dominating vitamin form, having about twice the concentration of pyridoxal-P. In the present work pyridoxal-P and pyridoxamine-P accounted for 80 per cent or more of the recovered isotope after the initial period. The amount of isotope in the pyridoxal-P fraction was at most times slightly larger than in the pyridoxamine-P fraction. Thus the distribution between these two vitamin B_6-forms is similar to that previously found in liver and carcass (JOHANSSON, LINDSTEDT and TISELIUS, 1968).

Small amounts of pyridoxal were detected in most extracts. BRAUNSTEIN and BUKIN (1956) isolated an enzyme which converted pyridoxine to pyridoxal. The appearance of free pyridoxal might be a result of such a reaction, but could also be explained by activity of phosphatases or by nonenzymic hydrolysis *in vitro*. The presence of a pyridoxal-P phosphatase in brain has been reported by BEGUM and BACHHAWAT (1960) and it has been purified from human brain by SARASWATHI and BACHHAWAT (1962).

After the administration of pyridoxine there occurred a rapid phosphorylation to pyridoxine-P followed by conversion to pyridoxal-P and more slowly to pyridoxamine-P. The last two reactions occurred after administration of pyridoxine-P. A similar time sequence has previously been observed in liver after the administration of [^{3}H]pyridoxine (JOHANSSON *et al.*, 1968) and [^{3}H]pyridoxine-P (Tiselius, to be published).

McCormick and co-workers (MCCORMICK and SNELL, 1961; MCCORMICK, GREGORY and SNELL, 1961) have studied the vitamin B_6 phosphokinase from brain. In the rat brain the activity is only slightly lower than in liver and kidney. The affinity of beef

brain phosphokinase for the substrate decreased in the order pyridoxal → pyridoxine → pyridoxamine. Several authors have reported studies on vitamin B_6 phosphokinase: in mouse brain (TSUBOSAKA and MAKINO, 1969), in rabbit brain (EBADI, MCCOY and KUGEL, 1970) and in bovine brain (NEARY and DIVEN, 1970). The enzymic conversion of pyridoxine-P to pyridoxal-P has been studied in some detail in liver (POGELL, 1958; WADA and SNELL, 1961), but to our knowledge not in brain. Comparison of the conversion of pyridoxine-P to pyridoxal-P in liver and brain *in vivo* shows a similar time course for the reaction in the two tissues (JOHANSSON *et al.*, 1968). Pyridoxal-P was shown to be converted to pyridoxamine-P by enzymic transamination (WADA and SNELL, 1962).

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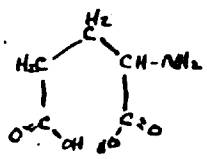
THE MONSTER-PRODUCING ACTION OF GLUTAMIC ACID

By

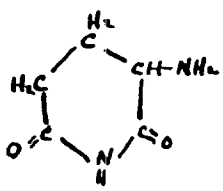
S. Tugrul

Paper received on 2/29/64

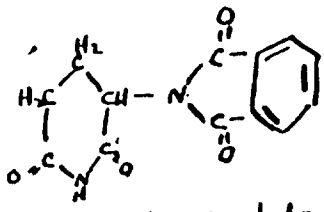
Thalidomide, whose chemical structure is shown below, is a derivative of glutamic acid and exercises a depressive effect on the central nervous system.



Glutamic Acid



Glutarimide



d- Phthalimido glutarimide
Thalidomide

This product, synthesized and studied from the pharmacological viewpoint in 1956, is said to be a sedative and hypnotic, not causing ~~the same~~ fatal toxic effect of strong doses administered by mouth, but certainly carried over to a state of sleep and severe migraine (18, 12, 16, 3). It elevates the threshhold of pain when it is administered with analgesics (10).

It potentializes the effects of depressive substances on the central nervous system, particularly those of the barbiturates, and it antagonizes stimulants of the central nervous system (17, 18,1,13).

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Several congenital anomalies have been observed which generally are related to the long bones of new babies born to women who have taken Thalidomide at the beginning of their pregnancy. Also observed have been abortions and fetal resorptions in the case of such women (4,16).

Preparations of glutamic acid ($HOOC-(NH_2)CH-CH_2-COOH$) are used in our clinic for well-defined therapeutic purposes such as states of hypoacidity, certain types of epilepsy (petit mal), various mental difficulties, etc. (15,6,8,19,1) without mention being made of the relative harm in administering this product to pregnant women or to those who are apt to become pregnant.

Once knowing the effects of Thalidomide on the fetus, we proposed to conduct research on glutamic acid--so commonly employed in different instances--to determine whether or not it is a monster-forming product.

Material and Methods

Experiments have been carried out on 28 rabbits, and at the same time, 28 other rabbits (20 females and 8 males) have served as controls during the experiments. The two groups have been subjected to normal nutritional conditions. The rabbits reserved for experimentation have been divided into three groups.

First Group. 14 rabbits (10 females, 4 males). The females only received glutamic acid. We have taken into account in the administration of this compound the case of females being in a state of pregnancy, and this is the reason only 2 out of 10 females were at the beginning of pregnancy; the other were in a

period of pre-pregnancy.

Second Group. 6 rabbits (4 females and 2 males). Both females and males received glutamic acid in combination with vitamin B6 for the following reasons:

a) Glutamic acid is administered in practice to males as well as females, and it is possible to obtain different results in connection with fertilization.

b) Vitamin B6 facilitates the transformation of glutamic acid to GABA and, in the case of a deficiency of this vitamin, it is possible to produce deformities in the following generation in connection with the response of the ovaries to gonadotropic hormones (7,11,22,14).

Third Group. 8 rabbits (6 females and 2 males). Glutamic acid has been administered alone in the case of the two sexes in order to compare the independent effects of vitamin B6. For the third group we have used Glutamidine,which is a specialty pharmaceutical based on the hydrochloride of glutamic acid, administered by mouth in doses of 25 mg/kg in the form of a pellet in caoutchouc.

We prefer the "by mouth" method because of the poor solubility of Thalidomide and glutamic acid in water and because ~~its~~ this therapeutic course is the one most frequently employed. Absorption of the product during its passage through the gastro-intestinal tract is rather easily accomplished.

Glutamidine has been used during a period of 40 ~~hours~~ days administering the product during 4 and 1 ~~hour~~ day intervals, being a

a total of 27 hours, at a rate of 25 mg/kg., vitamin B6 at the rate of 25 mg/kg subcutaneously.

At the conclusion of the experiment, the treated animals and their offspring have been observed from a morphological point of view, from an anatomic-pathological viewpoint, and by x-ray of all of the organs, systems, bones, and endocrine glands.

Results

First Group. The autopsy of 10 female and 4 male animals which had continued to live a normal communal life showed, at the end of the period of administration of glutamic acid, that 2 rabbits were in a state of pregnancy but the pregnancy was "arrested". At the uterine level there were diverse calcareous deposits in the region corresponding to the endometrium and infiltration of these elements as far as the myometrium. Finally, there was found a degenerated fetus in the interior of the uterus.

In the case of 2 other rabbits, there was found post-abortive endometritis of the uterus.

The abortus observed with Glutamidine (Fig. 1) corresponds to the observations with Thalidomide (4).

Two rabbits were delivered of young at a normal time, but the offspring had various malformations (Fig. 2). In the case of the two rabbits who did not deliver, hyperplastic uteri were found.

Regarding the young from this group, no pregnancies were observed during 7 months in the case of these offspring subjected to communal life two months after their birth. But we have been able to observe at the end of this period: a number of monstrosities

particularly of the extremities, which were long or short, of pathological form and position, and which caused the animals to use these organs with great difficulty (Fig. 3). This observation has been the same in the case of newly-born animals in the other groups subjected to the experiment. Curvature of the spine has been observed and accentuated in proportion to growth (Fig. 4)(4). The growth and development are sharply retarded compared to the control animals (Fig. 5).

Anatomic-pathological examination has shown serious atrophy of the adipose tissue, hyperplasia of the adrenal capsules, increase in the basophilic cells of the adenohypophysis, atrophy of the testes, and a cessation of spermatogenesis, and atrophy of the uterine endometrium.

Second Group. Composed of 4 female rabbits and 2 males living a communal life.

Two rabbits gave birth and their offspring showed monstrosities in the form of deformation of the extremities. (Fig.5). The three offspring died during their first hours; no abortions were observed in the animals of this group.

Anatomic-pathological examination of the animals having received glutamic acid showed: hyperplasia of the uterus and inhibition of spermatogenesis.

In the case of the offspring: Hyperplasia of the adrenal glands, increase in basophilic cells of the adenohypophysis, serious atrophy of the adipose tissue, atrophy of the testicles, arrestation of spermatogenesis, and atrophy of the uterine endometrium.

Third Group. Composed of six females and two males who lived a normal communal life.

Three rabbits were delivered of young. Distortions of the extremities and growth impairment was observed (Figure 8(. The other three rabbits did not deliver young. Pathological examination of the animals receiving the medication showed: uterine hyperplasia and inhibition of spermatogenesis in the case of testicles.

In the case of the offspring:

Serious atrophy of the adipose tissue, hyperplasia of the adrenals, atrophy of the endometrium of the uterus, atrophy of the testicles, and arrestation of spermatogenesis. Radiological examination of the long bones showed them to be more slender, their development mediocre, local deformities of the tendons, differences in symmetry of the two extremities of the same animal (Fig. 9(.

Autopsy of the young revealed almost total disappearance of adipose tissue of the whole organism.

Discussion

Our object was to investigate whether glutamic acid, of which ~~is a derivative of~~ Thalidomide is a derivative, has a monster-producing effect analogous to that of Thalidomide (4,20). Glutamidine was administered by mouth in doses of 25 mg/kg to three different groups of rabbits.

In the first group, Glutamidine was administered only to the females. It was this group which presented the anomalies resembling most those of Thalidomide when Glutamidine was administered in this manner to females in a pregnant condition.

Abortion and the arrest of pregnancy was observed only in this group.

We have thought that this state of affairs perhaps was a result of the presence of enzymatic transformation products of glutamic acid in the blood of father and mother (17), being a result of myotic intoxication (4).

Regarding the following generation, one observes mediocre growth of the long bones, a lengthening or shortening in comparison to the normal, hyperplasia of the adrenals and of the uterus, an atrophy of the testes, etc. These observations can arise by an alteration of the activity of oxaloacetic-glutamic transaminase and of pyruvic-glutamic transaminase on the organs of the systems (2,5) and the same on the genes, being an augmentation of the basophylic cells of the adenohypophysis (2,5,21,3).

This reasoning is confirmed by the fact that in the case of hereditary muscular atrophy, the long bones are shorter in comparison with the normal and the activity of oxaloacetic-glutamic transaminase is augmented. Never-the less, one might--with equal justification--think of these modifications of enzymatic activity (still not determined at this time) and also explain the observed results.

In the case of the offspring of the animals of the first group, congenital anomalies and malformations have been observed which (since they have been encountered in the two other groups) have been interpreted as being the toxic and monster-forming effect of glutamic acid administered over a long period.

In the second group, Glutamidine has been administered in association with vitamin B6 to males as well as females. Abortions and arrest of pregnancy at its onset has not been observed, but malformations and pathological results have been the same as those observed in the first and third groups.

In the third group, Glutamidine alone was administered to males and females, but the results parallel those of the second group in the case of the mothers and their young. Also the experiments have not revealed any beneficial or preventitive effect with the compound alone compared to administered in association with vitamin B6.

The effect of Glutamidine on the bone marrow merited a particular examination.

The harmful effects have not been observed on the nervous tissue of the mothers and offspring except as hyperemia. Hyperemia of all of the organs of the animals of the three groups arises as the inhibitive effect of glutamic acid on thrombokinase (8,9).

Disappearance of almost all fatty tissue of the offspring in all groups is due to the adsorption of glutamic acid to the lipid fraction of the liver and to the serum lipoproteins, being a mobilization of the lipids following the metabolism of glutamic acid itself, and an enzymatic transformation of the level of the fatty tissue (9) and finally a direct action on lipogenesis.

Inhibition of growth is related to augmentation of the basophils of the adenohypophysis (20).

The extent of mortality of the offspring and its relation to the organ malformations is distinctly greater than that of the controls.

The main difference between the groups is ~~xxxxxxxxx~~ evident when Glutamidine is administered to males at the same time it is given to females (first group).

In comparing reproduction between the control rabbits and the three groups comprising these experiments, one can observe that the amount of growth in the case of the latter is 30% less than that of the controls.

k This result perhaps can be explained by inhibition of spermatogenesis and by endometrial hyperplasia.

Malformations of the offspring of the second and third groups are less accentuated than those of the first group.

These experiments are presented as evidence of the monster-forming effect of glutamic acid and foreshadow modifications in future generations

of those receiving the product over a period of time which causes the effects of this substance on spermatogenesis, on the uterus, and on the adrenals and the hypophysis.

Resume

We have investigated the monster-forming effects of glutamic acid administered over a continuing period to parents and to their offspring, and we have used the rabbit has the experimental animal.

Summary

The administration of glutamic acid (25 mg/kg. per os) for one month to breeding rabbits was found to provoke the following changes: (1) in the parents: hyperplasia of the adrenals and endometrium, inhibition of spermatogenesis, generalized hyperemia, lowered reproduction rate, resorption of the foetus and abortion; (2) in the youngs: hyperplasia of the adrenals, increased basophils concentration in the adenohypophysis, atrophy of the endometrium and of the testes, blockade of spermatogenesis, atrophy of fat tissues, muscular anomalies, atrophy of bone tissue, resulting in malformations of limb bones and poor growth.

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L'auteur rémercie M. le Professeur A. Akçasu pour sa collaboration et ses encouragements.

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CAPTIONS FOR FIGURES

1. Dead fetus observed from an animal which had taken glutamic acid in continuing doses of 25 mg/kg/day (orally).

2. Malformations observed in the case of offspring of female rabbit on continuing doses of glutamic acid of 25 mg/kg/day, orally.

3. Hind extremity 1.5 times longer and unusable of offspring of mother receiving continuing doses of glutamic acid of 25 mg/kg/day.

4. Curvature of the spine during growth of a small deformed rabbit whose mother received continuing doses of glutamic acid of 25 mg/kg/day.

5. Left. Malformed hind extremities of a small rabbit born of parents receiving glutamic acid and vitamin B6 in continuing doses of 25 mg/kg/day. Right. Small rabbit born of the same group and showing malformations of the front paws.

6. Differences in development of baby rabbit (at left) born of parents receiving glutamic acid and vitamin B6 (25 mg/kg/day) compared with normal animal (right).

7. Radiological examination of the long bone of the control animal (right) and of offspring from parents receiving continuing doses of glutamic acid of 25 mg/kg/day.

8. Mediocre growth of offspring arising from parents receiving continuing doses of glutamic acid of 25 mg/kg/day.

9. Radiological comparison of the control animal (right) with rabbit of parents receiving glutamic acid at rate of 25 mg/kg/day.

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NSTITUT DE PHARMACOLOGIE, FACULTÉ DE MÉDECINE, UNIVERSITÉ DE L'ÉGÉE, IZMIR, TURQUIE

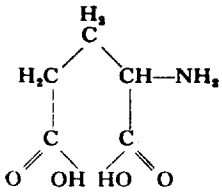
ACTION TERATOGÈNE DE L'ACIDE GLUTAMIQUE

PAR

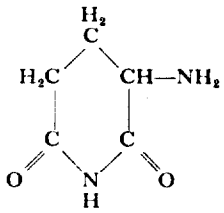
S. TUGRUL

(*Travail reçu le 29-2-1964*).

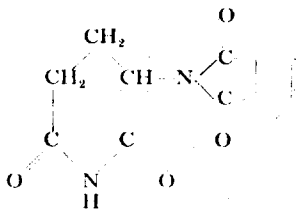
La « Thalidomide », dont la structure chimique est représentée ci-dessous, est un dérivé de l'Acide glutamique et exerce un effet dépressif sur le système nerveux central.



Acide Glutamique



Glutarimide



α-Phtalimido-Glutarimide
Thalidomide

Ce produit, synthétisé et etudié du point de vue pharmacologique en 1956, s'est avéré être un sédatif et hypnotique, ne causant pas d'effet toxique mortel même aux fortes doses administrées per os, qui n'entraînent qu'un état de sommeil et de fortes migraines (18, 12, 16, 3). Il élève le seuil de la douleur lorsqu'il est administré avec les analgésiques (10).

Il potentialise les effets des produits dépresseurs sur le système nerveux central, particulièrement ceux des barbituriques, et antagonise les stimulants du système nerveux central (17, 18, 1, 13).

Il a été observé des anomalies congénitales relatives surtout aux os longs, chez les nouveaux nés de femmes ayant pris la Thalidomide aux début de leur grossesse, ainsi que des cas d'abortus et de résorbtion foetale chez ces mêmes femmes (4, 16).

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Les préparations d'acide glutamique (HOOC. (H_2N) CH_2 .CH_2. COOH) sont employeés de nos jours en clinique dans certains buts thérapeutiques bien définis, tels qu'états d'hypoacidité, épilepsie petit mal, troubles mentaux divers, etc. (15, 6, 8, 19, 1), sans que mention soit faite des inconvénients relatifs à l'administration de ce produit aux femmes enceintes ou à celles qui sont susceptibles de l'être.

Une fois connus les effets de la Thalidomide sur le foetus, nous nous sommes proposés de rechercher si l'acide glutamique — si couramment employé dans différents buts — était ou non un produit tératogène.

Matériel et Méthode

Les expériences ont été réalisées sur 28 lapins, et parallèlement 28 autres lapins (20 femelles et 8 males) ont servi de témoins pendant les expériences. Les deux groupes ont été soumis aux conditions normales de nutrition.

Les lapins réservés à l'expérimentation avec l'acide glutamique ont été répartis en trois groupes.

Premier groupe : 14 lapins (10 femelles, 4 mâles). Les femelles seules ont reçu l'acide glutamique. Nous avons tenu compte de l'administration de ce produit chez les femelles avant l'état de grossesse, et c'est la raison pour laquelle seulement 2/10 lapins femelles étaint en début de grossesse, les autres étant en période d'avant-grossesse.

Deuxième groupe : 6 lapins (4 femelles, 2 mâles). Femelles et mâles ont reçu l'acide glutamique, en combinaison avec la vitamine B6, et ce pour les raisons suivantes :

a) l'acide glutamique est administré en pratique aux mâles aussi bien qu'aux femelles, et il pourrait y avoir des résultats différents en rapport avec la fécondation;

b) la vitamine B6 facilite la transformation de l'acide glutamique en GABA, et en état de carence de cette vitamine il peut se produire des déformations de la génération suivante en rapport avec la réponse des ovaires aux hormones gonadotropes (7, 11, 22, 14).

Troisième groupe : 8 lapins (6 femelles, 2 mâles). L'acide glutamique a été administré tout seul chez les deux sexes, ceci pour comparer les effets indépendamment de la vitamine B6. Pour les trois groupes nous avons utilisé la Glutamidine, qui est une spécialité pharmaceutique à base de chlorhydrate d'acide glutamique, administrée per os en doses de 25 mg/kg et au moyen d'une sonde mince en caoutchouc.

Nous avons préféré la voie per os, malgré la solubilité médiocre de la Thalidomide et de l'acide glutamique dans l'eau, puisqu'en thérapeutique courante c'est la voie la plus employée, et que l'absorption du produit durant son cheminement gastro-intestinal s'effectue assez facilement (13, 4).

La glutamidine a été employée pendant une durée de 40 jours, en administrant le produit pendant 4 jours et 1 jour d'intervalle, soit un total de 27 jours, à raison de 25 mg/kg, la vitamine B6 à raison de 25 mg/kg, par voie sous-cutanée.

A la fin de l'expérience, les animaux traités et leurs petits ont été observés, soit du point de vue morphologique, soit du point de vue anatomo-pathologique et radiologique (tous les organes, systèmes et osseux et glandes endocrines).

Resultats

Premier groupe. L'autopsie des 10 animaux femelles et 4 mâles avaient continué leur vie commune normale a montré qu'à la fin d

période d'administration de l'acide glutamique, deux lapins étaient en état de grossesse, mais de grossesse « arrêtée ».

Au niveau de l'utérus : diverses accumulations calcaires, dans les parties correspondant à l'endométrium, infiltration des éléments choriaux jusqu'au myometrium, et enfin le foetus dégénéré à l'intérieur de l'utérus.

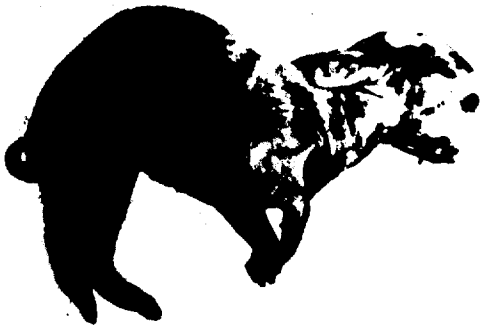


FIG. 1

Mort foetale observée chez les animaux ayant pris l'acide glutamique en doses continues de 25 mg/kg p.o. par jour.

Chez deux autres lapins : Endométrite postabortive de l'utérus.

L'abortus observé avec la Glutamidine (FIGURE 1) rejoint les observations avec la Thalidomide (4).

Deux lapins ont accouché en temps normal, mais leurs petits ont présenté diverses malformations (FIGURE 2).

a *b*

FIG. 2

Malformations observées chez les petits des lapins femelles ayant pris l'acide glutamique en doses continues de 25 mg/kg p.o. par jour.

Chez les lapins femelles n'ayant pas accouché : hyperplasie utérine.

Chez les petits provenant de ce groupe : Pas de grossesse observée pendant 7 mois chez ces petits soumis à une vie commune deux mois après leur naissance. Mais nous avons pu noter à la fin de cette période :

FIG. 3

Extrémité arrière 1,5 fois plus longue et presque inutilisable (petit dont la mère a reçu l'acide glutamique en doses continues de 25 mg/kg p.o. par jour).

de monstruosités, surtout aux extrémités, qui étaient longues ou courtes, de formes et positions pathologiques, les animaux pouvant se servir très difficilement de ces organes (FIGURE 3); cette observation a été la même chez les animaux nouveaux-nés des autres groupes soumis

FIG. 4

Scoliose formée pendant la croissance d'un petit lapin malformé (la mère ayant reçu l'acide glutamique en doses continues de 25 mg/kg p.o. par jour).

à l'expérience; la scoliose a été observée et accentuée au fur et à mesure de la croissance (FIGURE 4) (4); la croissance et le développement sont restés nettement inférieurs à ceux des animaux témoins (FIGURE 5).

L'examen anatomo-pathologique a montré : une atrophie séreuse du tissu adipeux, une hyperplasie des capsules surrénales, une augmen-

testicules et un arrêt de la spermatogénèse, une atrophie de l'endométrium de l'uterus.

FIG. 5

A gauche : Extrémités arrières malformées d'un petit lapin né de parents ayant pris de l'acide glutamique et de la Vit. B_6 s.c. en doses continues de 25 mg/kg par jour.

A droite : *b* : Petit lapin né du même groupe et présentant des malformations aux pattes avant.

Deuxième groupe. Composé de 4 lapins femelles et 2 mâles, ayant continué leur vie en commun.

Deux lapins ont accouché et les petits présentaient des monstruosités sous forme de déformation des extrémités (FIGURE 5) Les trois petits

FIG. 6

Différence de développement d'un petit (à gauche) né de parents ayant reçu l'acide glutamique et la Vit. B_6 (25 mg/kg par jour) comparé à celui d'un animal normal (à droite).

sont morts dans les premiers jours, pas d'abortion observée chez les animaux de ce groupe.

Développement médiocre des petits (FIGURE 6). L'examen comparatif des os longs a montré un amincissement évident de ces os (FIGURE 7).

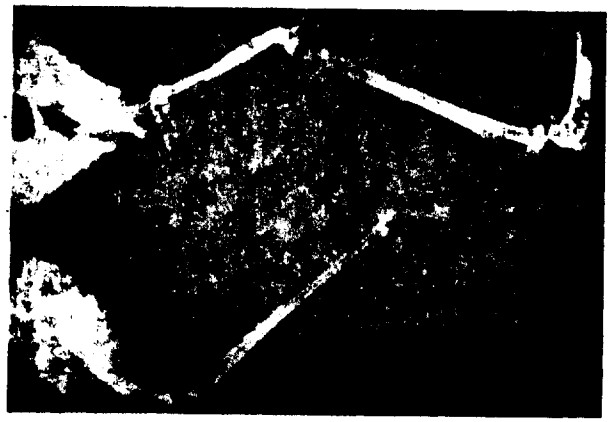


FIG. 7

Examen radiologique comparatif des os longs des témoins (à droite) et des petits provenant des parents ayant pris l'acide glutamique en doses continues de 25 mg/kg par jour.

L'examen anatomo-pathologique des animaux ayant pris l'acide glutamique a montré : une hyperplasie de l'utérus, une inhibition de la spermatogénèse;

Chez les petits :

hyperplasie des surrénales, augmentation des cellules basophiles à l'adéno hypophyse, atrophie séreuse du tissu adipeux, atrophie des

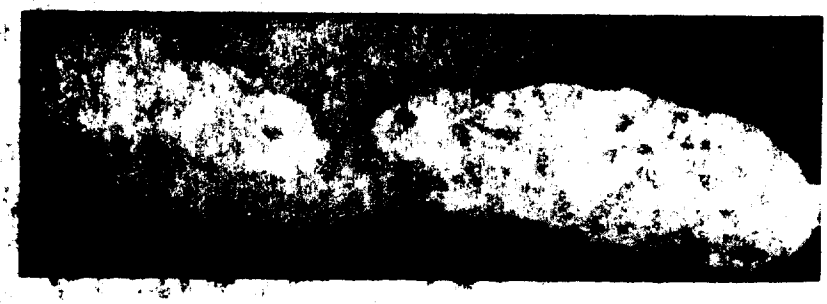


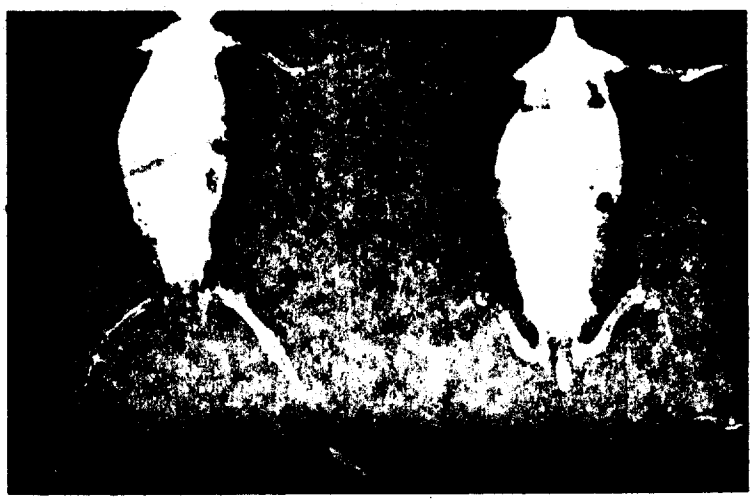
FIG. 8

[illegible] ayant reçu l'acide glutamique en

testicules et arrêt de la spermatogénèse, atrophie de l'endométrium de l'utérus.

Troisième groupe. Composé de 6 lapins femelles et 2 mâles ayant continué leur vie normale en commun.

Trois lapins ont accouché, déformation aux extrémités des petits et croissance (FIGURE 8). Les autres trois lapins n'ont pas accouché. L'examen pathologique des animaux ayant pris le médicament a montré : une hyperplasie de l'utérus, une inhibition de la Spermatogénèse aux testicules;



a *b*

FIG. 9

Comparaison radiologique des témoins (à droite) avec les lapins provenant de parents ayant pris de l'acide glutamique (25 mg/kg par jour).

Chez les petits :

atrophie séreuse du tissu adipeux, hyperplasie des surrénales, atrophie de l'endrométrium de l'utérus, atrophie des testicules et arrêt de la spermatogénèse, à l'examen radiologique les os longs étaient plus minces, leur développement médiocre, déformations locales du tissu compact, différence de structure aux deux extrémités symétriques du même animal (FIGURE 9).

L'autopsie des petits a révélé la disparition quasi totale du tissu [illegible] de tout l'organisme.

Discussion

Notre but était de rechercher si l'acide glutamique, dont la Thalidomide est un dérivé, avait des effets tératogènes analogues à ceux de la Thalidomide (4, 20). La Glutamidine a donc été administrée per os en doses de 25 mg/kg à 3 groupes différents de lapins.

Dans le premier groupe, la Glutamidine était administrée uniquement aux femelles. C'est ce groupe qui a présenté les anomalies ressemblant le plus à celles de la Thalidomide, lorsque la Glutamidine fut administrée d'une façon continue aux femelles en état de grossesse.

L'abortion et l'arrêt de la grossesse dans ses débuts a été observée seulement dans ce groupe.

Nous avons pensé que cet état de choses peut être le résultat soit de la présence de produits de transformation enzymatique de l'acide glutamique, dans le sang du père et de la mère (17), soit le résultat d'une intoxication myotique (4).

Quant à la génération suivante, on a pu observer une croissance médiocre, des os longs allongés ou raccourcis par rapport à la normale, une hyperplasie des surrénales et de l'utérus, une atrophie des testicules etc... Ces observations peuvent provenir, soit d'une altération de l'activité de la transaminase oxalo-acétique glutamique et de la transaminase pyruvique-glutamique, sur les organes et les systèmes (2, 5) et même sur les gènes, soit de l'augmentation des cellules basophiles de l'adéno-hypophyse (2, 5, 21, 3).

Ce raisonnement se voit confirmé par le fait que dans le cas d'atrophie musculaire héréditaire les os longs sont plus courts par rapport à la normale, et l'activité de la transaminase oxalo-acétique glutamique se trouve augmentée. Toutefois, on peut également penser à des modifications d'activité enzymatiques non déterminées jusqu'à ce jours, et expliquer ainsi les résultats observés.

Chez les petits provenant des animaux du premier groupe on avait observé des anomalies et malformations congénitales qui, puisqu'elles se rencontrent les deux autres groupes, ont été interpretées comme étant des effets nocifs et tératogènes de l'acide glutamique administré pendant une longue période.

Dans le deuxième groupe, on avait administré la Glutamidine, en association avec la vitamine B6, aux mâles aussi bien qu'aux femelles. Il n'a pas été observé d'abortus et d'arrêt de la grossesse à ses débuts, mais les malformations et les résultats pathologiques ont été les mêmes

Dans le troisième groupe, la Glutamidine seule fut administrée aux mâles et aux femelles, mais les résultats furent pareils à ceux du deuxième groupe, tant chez les mères que chez leurs petits. Aussi des expériences n'ont-t-elles pu révéler un effet bénéfique ou préventif indiscutables dûs a l'association de cette vitamine.

L'effet de la Glutamidine sur la moelle osseuse mérite un examen particulier.

On n'a pas observé des effets nocifs sur le tissu nerveux des mères et des petits, sauf de l'hyperémie.

L'hyperémie de tous les organes des animaux des trois groupes proviendrait de l'effet inhibiteur de l'acide glutamique sur la thrombokinase (8, 9).

La disparation quasi totale du tissu adipeux chez les petits de tous les groupes est dûe, soit à l'adhérence de l'acide glutamique à la fraction lipidique du foie et aux lipoprotéines du sérum, soit à la mobilisation des lipides pendant la métabolisation de l'acide glutamique lui-même, soit encore à des transformations enzymatiques au niveau du tissus adipeux (9), soit enfin à une action directe sur la lipogénèse.

L'inhibition de la croissance est en relation avec l'augmentation des basophiles de l'adénohypophyse (20).

Le taux de mortalité des petits à mettre en relation avec les malformations des organes, est nettement supérieur à celui des témoins.

La différence capitale entre les groupes est évidente lorsqu'on administre la Glutamidine aux mâles en même temps qu'aux femelles (premier groupe).

En comparant la reproduction entre les lapins-témoins et les trois groupes soumis à l'expérience, on observe que le taux des naissances chez ces derniers est inférieur de 30 pour cent par rapport aux témoins.

Ce résultat peut être expliqué par l'inhibition de la spermatogénèse et par l'hyperplasie de l'endométrium.

Les malformations des petits des deuxième et troisième groupes sont moins accentuées que celles du premier groupe.

Ces expériences ont mis en évidence l'effet tératogène de l'acide glutamique et montré que l'on peut prévoir des modifications dans les générations futures de ceux qui reçoivent le produit d'une façon continue, ceci a cause des effets de ce produit sur la spermatogénèse, l'utérus, les surrénales et l'hypophyse.

Résumé

Nous avons recherché les effets tératogènes de l'acide glutamique administré pendant une période continue aux parents et leurs petits, en nous servant du lapin comme animal d'expérience. Ces expériences ont montré : a) chez les parents : Hyperplasie des surrénales et de l'endométrium utérin, inhibition de la spermatogénèse, hyperémie de tous les organes, chute de la reproduction de 30 %, abortus et résorption foetale; b) chez les petits : Hyperplasie des surrénales, augmentation des basophiles de l'adénohypophyse, atrophie de l'endométrium, atrophie des testicules et arrêt de la spermatogénèse, atrophie du tissu adipeux, anomalies du tissu musculaire, atrophie du tissu osseux en général, suivie de malformations des os longs et croissance médiocre.

Summary

The administration of glutamic acid (25 mg/kg, per os) for one month to breeding rabbits was found to provoke the following changes : (1) in the parents : hyperplasia of the adrenals and endometrium, inhibition of spermatogenesis, generalized hyperemia, lowered reproduction rate, resorption of the foetus and abortion; (2) in the youngs : hyperplasia of the adrenals, increased basophils concentration in the adenohypophysis, atrophy of the endometrium and of the testes, blockade of spermatogenesis, atrophy of fat tissues, muscular anomalies, atrophy of bone tissue, resulting in malformations of limb bones and poor growth.

Remerciements

L'auteur rémercie M. le Professeur A. Akçasu pour sa collaboration et ses encouragements.

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STUDIES ON THE TOXICITY AND PHARMACOLOGY OF VITAMIN B_6 (2-METHYL-3-HYDROXY-4,5-BIS-(HYDROXYMETHYL)-PYRIDINE)

KLAUS UNNA

WITH THE TECHNICAL ASSISTANCE OF JOSEPH GRESLIN

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György (1, 2), in nutritional studies on rats, demonstrated that a characteristic dematitis for which he coined the name "rat acrodynia" is due to a new factor of the vitamin B complex. This factor, which he named vitamin B_6, has been isolated (3, 4, 5, 6) from rice bran and yeast and identified as 2-methyl-3-hydroxy-4,5-bis-(hydroxymethyl)-pyridine (Pyridoxine); its synthesis was accomplished in 1939 by Harris and Folkers (7) and simultaneously by Kuhn and coworkers (8).

The importance of vitamin B_6 in the nutrition of animal species other than the rat has been pointed out by several investigators. Fouts, Helmer, Lepkovsky, and Jukes (9, 10, 11) showed that dogs maintained on a diet free of vitamin B_6 develop a microcytic, hypochromic anemia which is not relieved by iron but responds readily to the administration of vitamin B_6. These results have been confirmed by McKibbon, Madden, Black and Elvehjem (12). The rôle of vitamin B_6 in the nutrition of pigs has been suggested by the work of Chick et al. (13) and Wintrobe (14), and Jukes (15) has established the need for this vitamin in the chick. Recent reports (16, 17, 18, 19) indicate the necessity for vitamin B_6 in the nutrition of man.

Synthetic material was used exclusively in the present investigation since we found in a previous study (20) on B_6 deficient rats that synthetic and pure natural vitamin B_6 are identical in their physiological actions. The vitamin both as the free base and in the form of the hydrochloride is readily soluble in water. Solutions of the base are neutral in reaction, whereas the pH of a 10 per cent solution of vitamin B_6 hydrochloride is 2.5. Hence, in experiments which required large amounts of vitamin B_6, the base rather than the hydrochloride was used to avoid complications from the high acidity of the solution.

ACUTE TOXICITY

Acute toxicity was studied in rats, rabbits and dogs.

Figure 1 shows the toxicity curves of vitamin B_6 and its hydrochloride in rats following subcutaneous and oral administration, 10 rats weighing between

120 and 150 grams being used for each dose level. The L.D. 50 following subcutaneous administration is 3.1 grams per kilogram for the vitamin B_6 base and 3.7 grams per kilogram for the hydrochloride. This difference in toxicity corresponds with the difference in molecular weight. Following oral administration, the L.D. 50 is 5.5 grams per kilogram for vitamin B_6 hydrochloride.[1] The difference between oral and subcutaneous toxicity is slight and indicates that the vitamin is readily absorbed from the gastrointestinal tract.

Doses up to 1 gram per kilogram were tolerated by rats, rabbits, and dogs without untoward effects. Larger doses produced rather peculiar delayed toxic symptoms which were similar in all three species. These manifestations began with marked impairment of the coördination and of the righting reflexes, occurring two or three days after administration of the drug, and pro-

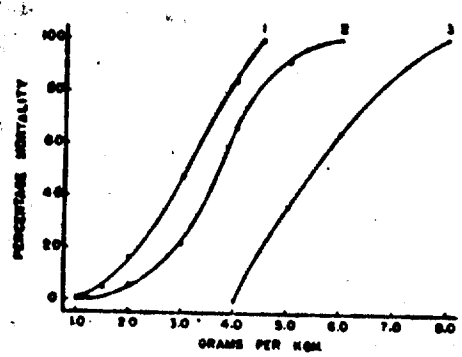

FIG. 1. TOXICITY OF VITAMIN B_6 IN RATS
1. Vitamin B_6 (base) subcutaneously. 2. Vitamin B_6 hydrochloride subcutaneously. 3. Vitamin B_6 hydrochloride orally.

gressing through severe convulsions to death in the stage of paralysis. Following a dose of 1 gram per kilogram, dogs showed an ataxic gait, but recovered slowly within one week. Rats receiving doses of 4 to 6 grams per kilogram showed convulsions of a tonic type, during which the hindlimbs were rigidly extended and the forelimbs mostly bent under the body. Opisthotonus was frequently observed. Between attacks the rats were able to crawl and to take some food. Animals which survived these toxic doses exhibited convulsive seizures over a period of 1 or 2 weeks. Autopsies revealed enlargement of the adrenals with an occasional animal showing hemorrhages into the cortex of this organ. Since the type of toxic manifestations suggest involvement of certain parts of the brain, neuropathological studies are being carried out and the results will be published elsewhere by Dr. William Antopol.

[1] These values are in agreement with our previous findings (21) recently confirmed by Weigand, Eckler and Chen (22).

The low toxicity of vitamin B_6 agrees well with the low toxicity recorded of other members of the B complex, as thiamin (23), riboflavin (24), and nicotinic acid (25). However, vitamin B_6 is the only member of this group which produces characteristic toxic manifestations when given in excessive doses.

CHRONIC TOXICITY

Chronic toxicity was studied in rats, dogs, and monkeys. Daily doses of 0.5, 1.0 and 2.5 mgm. respectively were fed to young rats in groups of 15 over a period of 80 days. The animals developed normally and their weights increased at the same rate as those of a control group (table 1). Autopsies at the end of the feeding period did not show any gross or microscopic changes

TABLE 1

Growth of young rats receiving 2.5 mgm. of vitamin B_6 daily by mouth

	AVERAGE WEIGHT							
	at start		20 days		50 days		80 days	
	Males	Females	Males	Females	Males	Females	Males	Females
	grams	grams	grams	grams	grams	grams	grams	grams
Stock diet only. 14 rats: 6 males, 8 females. Control	27	27	79	72	193	136	267	186
2.5 mgm. vitamin B_6 daily. 13 rats: 9 males, 4 females. 1st generation	29	28	86	76	177	137	262	177
2.5 mgm. vitamin B_6 daily. 13 rats: 7 males, 6 females. 2nd generation	32	32	81	74	183	136	252	181
2.5 mgm. vitamin B_6 daily. 19 rats: 8 males, 11 females. 3rd generation	34	32	85	79	193	144	267	184

in the organs. A group of 6 animals, 3 males and 3 females, were kept to maturity on a daily dose of 2.5 mgm. Two litters, one of 5 and one of 8, averaging 5 grams birth weight, were obtained. At 21 days of age, daily feeding of 2.5 mgm. was started. The weight record of this group (2nd generation) shows no significant difference from that of the parent generation. Three litters of the 2nd generation were again fed daily with 2.5 mgm. of vitamin B_6 and this group also developed normally (3rd generation).

Another group of 20 young rats was fed daily 25 mgm. of the vitamin over a period of 100 days. No toxic symptoms were observed. The growth rate of this group was practically the same as that of the animals receiving 2.5 mgm. reported in table 1.

A group of 3 litter-mate puppies received 20 mgm. of B_6 per kilogram by

mouth for a period of 80 days. Their weight increase, as compared to a littermate control dog, was normal. Hemoglobin, erythrocyte, leucocyte, and differential blood counts taken at weekly intervals gave no evidence of significant changes. Histological examination at the end of the feeding period revealed no pathological changes.

One monkey weighing 3 kgm. was given orally 10 mgm. per kilogram daily over a period of 39 days. A second monkey (weight 2.8 kgm.) was injected subcutaneously with the same dose of the vitamin daily for 106 days. Neither animal evidenced any toxic symptoms. Hemoglobin and blood cell counts, as well as the differential picture of the sternal bone marrow obtained by puncture,[3] did not fluctuate more than those of a control monkey during the same period.

EFFECTS ON METABOLISM

The action of vitamin B_6 on the metabolism of rats was studied by the method of Richards and Collison (26). In 24 experiments in which the vitamin was given orally in doses ranging from 10 to 100 mgm. per kilogram, no change in oxygen consumption was found on observing the animals for three hours after the administration of the vitamin.

The effect of vitamin B_6 on water metabolism was studied in 30 rats in groups of 10. The normal excretion following 5 cc. of tap water per 100 grams body weight was first established for each group. One hundred milligrams per kilogram of the vitamin given simultaneously with the water changed neither the rate nor the amount of urine excreted. Rats injected daily with 100 mgm. per kilogram of vitamin B_6 subcutaneously likewise failed to show any change in water metabolism.

EFFECTS ON CIRCULATION AND SMOOTH MUSCLES

The blood pressure of rabbits and cats under urethane and chloralose anesthesia was not influenced by the intravenous injection of vitamin B_6 in doses ranging from 1 to 50 mgm. per kilogram (fig. 2). Respiration and heart rate remained unchanged.

On the smooth muscles of the isolated intestine of the rabbit, the vitamin was likewise without effect. Concentrations up to 1:10,000 did not alter the intestinal tone nor the rhythmic contractions (fig. 3). Spasms of the intestine induced either by barium chloride or acetyl choline were not influenced by the vitamin. No effect on the isolated uterus of rabbits could be observed; a recent statement that vitamin B_6 hydrochloride causes contraction of the isolated uterus and inhibition of the intestine (22) could not be confirmed.

[3] We are indebted to Dr. L. Goldman of the Newark Beth Israel Hospital for the hematological findings.

EXCRETION OF VITAMIN B_6

An attempt has been made to determine the urinary excretion of vitamin B_6 under normal conditions in men, dogs, and rats using a rat assay method (20). The dogs and rats were maintained on a diet of purina dog chow; the human urines were pooled samples from a large group of apparently healthy individuals. Since the concentration of the vitamin is very low, it

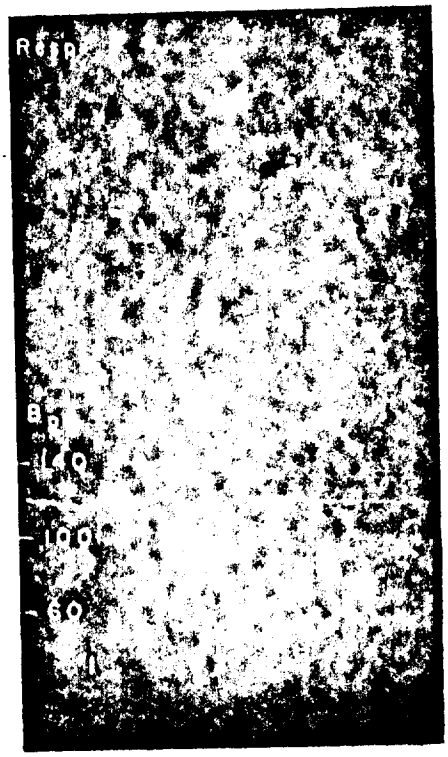

FIG. 2. EFFECT OF VITAMIN B_6 ON RESPIRATION AND BLOOD PRESSURE

Cat 2.5 kgm. body weight; 0.6 gram per kilogram urethane and 0.06 gram per kilogram chloralose.

Resp.—Respiration; Bp—carotid blood pressure mm. Hg; time in 30 seconds. 1—10 mgm. of vitamin B_6 per kilogram, 2—20 mgm. of vitamin B_6 per kilogram.

was necessary to concentrate the urine. This was accomplished by acidifying to pH 4.5 and evaporating to one-tenth of the original volume, then diluting with 10 volumes of alcohol, removing the insoluble salts by filtration and again distilling to remove excess alcohol.[3]

The urine concentrates were administered in graded doses daily over a

[3] We are indebted to Drs. J. C. Keresztesy and J. V. Seffch for the preparation of the urine concentrates.

period of seven days to groups of 4 to 6 rats that were deficient in vitamin B_6. A total of 126 rats were used for these assays. Weight and deficiency symptoms were recorded daily. The effect of various urine concentrates compared

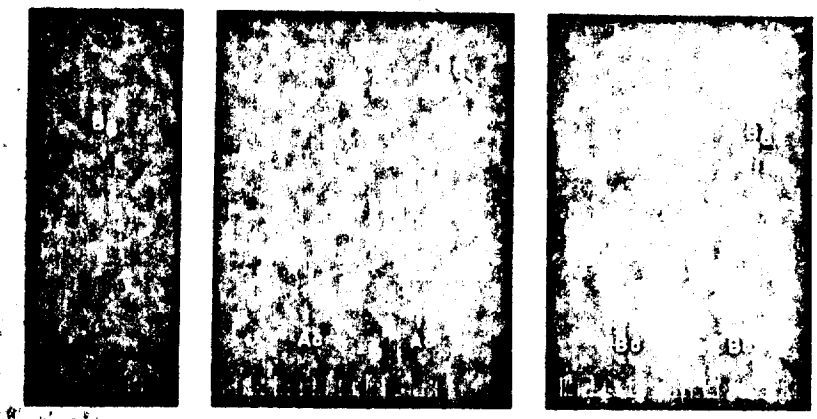

FIG. 3. EFFECT OF VITAMIN B_6 ON THE ISOLATED INTESTINE OF A RABBIT

Time in minutes. Concentration of the compounds added at the arrows: B_6—vitamin B_6, 1:50,000; Ac—acetyl choline 1:2,000,000; Ba—barium chloride 1:20,000.

TABLE 2

Seven-day bioassay of urine concentrates in vitamin B_6 depleted rats

0.1 cc. of concentrate equals 1.0 cc. of urine

	DAILY DOSE	WEIGHT GAIN	HEALING OF DEFICIENCY LESIONS
	micrograms	*grams*	
Vitamin B_6	2	0–2	None
	3	4–9	Slight
	5	8–10	Marked
Urine concentrates:	*cc.*		
Human urine*	0.6	0	
Human urine†	0.6	0	
Dog urine‡	0.6	0	
Rat urine§	0.3	5–6	Very slight
	0.6	9–14	Marked

* Pooled sample from a large group.
† Individual sample following the ingestion of liver.
‡ Pooled sample from 2 dogs.
§ Pooled sample from 12 rats.

with the effect of the daily administration of synthetic vitamin B_6 are recorded in table 2.

From these experiments it is evident that the urine of rats contains nor-

mally between 0.5 and 1.0 micrograms per cubic centimeter. On the other hand, because of toxic reactions produced by larger doses of the concentrates, we were unable to feed urine of men and dogs in sufficient amounts to influence the vitamin B_6 deficiency lesions. Since the results obtained with an equivalent of 6 cc. of undiluted urine were negative, it may be concluded that these urines normally contain less than 0.5 microgram per cubic centimeter. The ingestion of half a pound of fried liver did not augment the excretion in man to amounts measurable by this method. However, when the vitamin was given in doses of 100 and 500 mgm. to dogs, their urine was found to contain 3 mgm. per cubic centimeter. These results were checked by a colorimetric method (27) which was used for a study of the urinary excretion of vitamin B_6 in men and dogs (28). Following oral and intravenous administration the vitamin appeared rapidly in the urine, but only fractions of the amounts administered could be recovered. Thus, it seems likely that an excess of vitamin B_6 is metabolized in the organism.

Acknowledgment

Appreciation is expressed to Dr. W. Antopol for the histological studies and to Mr. Leo Fernandez and Miss Josephine Dawson for valuable technical assistance.

SUMMARY

1. The L.D. 50 of vitamin B_6 hydrochloride in rats is 3.7 grams per kilogram following subcutaneous injection and 5.5 grams per kilogram following oral adminstration.

2. Doses above 1 gram per kilogram of vitamin B_6 produce toxic manifestations in dogs, rabbits, and rats characterized by impairment of the coordination and tonic convulsions. The dose required to produce these symptoms is at least 1000 times greater than the therapeutic dose.

3. Daily administration of vitamin B_6 over a period of 80 days to dogs (20 mgm. per kilogram) and rats (25 mgm. per rat) failed to produce any toxic manifestations or pathological changes of the organs. Rats receiving 2.5 mgm. daily were raised through three generations.

4. The urine of apparently healthy men and dogs contains less than 0.5 micrograms of vitamin B_6 per cubic centimeter as determined by bioassay.

5. The metabolism, the circulatory and respiratory system, and isolated smooth muscle organs of normal animals are not influenced by vitamin B_6.

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Toxicity of Vitamin B_6.

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The existence of vitamin B_6, the rat acrodynia factor was first established by György.[1] The compound has since been isolated[2-5] and synthesized.[6, 7] Sufficient amounts of the synthetic vitamin, 2-methyl-3-hydroxy-4,5-dihydroxymethylpyridine, are now available for the study of the effect of large doses in animals.

The following investigation of the toxicity of the synthetic vitamin B_6 has been carried out on rats, dogs, and monkeys. All animals were maintained on completely adequate diets with the exception of one series of rats which was kept on a modified Sherman-Spohn diet supplemented with 40 micrograms of thiamin and of riboflavin per rat per day. This group developed typical symptoms of rat acrodynia.

The vitamin was administered both as the base and as the hydrochloride. The solutions of the base are neutral in reaction; those of the hydrochloride react acid, the pH of a 20% solution being 2.3.

Acute toxicity following oral and subcutaneous administration was studied in rats, 10 animals being used for each dose level. Doses up to 1 g per kg were tolerated without untoward effects. Higher doses produced rather peculiar toxic symptoms. Twenty-four hours after dosing, the rats showed tonic convulsions, the hind limbs stretched away from the body, the fore limb bent under the body and the paws closed. Between convulsive attacks the animals were able to move slowly and awkwardly and showed marked impairment of the righting reflexes. Animals receiving sublethal doses exhibited these convulsive reactions over a period extending from several days to as much as 3 weeks. With lethal doses the animals died in tonic convulsions within 36 to 72 hours. The L.D. 50* following subcutaneous injection was 3.1 g per kg for the free base and 3.7 g

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2 György, P., *J. Am. Chem. Soc.*, 1938, **60**, 983.
3 Keresztesy, J. C., and Stevens, J. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 64.
4 Kuhn, R., and Wendt, G., *Ber.*, 1938, **71**, 780.
5 Lepkovsky, S., *Science*, 1938, **87**, 168.
6 Harris, S. A., and Folkers, K., *Science*, 1939, **89**, 347.
7 Kuhn, R., Wendt, G., Westphahl and Westphahl, K., *Naturwissenschaften*, 1939, **27**, 469.
* L.D. (lethal dose) 50 is the dose which is fatal to 50% of the animals.

per kg for the hydrochloride. This difference of 16% in toxicity is in good agreement with the difference of 18% in the molecular weights of the respective compounds. The L.D. 50 following oral administration was approximately 4 g per kg for the base and 6 g per kg for the hydrochloride. Autopsies of animals exhibiting various degrees of toxic manifestations failed to show pathologic changes on gross examination except for enlargement of the adrenals with occasional massive hemorrhages particularly in the cortex. Histological studies are in progress.

Three-week-old rats in groups of 15 were fed orally 0.25, 1.0, and 2.5 mg of B_6 hydrochloride daily over a period of 87 days. The animals developed normally and their weights increased at the same rate as that of the normal control group. Autopsies at the end of the 87-day feeding period showed no gross or microscopic changes in the organs. A group of 6 animals, 3 males and 3 females, were kept to maturity on a daily dose of 2.5 mg of the hydrochloride. Two litters, one of 5 and the other of 8, averaging 5 g birth weight, were obtained.

In dog studies 20 mg of B_6 per kg body weight was given orally to 3 litter mate puppies over a period of 75 days. Their weight increase was normal and did not differ from that of a control dog from the same litter. Hemoglobin, erythrocyte, leukocyte, and differential blood counts were taken at regular intervals and did not show any significant changes. Histological examination after 75 days revealed no pathological changes.

Monkeys of 2.5 and 4 kg body weight were fed or injected daily with 10 mg per kg B_6 hydrochloride. Unfortunately, most of the animals succumbed to tuberculosis during the test period. One monkey was dosed orally for 39 days, another subcutaneously for 106 days without showing any toxic symptoms. Hemoglobin and blood cell counts as well as the differential picture of the sternal bone marrow obtained by puncture did not fluctuate more than in a control monkey during the same period.†

Eight rats in severe stages of vitamin B_6 deficiency, weighing between 40 and 50 g, were given 9 mg of B_6 hydrochloride orally on two consecutive days. This dose is far in excess of the minimum curative dose of 50 micrograms.[8] Prompt cures were effected, and no toxic symptoms were observed.

† We are grateful to Dr. Lester Goldman for these data which will be presented in full in another communication.

8 Reedman, E. J., Sampson, W. L., and Unna, K., PROC. SOC. EXP. BIOL. AND MED., 1940, 43, 112.

The results of these studies characterize vitamin B_6 as a substance of very low toxicity corresponding with the low toxicity found for other members of the vitamin B complex, thiamin,[9] riboflavin,[10] and nicotinic acid.[11] Excessively large doses (3.0 g per kg) of vitamin B_6 produced convulsions and death.

The difference in subcutaneous and oral toxicity, as shown by the L.D. 50, is small and suggests a rapid and complete absorption from the intestinal tract.

Prolonged feeding of sublethal doses failed to produce toxic symptoms, thus indicating that excessive doses of vitamin B_6 are either rapidly excreted or destroyed. Studies on this problem are now in progress.

We wish to acknowledge the technical assistance of Mr. Joseph Greslin.

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Vitamin B_6: Pyridoxine, Pyridoxal and Pyridoxamine

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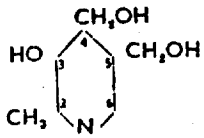
VITAMIN B_6, also known as pyridoxine, or adermin, is a derivative of pyridine and in that respect is similar to nicotinic acid and nicotinamide, which also belong to the complex mixture originally called vitamin B.

According to our present knowledge, vitamin B_6 is a group of three substances: an alcohol, pyridoxine, an aldehyde, pyridoxal and an amine, pyridoxamine. All three are derivatives of β-hydroxypyridine, and thus there is a profound similarity between the vitamin B_6 group and nicotinic acid and its derivatives: in all of them the β-position in the pyridine ring is substituted by a group strongly influencing the character of the molecule—the phenolic group in vitamin B_6 and the carboxylic group in nicotinic acid.

The principal component of vitamin B_6, pyridoxine, has the structure 2-methyl-3-hydroxy-4,5-di(hydroxymethyl) pyridine, *I*.



(*I*)

This was elucidated in 1939 by experiments described below, but the first mention of the relationship between β-hydroxypyridine and vitamin B was given by R. R. WILLIAMS[1] in 1921 *i.e.* long before vitamin B_6 was isolated.

A few years later J. GOLDBERGER and R. D. LILLIE[2] found certain types of dermatitis in rats fed on diet free from vitamin B_2 (lactoflavin). The dermatitis was produced by an unknown factor present in vitamin B_2, since the rats did not recover on administration of pure vitamin B_2.

Then S. J. OHDAKE[3] isolated a new crystalline substance from rice bran. He found the empyrical formula of the hydrochloride of this product to be $C_8H_{11}O_3N.HCl$. It was certainly pyridoxine, although Ohdake did not recognize the vitamin character of the substance.

The name 'vitamin B_6' appeared in 1934, bestowed by P. GYÖRGY[4] who isolated this substance from yeast, rice bran and liver. He found it to be an antipellagra factor, which can accompany vitamin B_2 in yeast, liver and other 'vitamin B' sources. The same author, together with T. W. BIRCH[5], found vitamin B_6 to be a nitrogen base which forms a hydrochloride.

In a series of papers[6-10] published in 1938 improved methods of isolating vitamin B_6 were described. Further experiments showed that vitamin B_6 is present in animal or vegetable tissue in the form of a complex with proteins or carbohydrates which is subject to cleavage by the action of enzymes or heat. The richest sources of vitamin B_6 are yeast and rice bran. Various seeds including corn also contain a considerable quantity, while lesser amounts occur in molasses and liver. Milk, egg yolk, spinach and green vegetables are poor sources of vitamin B_6.

CHEMICAL PROPERTIES AND STRUCTURE OF PYRIDOXINE

The free base pyridoxine has the empirical formula $C_8H_{11}O_3N$. It is a white crystalline substance with m. pt 160°C. It is soluble in water and most organic solvents, and forms several salts *e.g.* the hydrochloride (m. pt 204–206°C with decomposition). R. KUHN and his co-workers[11] determined its structure in an extremely skilful way within a very short time. The main facts which led to their conclusions were:

1 The substance contains three hydroxyl groups, but only one of them can be methylated with diazomethane; thus the substance has one phenolic and two alcoholic groups.

2 If pyridoxine methylated with diazomethane was subjected to heating with lead tetracetate, no oxidation occurred. This indicates that the alcoholic groups are not attached to the neighbouring carbon atoms since lead tetracetate is a reagent which oxidizes α-glycols.

3 Pyridoxine methylated with diazomethane can be oxidized with cold potassium permanganate. One of the alcoholic groups is oxidized to a carboxylic group which readily forms a lactone with the second alcoholic group. As only γ- and sometimes δ-lactones can be formed in this way, the alcoholic groups can only occupy the 1.4 or 1.5 positions.

4 The ultraviolet absorption spectrum shows a great similarity between pyridoxine and β-hydroxypyridine. Both pyridoxine and β-hydroxypyridine give a cherry red colour with ferric chloride, which indicates the phenolic character of their hydroxyl groups: α- and γ-hydroxypyridine are not phenolic in character and do not become coloured with ferric chloride.

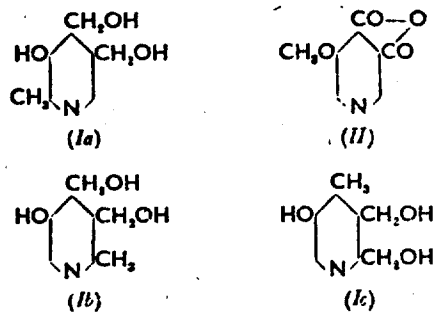
5 If pyridoxine methylated with diazomethane is oxidized with hot potassium permanganate in alkaline medium a tribasic acid is formed which gives a brown colour with ferrous sulphate. This colour reaction is characteristic of pyridinic acids, which contain a carboxylic group in α-position. On heating the acid looses a molecule of water and a molecule of carbon dioxide, forming the anhydride of a dibasic acid. The acid produced from this anhydride does not give the colour reaction with ferrous sulphate.

These facts, together with the analytical data, brought Kuhn to the following conclusions :

a the three carboxyl groups are formed by the oxidation of two primary alcoholic and one methyl group ;

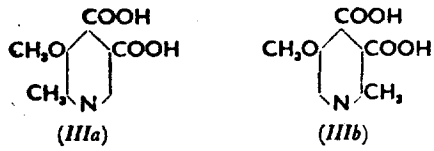
b the carboxyl group, which is eliminated as CO_2 on heating, occupies the α-position ;

c the two remaining carboxyl groups are attached to the neighbouring carbons. On this basis Kuhn gives the structure of the anhydride *II* and three possible structures of pyridoxine, *Ia*, *Ib* and *Ic* :



6 Kuhn and co-workers synthesized the anhydride which possesses the structure *II*. It proved to be identical with that obtained from the methyl ether of pyridoxine.

7 Careful oxidation of the methyl ether of pyridoxine with barium permanganate led to a dibasic acid with an untouched methyl group. This acid does not give any colour with ferrous sulphate. It has therefore two possible structures, *IIIa* and *IIIb* :



Consequently the structure *Ic* for pyridoxine should be rejected.

8 Kuhn and his co-workers synthesized a dibasic acid *IIIa*. It proved to be identical with the acid formed from the methyl ether of pyridoxine, so formula *Ib* was therefore rejected.

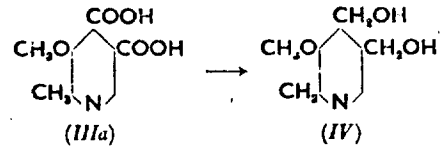
Independently the structure of pyridoxine was also established by a team of workers in the U.S.A. Thus the acid *IIIa* was obtained[12] by oxidizing the methyl ether of pyridoxine *IV*. This acid was subsequently synthesized[13] by a different method. Both syntheses of the acid *IIIa* were important in providing a basis for the preparation of pyridoxine, which both groups of investigators achieved shortly afterwards.

SYNTHESIS OF PYRIDOXINE

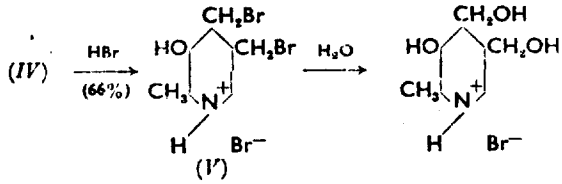
R. Kuhn and his co-workers[14] synthesized pyridoxine by starting from the acid *IIIa* and transforming the carboxyl groups according to the scheme :

$$-COOH \rightarrow -CONH_2 \rightarrow -CN \rightarrow -CH_2NH_2 \rightarrow -CH_2OH.$$

Thus the acid *IIIa* gave the methyl ether of pyridoxine *IV* :

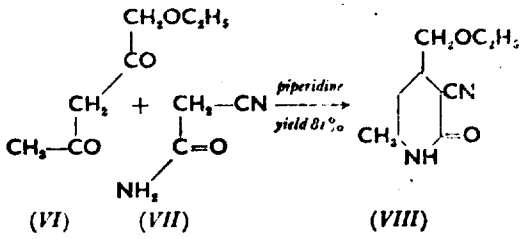


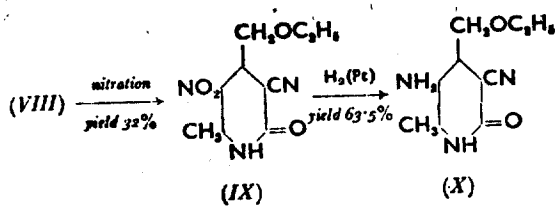
which was demethylated to give the hydrobromide :



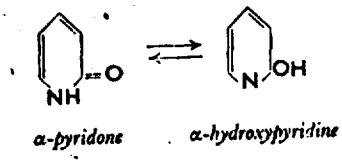
An identical synthesis was also described by A. Ichiba and K. Michi[15].

The method of synthesizing pyridoxine developed by S. A. Harris and K. Folkers[16] gave the best practical results from the viewpoints of yield and cost of production. They started from ethoxyacetylacetone *VI* and cyanacetamide *VII* which with piperidine in alcoholic solution underwent ring closure and formed a pyridine derivative *VIII* :

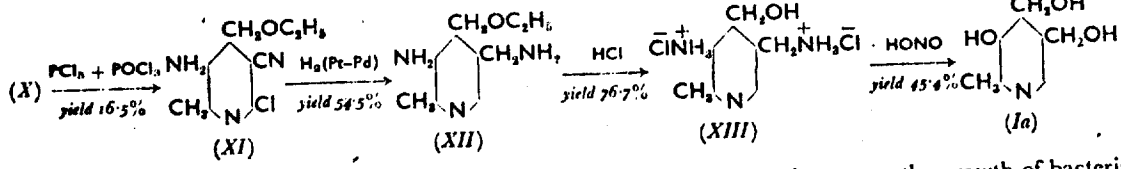




Substance *VIII* was nitrated to *IX* and then reduced by platinum black to the amine *X*. Substance *X*, which is an α-pyridone derivative, can be transformed by phosphorus pentachloride and phosphorus oxychloride into an α-chloropyridine derivative, because α-pyridone reacts tautomerically as α-hydroxypyridine:

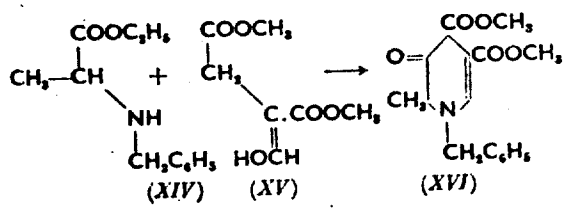


Thus product *X* can be transformed into *XI*:

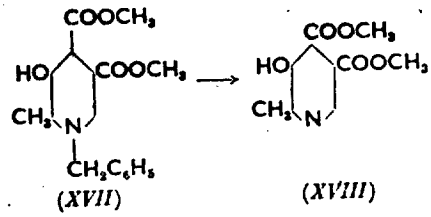


This was reduced by hydrogen on platinum and palladium with the simultaneous removal of chlorine, giving the amine *XII*. Hydrolysis with hydrochloric acid at 175–180°C yielded the aminoalcohol *XIII* which on diazotization finally gave pyridoxine. An almost identical method was adopted by Merck A.G. in Germany for the commercial synthesis of pyridoxine[17].

Recently A. COHEN[18] proposed a new method of preparing pyridoxine by starting from α-alanine derivatives: N-benzyl-α-alanine ester *XIV* and α-hydroxymethylenesuccinic ester *XV* were cyclized to a 3-keto-1.2.3.4-tetrahydropyridine derivative *XVI*:



The product was dehydrogenated to *XVII* and after hydrogenolysis of the N-benzyl group the substance *XVIII* was obtained.



The product *XVIII* can be converted into pyridoxine in the conventional way by stepwise transformation of $-COOCH_3$ into $-CH_2OH$. This synthesis suggested the possible functioning of α-alanine as a natural precursor of pyridoxine and it is interesting to note that E. E. SNELL[19] found alanine capable of replacing vitamin B_6 for the growth of *Lactobacillus casei* and *Streptococcus faecalis* R.

PYRIDOXAL AND PYRIDOXAMINE (PSEUDOPYRIDOXINE)

It was found in 1938–39 that vitamin B_6 is a growth-promoting agent for yeast and bacteria. A biological method for the assay of pyridoxine was then developed[20]. Experiments on the growth of bacteria on a medium containing pyridoxine led to some important conclusions. E. E. SNELL, B. M. GUIRARD and R. J. WILLIAMS[21] found that *Strep lactis* R would grow on such a medium many times faster than could be accounted for on the basis of actual pyridoxine content. The explanation is that pyridoxine was converted into a more highly active metabolite prior to utilization by the bacterial organism. This hypothetical metabolite was called pseudopyridoxine.

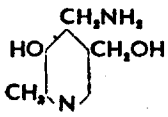
In his later experiments E. E. SNELL[22] found that media containing pyridoxine could be activated by simple heating in an autoclave. He advanced a suggestion that pseudopyridoxine was formed by the interaction of pyridoxine and aminoacids present in the mixtures. He developed[23] his experiments further and heated pyridoxine *a* with ammonia in an autoclave *b* with manganese dioxide.

In both instances he obtained products much more active than pyridoxine. In the experiments *a* they were 140 times more active than pyridoxine, and in *b* 50 times. He therefore suggested that amination and partial oxidation are possible reactions which can transform pyridoxine into pseudopyridoxine. Pseudopyridoxine is therefore a mixture

of two substances, the product of oxidation, which Snell called pyridoxal, and the product of amination, which he called pyridoxamine. The following reaction scheme represents this transformation :

$$\underset{\textit{Pyridoxine}}{R.CH_2OH} \overset{[O]}{\rightleftarrows} \underset{\textit{Pyridoxal}}{R.CHO} \overset{NH_3}{\rightleftarrows} \underset{}{[R.CH{=}NH]} \overset{[H]}{\rightleftarrows} \underset{\textit{Pyridoxamine}}{R.CH_2NH_2}$$

Snell's metabolic experiments with rats and human beings also led to the conclusion that pyridoxine is partially converted by animal organisms into pseudopyridoxine.

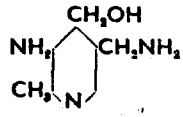


(*XIX*)

Independently R. KUHN[24] found that patients cured with pyridoxine excreted in their urine a new substance much more potent than pyridoxine, which possesses the structure *XIX*.

No details were given of how he arrived at this conclusion.

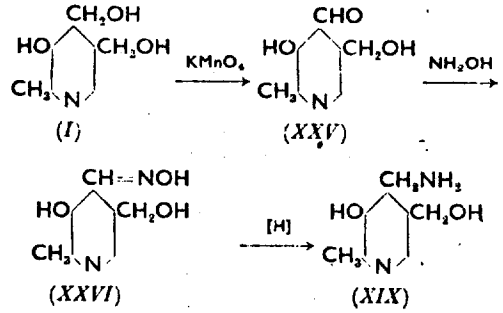
The structures of pyridoxal and pyridoxamine were established by S. A. HARRIS, D. HEYL and K. FOLKERS[25] by synthesizing these two products and their isomers. They started from substance *XX*, the methyl ether of the alcohol *XIIa*, homologous with substance *XII*, the ethyl ether of the same alcohol. Diazotization of *XX* gave the product *XXI*. On action of thionyl chloride the alcoholic group was replaced by chlorine *XXII* which was then substituted by the amino group *XXIII*; finally the ether group was hydrolysed with hydrochloric acid at 170–180°C and product *XXIV* was obtained. There was no doubt about the structure of this compound, which on bio-assay proved to be non-identical with pyridoxamine and did not possess its activity.



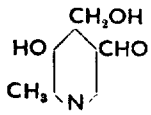
(*XIIa*)

The product possessed the properties of pyridoxamine, as determined from its action on the growth of *Lact casei* and *Strep lactis* R.

Harris, Heyl and Folkers also prepared pyridoxal *XXV* and transformed it into pyridoxamine, providing evidence that the same 4-hydroxymethyl group was transformed into an aldehyde and methylamino group respectively. Pyridoxal was prepared by a careful oxidation of pyridoxine in an alkaline medium :



The aldehyde *XXV* was transformed into the oxime and this was catalytically reduced to pyridoxamine.

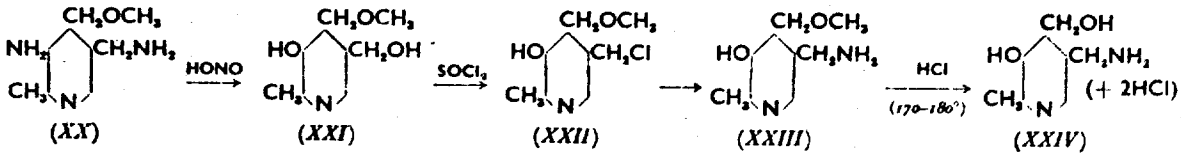


(*XXVII*)

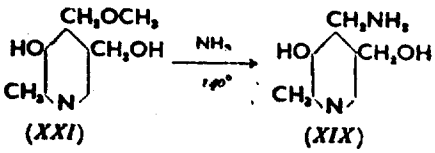
Final confirmation of the structure of pyridoxal was obtained when the same authors synthesized the isomeric aldehyde *XXVII*, which does not possess the growth promoting activity of pyridoxal.

Further, E. E. SNELL[26] found that all three members of the vitamin B_6 group occur in natural tissues. In various papers E. E. SNELL and his co-worker[27] showed that the three compounds pyridoxine, pyridoxal and pyridoxamine each behave very differently towards different organisms.

Thus they found pyridoxal has 5,000–8,000 times and pyridoxamine 6,000–9,000 times the activity of



Acting on substance *XXI* with ammonia at 140°C in methyl alcohol, they obtained the substance *XIX* identical with pyridoxamine :



pyridoxine as a growth factor for *Strep lactis* R. For other organisms, such as *Lact casei*, pyridoxal was also found to be highly active, but pyridoxine and pyridoxamine almost inactive. On the contrary, for *Saccharomyces carlsbergensis* all the compounds have approximately equal activities. It is assumed that the variation in activity is due to differences in the ease with which the various organisms convert pyridoxine into pyridoxamine and pyridoxal.

The original presence or formation of the members of vitamin B_6 groups renders microbiological assay for vitamin B_6 invalid if the growth of unsuitable organisms is examined. For that reason it is preferable to use organisms which respond equally to all three members of the group *e.g. Sacch carlsbergensis* and *Neurospora sitophila*.

PHYSIOLOGICAL ACTION OF VITAMIN B_6

None of the members of the vitamin B_6 group produces a definite pharmacological action and this is the main reason why the part played by this vitamin was obscure. The earliest experiments showed a connection between vitamin B_6 and certain skin disorders, so typical for most B group vitamins. This was confirmed by later experiments. A special type of dermatitis, *acrodinia*, occurring on peripheral parts of the bodies of animals is due to lack of vitamin B_6. It was also suggested that vitamin B_6 is an agent in the metabolism of unsaturated fatty acids[28]. Experiments with rats gave evidence of the importance of pyridoxine in the process of transformation of proteins into fats[29].

More recently a relation between the vitamin B_6 deficiency and anaemia has been clearly shown by several authors. S. LEPKOVSKY[30] found that anaemia produced by the lack of pyridoxine does not respond to iron and copper, but prompt improvement follows administration of pyridoxine. M. M. WINTROBE *et alii*[31] showed that pigs fed on a diet supplemented with vitamins A and D and all known vitamins B except B_6 develop a severe microcytic anaemia frequently accompanied by epileptic convulsions. Administration of pyridoxine produced rapid regeneration of blood.

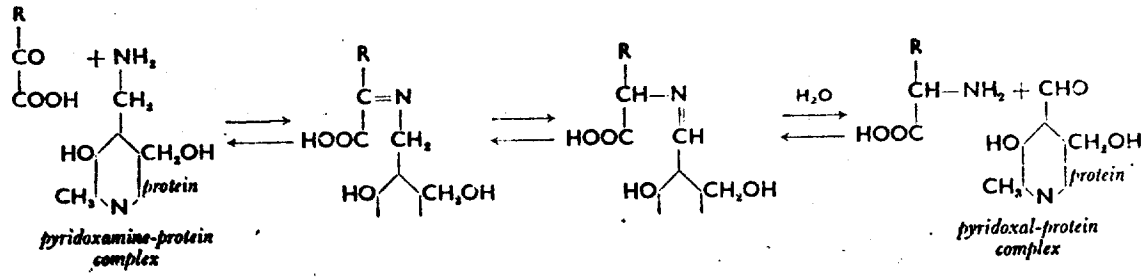
It is known that kynurenine, an amino acid, and xanthurenic acid, a quinoline derivative, can be excreted by animals suffering from nutritional disorders. It has also been shown that both substances are products of incomplete metabolism of tryptophane. S. LEPKOVSKY *et alii*[32] established the exact dietary deficiency necessary to produce the excretion of xanthurenic acid. They found that pyridoxine deficient rats excreted in their urine a complex of xanthurenic acid and iron. This excretion of iron might be one of the factors causing anaemia. On the basis of this and some later work the role of pyridoxine was established as a factor producing metabolism of tryptophane and particularly of kynurenine and xanthurenic acid. This property of pyridoxine was later elucidated in the course of work on the coenzyme action of phosphorylated pyridoxal quoted[10] below.

The discovery that vitamin B_6 is closely related to certain enzymes was the most important part of the research on its biological action. The first observation was related to the role of vitamin B_6 in the transformation of certain amino acids to amines *i.e.* of their decarboxylation by decarboxylase. Thus W. D. BELLAMY and I. C. GUNSALUS[33] found that addition of pyridoxine and nicotinic acid or pyridoxal stimulated production of tyrosine decarboxylase. Then J. BADDILEY and E. F. GALE[34] and, almost simultaneously, W. W. UMBREIT and I. C. GUNSALUS[35] discovered that phosphorylated pyridoxal can act as co-decarboxylase. In addition, others[36] prepared phosphorylated pyridoxal with 40 to 50 per cent coenzyme activity from pyridoxal and phosphorus oxychloride. The exact position of the phosphoric acid group in this coenzyme is not yet known.

A further discovery was the function of pyridoxine derivatives as agents playing important parts in biological transaminations. Previously E. E. SNELL[37] had suggested that the possible conversion of pyridoxal into pyridoxamine may be explained by a transamination reaction. In a number of papers he showed that a relation existed between members of vitamin B_6 group and the process of transamination. Then H. C. LICHSTEIN, I. C. GUNSALUS and W. W. UMBREIT[38] found that pyridoxal phosphate functioned as the coenzyme of the transaminase of certain acids.

The process of transamination shown below was suggested by F. SCHLENK and A. FISCHER[39].

Another function of pyridoxal phosphate was found by W. W. UMBREIT, W. A. WOOD and I. C. GUNSALUS[40]. They proved it to be the coenzyme



for tryptophanase which catalyses the breakdown of tryptophane to indole, pyruvic acid and ammonia.

The complex behaviour of the vitamin B_6 group gives no clear indication of its use in therapy. They may be useful remedies against some types of anaemia and some skin disorders, including such symptoms of pellagra which are not relieved by the usual treatment with vitamin B_1, B_2 and nicotinic acid. It is certain that pyridoxine is an invaluable remedy against certain nervous disorders accompanied by insomnia and irritability, and also against vomiting in pregnancy. It is of value in treating muscular dystrophy, paralysis agitans and chorea.

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Weigand-'40
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11384

Action and Toxicity of Vitamin B_6 Hydrochloride.

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In view of the increasing interest in vitamin B_6[1-4] as a nutritional accessory, the present investigation was undertaken in order to determine the degree of potency and limits of toxicity. All experiments were carried out with crystalline vitamin B_6 hydrochloride, m.p. 212°C (corrected) with effervescence.

General Properties. Vitamin $B_6 \cdot HCl$ is easily soluble in water. Its aqueous solutions are acid in reaction. A 1% solution has a pH of 2.44. *In vitro* a quantity of 4 mg caused hemolysis of sheep's washed erythrocytes, but if it was previously neutralized, no laking took place. Obviously the hemolytic effect was due to the acidity. When a 1% solution of $B_6 \cdot HCl$ in the amount of 0.5 cc was injected both subcutaneously and intramuscularly into 3 rabbits, practically no irritation occurred, but when 0.1 cc of the same

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[4] Kark, R., Lozuer, E. L., and Meiklejohn, A. P., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 97.

solution was injected intracutaneously, congestion was evident for more than 4 days in 2 out of 3 animals.

Potency. In addition to physical and chemical characterization, crystalline vitamin B_6 may be assayed biologically. For this purpose, young rats of 21 to 23 days of age were fed a ration composed of sucrose 67%, vitamin-free casein 20%, McCollum's salt mixture No. 185 4%, Crisco 3%, liver filtrate free from B_6 2%, cod liver oil 2%, and agar 2%. In addition, each animal received daily doses of 20 γ each of thiamin chloride and riboflavin. Towards the end of 6 to 8 weeks, these rats developed typical signs of B_6-avitaminosis—acrodynia and inhibition of growth. They were then treated individually with single doses of various size by mouth, and examined daily for 2 weeks. In a group of 60 rats, it was found that a dose of 40 to 60 γ cured acrodynia in an overwhelming majority of the rats within 14 days. Meanwhile, there was a decisive increase in their body weights. These results are comparable to those of Reedman, Sampson, and Unna.[5] For testing the potency of a new lot of $B_6 \cdot HCl$, it has been the practice of this laboratory to determine the median curative dose for acrodynia (CD_{50}), and compare it with the CD_{50}, determined simultaneously, of the purest lot which is being preserved as a standard.

Acute Toxicity. By intravenous injection, the median lethal dose (LD_{50}) of $B_6 \cdot HCl$ in mice was found to be 545.3 ± 42.9 mg per kg, and that in rats 657.5 ± 18.3 mg per kg. The data are shown in Table 1. Tonic and then clonic convulsions occurred, and death followed rapidly. Animals either completely recovered or succumbed within 5 minutes after injection. No after effects were noted in the surviving rats. The acute toxicity of $B_6 \cdot HCl$ in rats

TABLE 1.
Toxicity of Vitamin $B_6 \cdot HCl$ in Mice and Rats by Intravenous Injection.

Animal	Conc. of solution, %	Dose, mg per kg	No. died / No. used	LD_{50} ± Standard error, mg per kg
Mice	1	300	0/5	545.3 ± 42.9
		400	1/5	
		500	1/5	
		600	3/5	
		700	3/3	
Rats	5	500	0/3	657.5 ± 18.3
		600	1/5	
		650	4/10	
		700	4/5	

[5] Reedman, E. J., Sampson, W. L., and Unna, K., PROC. SOC. EXP. BIOL. AND MED., 1940, 48, 112.

by subcutaneous and oral administration has been estimated by Unna and Antopol.[6]

Repeated Administration. Five mice were injected by the tail vein with daily doses of 100 mg per kg except Saturdays and Sundays for 2 weeks. All of them gained weight during the course of medication. Upon sacrifice, no pathological changes were detected.

In Men. Twelve young male adults consented to take $B_6 \cdot HCl$ by different routes as shown in Table II. The drug was dispensed in capsules for oral use, but made into 2.5% solution for injection purposes. Briefly, it may be stated that no ill effects were observed when $B_6 \cdot HCl$ was given either by mouth or by intravenous injection in the dosage of 100 to 200 mg. Pain uniformly occurred when the drug was injected intramuscularly, perhaps due to its

TABLE II.
Action of Vitamin $B_6 \cdot HCl$ in Men by Various Routes of Administration.

Subject No.	Age	Dose, mg			Reactions noted
		Oral	Intra-muscular	Intra-venous	
1	22	100			None
			50		"Sore" at site of injection for 1½ hours
				100	None
2	42	100			"
3	35	100			"
4	40	100			"
5	24	100			"
			50		"Aching" for 15 min
				100	None
6	33		50		"Burning" for 15 min
				100	None
7	28		50		"Sore" for 2 hrs
				100	None
8	30		50		"Sore" for 15 min
				100	None
9	28		50		"Aching" for 2 hrs
				100	None
10	21			200	"
11	29			200	"
12	21			200	"

[6] Unna, K., and Antopol, W., PROC. SOC. EXP. BIOL. AND MED., 1940, 43, 116.

acidity. In 3 out of 6 cases, it lasted as long as 1½ to 2 hours. No other toxic manifestations were noted.

Other Effects. Casual observations were made on cats' blood pressure and respiration. Doses of 100 mg did not alter the pulse nor the respiratory rate; nor did they change the height of carotid pressure. Appropriate concentrations (1:8000) of $B_6 \cdot HCl$ caused brief inhibition of isolated rabbit's small intestines with prompt recovery, and contraction of the isolated guinea pig's uterus. The response here was not due to a low pH, because controls with the same acidity did not reproduce these results.

Summary. 1. Vitamin $B_6 \cdot HCl$ is acid in reaction which may be responsible for certain irritating effects in body tissues. 2. A method of bioassay has been described based upon the curing of rats' acrodynia. 3. The acute toxicity of $B_6 \cdot HCl$ in mice and rats by intravenous injection has been determined. 4. Mice can tolerate repeated doses of 100 mg per kg. given intravenously, without pathological

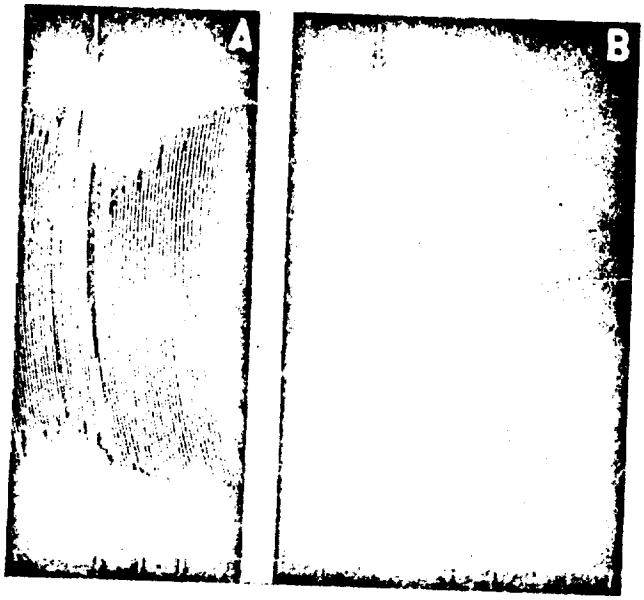

FIG. 1.
Action of Vitamin B_6-HCl on Isolated Smooth Muscle Organs.

A. The peristaltic movements of a strip of a rabbit's small intestines immersed in Tyrode's solution at 38°C. At arrow, 0.5 cc of 2.5% solution of B_6-HCl was applied (making the concentration 1:8000). There was a brief inhibition followed by prompt recovery.

B. The myogram of a horn of a virgin guinea pig's uterus immersed in Tyrode's solution at 38°C. At arrow, the same amount of B_6-HCl as above was added. It resulted in a contraction.

changes. 5. No ill effects occur in men when $B_6 \cdot HCl$ is administered orally or intravenously in the dosage of 100 to 200 mg. Pain occurs when the drug is injected intramuscularly. 6. In the concentration of 1:8000, $B_6 \cdot HCl$ inhibits isolated rabbit's small intestines, but contracts the isolated guinea pig's uterus.

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Role of Pyridoxine in the Production of Leucocytes in Normal and Leukemic Mice.* (17467)

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Morgan, Groody, and Axelrod[1] showed that pyridoxine-deficient dogs developed a leucocytosis with an absolute increase in the numbers of circulating neutrophiles and an absolute decrease in the numbers of lymphocytes. These abnormalities could be corrected by giving adequate doses of pyridoxine. In the same year McCall, Waisman, Elvehjem, and Jones[2] described similar leucocyte changes in pyridoxine-deficient and in pantothenic acid-deficient monkeys. They stated that the reversal of the polymorphonuclear to lymphocyte ratio also occurred in riboflavin deficiency. In the pyridoxine-deficient animals the reversal could not be corrected completely by pyridoxine but could be corrected by whole liver. Wintrobe and his associates[3] observed a tendency of the granulocytes to increase in pyridoxine-deficient swine, but there was no reversal of the ratio of polymorphonuclears to lymphocytes. Stoerk, Eisen, and John[4] described thymus atrophy and a reduction in the number of fixed and circulating lymphocytes, and Stoerk[5] described lymphoid atrophy and regression of rat lymphosarcoma in pyridoxine-deficient rats. In order to facilitate the production of pyridoxine deficiency, Ott[6] investigated the antipyridoxine activity of desoxypyridoxine hydrochloride (2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine). He found that two molecules of the analogue were sufficient to offset the vitamin activity of one molecule of pyridoxine, as measured by chick growth. Under other conditions[7] much larger quantities of the analogue are necessary. These observations prompted us to investigate the effect of both pyridoxine deficiency and pyridoxine excess on the circulating leucocytes and hematopoietic organs of normal mice and of mice having various abnormalities of the leucocyte pattern.

Pyridoxine Deficiency in Normal Mice.

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[2] McCall, K. B., Waisman, H. A., Elvehjem, C. A., and Jones, E. S., *J. Nutrition*, 1946, **31**, 687.

[3] Wintrobe, M. W., Follis, R. H., Miller, M. H., Stein, H. J., Alcayaga, R., Humphreys, S., Suksta, A., and Cartwright, G. E., *Johns Hopkins Hosp. Bull.*, 1943, **72**, 1.

[4] Stoerk, H. C., Eisen, H. N., and John, H. M., *J. Exp. Med.*, 1947, **85**, 365.

[5] Stoerk, H. C., *Fed. Proc.*, 1948, **7**, 281.

[6] Ott, W. H., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 125.

[7] Umbreit, W. W., and Wadell, J. G., Proc. Soc. Exp. Biol. and Med., 1949, **70**, 293.

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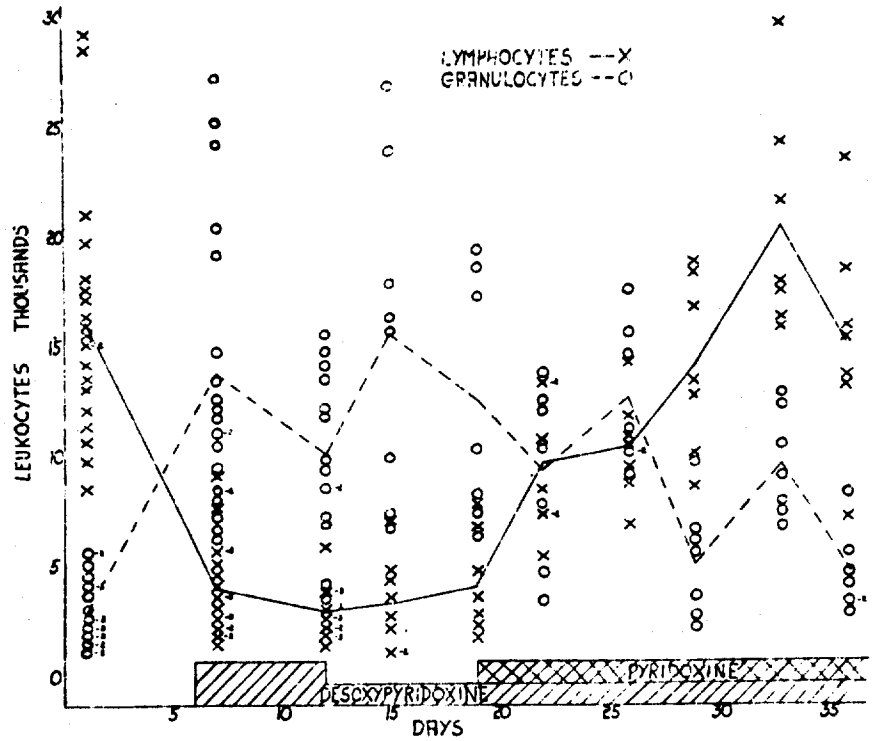

FIG. 1.
Leucocyte changes in pyridoxine-deficient normal mice.

Weanling mice of a Swiss strain were placed in cages with wire-mesh bottoms. They were fed *ad libitum* a modification of a pteroylglutamic acid-deficient diet which has been described previously:[8] pyridoxine and succinylsulfathiazole were not included. The animals were given pteroylglutamic acid intraperitoneally in daily doses of 20 μg. In addition desoxypyridoxine† was given subcutaneously as a 2% solution of the hydrochloride. In a preliminary experiment, daily doses in excess of 1 mg were fatal within ten days. On a 1 mg dose the mean survival time was 20 days and weight loss was marked. Many of the animals developed the expected granulocytosis and lymphopenia but several did not. In a second experiment the animals were given 2 mg daily for a week and then 1 mg daily. Again the leucocyte response was variable and weight loss occurred rapidly. Also the leucocyte changes could not be corrected completely by the administration of pyridoxine. These unsatisfactory results using a purified diet suggested a trial of a crude diet (Purina dog chow) supplemented by larger amounts of desoxypyridoxine given parenterally, as a possible means of producing the desired alteration in the leucocyte pattern. This approach proved successful. Twenty Swiss mice were fed Purina dog chow *ad libitum*. On the 6th day subcutaneous injections of desoxypyridoxine HCl in a 2% solution were started. From the 6th to the 12th day 8 mg were given daily in 2 doses of 4 mg each at an interval of 8 hours. Because of a high mortality in the animals the doses were then halved to supply 4 mg daily, and this was continued to the end of the experiment; during this period only one mouse died. Supplementary pyridoxine HCl (0.2%) was added to the ground diet on the 19th day. Since the mice ate from 3 to 6 g of dog chow daily, they were supplied with 6 to 12 mg of supplementary vitamin daily. Smaller amounts used in preliminary experiments had failed to restore the

[8] Weir, D. R., Heinle, R. W., and Welch, A. D., PROC. SOC. EXP. BIOL. AND MED., 1948, 69, 211.

† Kindly furnished by Merck & Co., Inc.

leucocyte pattern to normal. Total and differential leucocyte counts were done twice weekly. At autopsy, sections of liver, spleen, marrow, thymus, lymph nodes, and both adrenals were taken for histologic study.

As shown in Fig. 1, a complete reversal of the ratio of granulocytes to leucocytes occurred very promptly after desoxypyridoxine was started, with development of absolute granulocytosis and lymphopenia. Within 5 days after the pyridoxine supplement was started restoration of the normal leucocyte pattern had begun, and was complete 14 days later (36th day). After the reduction of the dose of desoxypyridoxine during the initial period, only one animal died and the others remained in apparent good health. There was no significant weight loss, and they did not develop ruffled fur, convulsions, or obvious neurologic disturbances. Red blood cell counts were not done in this experiment, but, in a preliminary experiment which was conducted under identical conditions except for a lower dose of supplementary pyridoxine, the mean erythrocyte count did not change significantly.

At autopsy on the 36th day no gross or microscopic abnormality could be detected in the tissues. In order to obtain material for histologic study at a time when the reversal of the granulocyte to lymphocyte ratio was established, 20 mice were maintained on the regimen described above, but were not given the supplementary pyridoxine. Fifteen of the 20 survived to be killed on the 37th day, at which time granulocytosis and lymphopenia, with a complete reversal of the ratio, had been present for 32 days. None of these animals showed any evidence of lymphoid atrophy. The thymus glands and lymph nodes were normal in appearance, and the splenic follicles were, if anything, slightly hyperactive. There was no increase in the size of the adrenal glands or demonstrable change in their cytological structure. The marrows showed possible increase in cellularity due to increased numbers of granulocytes without increase in immature cells, although this change was not marked. The liver of one animal that died early showed extensive hemorrhagic necrosis. The liver of another that survived to be killed showed a few small foci of necrosis. There is reason to believe that a generalized tissue deficiency of pyridoxine had not been established. Growth was not impaired, convulsions did not occur, there was very slight anemia, and lymphoid regression or atrophy was not present at autopsy. It is likely that a partial deficiency is sufficient to produce the leucocyte shift without producing other demonstrable changes.

Effect of Pyridoxine Deficiency on the Leukemia of Ak Mice. In mice of the Ak strain the intravenous injection of cells from diseased older animals produces leukemia in 100% of younger animals.[9] In the animals now being bred in this laboratory this is believed to be a mixed type of leukemia, in which both adult granulocytes and adult lymphocytes participate, with the appearance of small numbers of immature blastic cells. About 10 to 15 days after transplanting, the total leucocyte count begins to rise. It remains elevated for 10 to 20 days and then falls toward normal levels shortly before the animal dies. Death usually occurs 25 to 35 days after the transplant. Differential counts show that the initial rise is due to an increase of granulocytes. The ratio of these cells to lymphocytes may be as great as 4 to 1. As the leukemia progresses the lymphocytes increase and, coincidentally with this rise, the granulocytes begin to fall and reach normal levels when the lymphocytes are still elevated. At death any elevation of counts above normal is due to increased numbers of lymphocytes. The immature blastic cells appear in small numbers at the height of the leucocytosis and persist until death. The effect of pyridoxine deficiency on this leucocyte pattern was investigated. Fourteen weanling Ak mice were placed on a diet of Purina dog chow. On the first day all animals were given intravenous injections of a suspension of cells from lymph node and spleen of a diseased older animal. On the 8th day a group of 9 was started on desoxypyridoxine hydrochloride. Each was given 1.5 mg subcutaneously twice daily. Total and differential leucocyte counts were done at the end of the first week and twice weekly

[9] Weir, D. R., and Heinle, R. W., PROC. SOC. EXP. BIOL. AND MED., 1947, 66, 268

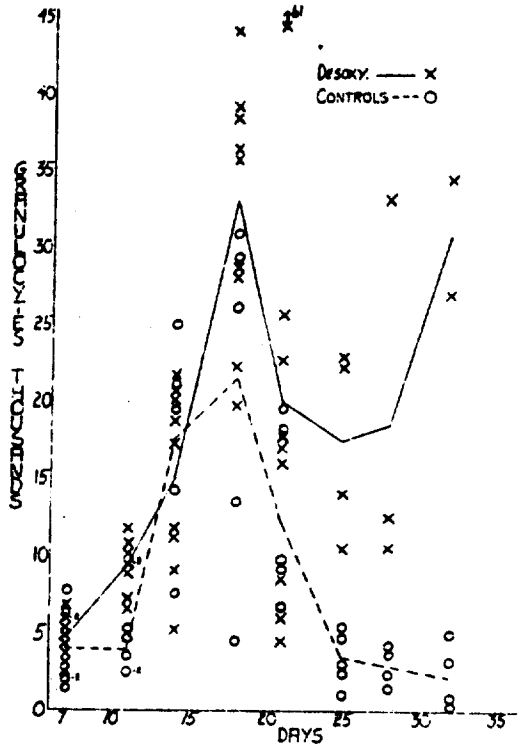

FIG. 2.
The effect of pyridoxine deficiency on the granulocytosis of Ak mouse leukemia.

thereafter. The mean survival time of the animals receiving desoxypyridoxine was 24.6 days as compared to 31.8 days for the controls. This decrease in survival time is statistically significant. The value for P is less than 0.02.[10] The leucocyte patterns are shown in Fig. 2. In the early stages of the leukemia the granulocyte counts of the experimental animals were generally higher than those of the controls. On the 25th day and thereafter all the granulocyte counts of the experimental animals were 2 to 6 times as great as the highest count shown by the controls during the same period. The lymphocyte counts of the experimental animals were generally lower than those of the controls but not significantly so. Histologic study of sections of liver, spleen, bone marrow and lymph nodes taken at autopsy failed to reveal any difference between the experimental and control groups.

[10] Fischer, R. A., Statistical Methods for Research Workers, Oliver and Boyd, Edinburgh, 1934.

Preliminary Studies of the Effects of Pyridoxine in Excess. Groups of normal mice were given parenteral pyridoxine HCl in divided daily doses of 8 and 16 mg for a period of 48 days. None of the animals showed any significant change in the leucocyte pattern. Three groups of 5 Ak mice were injected intravenously with a leukemic transplant. All were maintained on dog chow diet. One group received 2 mg of parenteral pyridoxine HCl in a single daily dose; another group received 4 mg; and the third group served as controls. The pyridoxine supplement failed to influence the course of the leukemia or to alter the histologic findings at autopsy. During the study of a myeloid metaplasia factor from human urine Heinle, Hirschmann, and Wearn[11] observed that the injection of certain extracts of urine from patients with chronic myeloid leukemia, produced a moderate to marked granulocytosis in mice. The effects of excess amounts of pyridoxine on this granulocytosis was studied. Ten adult Swiss mice were given for 10 days daily injections of a urine extract known to be potent. Five of these also were given pyridoxine hydrochloride parenterally in doses of 8 mg twice daily. The mean polymorphonuclear count of the 10 animals at the beginning of the experiment was 3370 per cu mm. At the end of the 10 day injection period the mean for the group receiving pyridoxine was 32.615; for those not receiving pyridoxine it was 43.544. The difference is not statistically significant.

Discussion. The results indicate that a deficiency of pyridoxine causes an increase in granulocyte production in normal mice and in mice dying from transplanted leukemia. It is known that vitamins of the B_6 group take part in transamination and decarboxylation,[12-14] and that the active compound is a phosphorylated form of pyridoxal acting as co-

[11] Heinle, R. W., Hirschmann, H., and Wearn, J. T., Approaches to Tumor Chemotherapy, 1947, 77.

[12] Schlenk, F., and Snell, E. E., *J. Biol. Chem.*, 1945, **157**, 425.

[13] Bellamy, W. D., and Gunsalus, I. C., *J. Bact.*, 1943, **46**, 573.

[14] Gunsalus, I. C., and Bellamy, W. D., *J. Bact.*, 1944, **47**, 413.

transaminase and co-decarboxylase.[15-18] In the intact normal animal the various compounds of the group presumably are freely interconvertible.[19,20] From these considerations, the hypothesis is suggested that the granulocytosis in pyridoxine-deficient mice is due to the failure on the part of the animals to transaminate, decarboxylate, or otherwise modify unneeded supplies of granulocyte-building substances, and that the animals thereupon utilize these unneeded supplies in producing excess numbers of granulocytes. It is not necessary to assume that the reverse would be true. A further hypothesis may be made that the granulocytosis of a disease state such as human myeloid leukemia may be due to a defect in the absorption or utilization of vitamins of the B_6 group. Although freely interconvertible in the intact animal, this may not be true of them in the diseased animal. Further investigations in mice and in leukemic human beings are in progress to test the validity of these hypotheses. It has been demonstrated that adrenocorticotropic and adrenal cortical hormones will cause granulocytosis and lymphopenia in animals.[21,22] In our histologic material we could not demonstrate evidence of adrenal cortical hyperplasia.

Summary. 1) Pyridoxine deficiency in mice produces granulocytosis and lymphopenia. 2). The leukemia of the Ak mice now being bred in this laboratory is participated in by both granulocytes and lymphocytes. In pyridoxine deficiency the leukemic granulocytosis is more severe and the survival time of the animals is decreased. 3) The hypothesis is advanced that the granulocytosis in pyridoxine-deficient mice is due to the failure on the part of the animals to perform metabolic functions in which pyridoxine derivatives play a catalytic role; thus, supplies of compounds used in the formation of granulocytes accumulate, and, as a result, the animals utilize this plethora in producing excess numbers of granulocytes. The implications of this hypothesis are discussed.

[15] Gunsalus, I. C., Bellamy, W. D., and Umbreit, W. W., *J. Biol. Chem.*, 1944, **155**, 685.

[16] Liebstein, H. C., Gunsalus, I. C., and Umbreit, W. W., *J. Biol. Chem.*, 1945, **161**, 311.

[17] Ames, S. R., Sarma, P. S., and Elvehjem, C. A., *J. Biol. Chem.*, 1947, **167**, 135.

[18] Beiler, J. M., and Martin, G. J., *J. Biol. Chem.*, 1947, **169**, 345.

[19] Sarma, P. S., Snell, E. E., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 284.

[20] Junqueira, P. B., and Schweigert, B. S., *J. Biol. Chem.*, 1948, **174**, 605.

[21] Valentine, W. N., Craddock, C. G., Jr., and Lawrence, J. S., *Blood*, 1948, **3**, 729.

[22] Hills, A. G., Forsham, P. H., and Finch, C. A., *Blood*, 1948, **3**, 755.

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Wynn'60
Bib No. 2867

Journ. Nutrition 70(1):69-71. 1960.

Dental Caries in the Albino Rat Fed High Sucrose Diets of Relatively High and Low Pyridoxine Content[1]

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Pyridoxine as a dietary supplement is believed by Strean and his co-workers to have a suppressive effect on the initiation and spread of dental caries. This deduction has been drawn from experiments using hamsters, as well as children. Hamsters fed a cariogenic diet containing pyridoxine in a concentration of 10 ppm had fewer dental caries than a similar group fed the same diet with a concentration of 0.5 ppm (Strean, Gilfillan and Emerson, '56; Strean, Bell, Gilfillan, Emerson and Howe, '58). The later report included an experiment using 28 children divided into two groups of 14 each. One group received a lozenge containing 3 mg of pyridoxine three times a day after meals. At the end of a year this group, on the basis of their DMF (decayed, missing, filled) rating, had a lower caries score than the control group. In a preliminary report of a study scheduled to be conducted over a period of several years, Cohen and Rubin ('58) observed a slightly favorable trend in caries experience of a group of children receiving pyridoxine-containing lozenges as compared with another group receiving a placebo.

Strean ('57) has postulated that pyridoxine may effect a change in the oral flora whereby the lactobacilli requiring this vitamin as an essential nutrient, and not associated with dental caries, would multiply at the expense of *L. acidophilus*, *S. salivarius*, *S. mitis* and *S. mitior*, which some believe to be associated with tooth decay.

The interesting suggestion has been offered (Strean, Bell, Gilfillan, Emerson and Howe, '58) that the difference in cariogenicity of two high-sucrose diets containing the same amount of sugar which has been reported from our laboratories (Wynn, Haldi, Shaw and Sognnaes, '53) may have been due to a difference in the concentration of pyridoxine in the two diets. The experiments reported in this paper were undertaken to test the validity of this supposition.

PROCEDURE AND RESULTS

The Harvard high-sucrose diet, the composition of which is given elsewhere (Sognnaes, '48; Wynn, Haldi, Shaw and Sognnaes, '53) was used in all experiments. This diet contains 3.5 ppm of pyridoxine. Two sets of experiments were conducted: one on sialoadenectomized albino rats of our Emory-Wistar strain which has proved to be fairly resistant to dental caries, and another on intact animals of a more susceptible strain. In the first experiment 8 groups of triplicate litter mates were selected at weaning and sialoadenectomized by our usual procedure (Haldi, Wynn, Shaw and Sognnaes, '53). Whereas intact animals of this strain are fairly resistant to dental caries, when sialoadenectomized they develop caries on a cariogenic diet within a relatively short time. Beginning immediately after weaning, the three groups of litter mates were fed throughout the experiment the Harvard diet to which was added for each group, respectively, 1, 10 and 100 ppm of pyridoxine. At the end of a 70-day feeding period, the animals were killed and the teeth scored for dental caries by the method customarily followed in our laboratories (Haldi and Wynn, '52).

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[1] This investigation was supported by Contract DA-49-007-MD-675 with the U. S. Army Medical Research and Development Command, Office of the Surgeon General of the U. S. Army, and by research grant D-103 (C3) from the National Institute of Dental Health of the National Institutes of Health, U. S. Public Health Service.

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TABLE 1

Carious lesions and caries scores of sialoadenectomized caries-resistant albino rats fed a cariogenic diet with different concentrations of pyridoxine

No. animals	Days on diet	Additional pyridoxine	Carious lesions[1]	Caries score[1]
		ppm		
8	70	1	24	59
8	70	10	24	61
8	70	100	22	55

[1] Average per animal.

The results of this experiment are presented in table 1.

From these data it is obvious that the addition of pyridoxine in a relatively large amount to a cariogenic diet did not affect its cariogenicity when fed to sialoadenectomized albino rats of the Wistar strain.

Strean, as stated previously, believes that pyridoxine may bring about a change in the oral flora. In this connection, it may be noted that the addition of pyridoxine to a synthetic medium increases the population of *L. casei* proportional to the quantity of this vitamin added to the medium (Bohonas, Hutchings and Peterson, '42) and that *L. casei* is the test organism for the biological assay of pyridoxine (Rabinowitz, Mondy and Snell, '48).

In view of the possibility that pyridoxine, once absorbed, may be returned to the mouth by way of the salivary glands, thereby having a more prolonged effect on the oral bacteria, we decided to conduct another experiment on intact rats of a strain more susceptible to dental caries. For this purpose we used albino rats from a colony developed from animals that had been supplied to us several years ago by the laboratories of the Naval Medical Research Institute. We had found that these animals, when fed a cariogenic diet with their salivary glands intact, develop caries in a relatively short time.

The animals were selected at weaning, using triplicate litter mates, with 20 animals placed in each group, and fed immediately the prescribed diets. The control group was fed the Harvard diet without supplementation and the other two groups, the same diet to which was added, respectively, 10 and 100 ppm of pyridoxine. When the animals had been fed the diets 120 days, they were killed and the teeth were given a score for dental caries in the same manner as in the preceding experiments. The results are given in table 2. In these experiments, as in those on the sialoadenectomized rats of the Emory-Wistar strain, the addition of pyridoxine to the diet did not reduce its cariogenicity.

TABLE 2

Carious lesions and caries scores of intact caries susceptible albino rats fed a cariogenic diet with different concentrations of pyridoxine

No. animals	Days on diet	Additional pyridoxine	Carious lesions[1]	Caries score[1]
		ppm		
20	120	0	17	40
20	120	10	18	35
20	120	100	18	38

[1] Average per animal.

DISCUSSION

In our previous studies, we found that the high-sucrose diet referred to as the Harvard diet is much more cariogenic than the Emory diet which contains the same amount of sucrose. These two diets differ in their pyridoxine content with a concentration of 3.5 ppm in the Harvard and 10 ppm in the Emory diet. If Strean's supposition were correct that the difference in pyridoxine concentration may account for the difference in cariogenicity of the two diets, one would reasonably expect that the addition of pyridoxine to the Harvard diet would make it less cariogenic. This proved not to be the case. The cariogenicity of the Harvard diet was not diminished even though the pyridoxine content was brought well above the level of that which obtained in the Emory diet.

The results of our experiments are not in agreement with those of Strean and his co-workers ('56; '58). These investigators found that increasing the pyridoxine concentration in the diet from 0.5 to 10 ppm resulted in a decrease in the cariogenicity of the diet. It is possible that the response of the hamster to the addition of pyridoxine to the diet may differ from that of the albino rat.

Another possibility should also be taken into account, namely, that the diet fed the hamsters, containing 0.5 ppm of pyridox-

ure, may have been deficient in this factor. If this were the case, the higher incidence and greater extent of caries observed when using this diet as compared with the one containing 10 ppm, may have been related to a pyridoxine deficiency. It seems unlikely, however, that the hamsters suffered from a pyridoxine deficiency inasmuch as they survived over an experimental period of 7 to 10 months. Furthermore, the authors did not report acrodynia or other signs of pyridoxine deficiency. In our experiments, the basic Harvard diet provided 35 to 50 μg of pyridoxine in the normal daily food intake of 10 to 15 gm which doubtless satisfied the rat's pyridoxine requirements. Lepkvosky ('38) obtained optimal growth in rats with a daily dose of 10 μg. Sure ('40) reported later that 10 to 25 μg/day are adequate for continuous growth.

CONCLUSION

The addition of pyridoxine in relatively large amounts to a synthetic high-sucrose diet, originally adequate in its pyridoxine content, did not reduce the cariogenic properties of the diet when fed to sialoadenectomized albino rats of a fairly resistant strain nor to intact rats of a much more susceptible strain.

The difference in the cariogenicity of two high-sucrose diets containing the same amount of sugar, previously reported from our laboratories, cannot be attributed to the difference in pyridoxine concentration of the two diets.

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