Monograph on **Glycerine & Glycerides**-Volume 1 2/2/73

MONOGRAPH ON GLYCERINE AND GLYCERIDES

Volume I

TR-72-1552-18

Submitted Under:
Contract No. FDA 72-104

February 2, 1973

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GLYCERINE AND GLYCERIDES

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GLYCERIN AND GLYCERIDES

Summary

Glycerine is a common energy yielding food; the average fat or oil molecule of animal or vegetable origin contains about 10% by weight of glycerol in combined form. Although, combined in fats and phosphatides, glycerine is present in animal tissues to the extent of about 1% of the body weight, it is not essential in the diet since it is readily synthesized in the animal body. Aside from furnishing energy by contributing to the general pool of oxidizable organic compounds, it plays no special role in nutrition. Mono- and diglycerides are present in food fats to an extent of approximately 1% and are therefore also a regular part of the diet. Furthermore, the triglycerides which are the main components of the food fats are transformed into mono- and diglycerides in the body during digestion.

Hoeber and Hoeber (656) and Johnson et al. (740) reported that glycerine is well absorbed from the intestines of rats and dogs.

Kayden et al. (775) reported that the analysis of the lumen of men after fat feeding showed a separation of absorbed glycerides into two phases: an oil phase which contained mainly diglycerides and triglycerides and some fatty acids, and a micellar phase which was a combination of bile salts, free fatty acids and monoglycerides. The review of the literature by Johnston (741), indicates that the monoglycerides and free fatty acids in the micellar solution are absorbed into the intestinal wall, leaving the bile salts within the lumen to form additional micelles. The monoglycerides and fatty acids migrate to the endoplasmic reticulum where resynthesis occurs (741). Fatty acids are activated enzymatically and resynthesized into triglycerides by the acylation of monoglycerides, the monoglycerides are esterified to triglycerides, and then the triglycerides are incorporated into chylomicrons for delivery to the intestinal lymphatics (741). Thus diglycerides and triglycerides are digested through enzymatic action to monoglycerides, absorbed into the lumen of the intestinal wall, resynthesized into the triglycerides and delivered to the lymphatic system for final distribution to the extracellular fluids and to the fat depots (741).

Catron and Lewis (214) reported that glycerine is readily metabolized and deposited as glycogen in the liver of young white rats. Using cats and human subjects, Holst (669) found that the rate of metabolism of ingested glycerine is constant and independent of the blood concentration, the metabolism of glycerine occurs almost exclusively in the liver, and the oxidation in the liver is incomplete. The hypothesis that glycerine is converted to glucose was confirmed by Chambers and Deuel (228) in their tests using phlorhizinized dogs. The fate of ^{14}C-glycerine

in the rat, following administration of up to 238 mg, as determined by Gidez and Karnovsky (526) is shown in the figure on page 54.

Fatty acids play an important role as an energy source (913). Their oxidation is prominent in higher animals which can store large amounts of neutral fat as a fuel reserve (913). It is estimated that fatty acid oxidation provides at least half of the oxidative energy in the liver, kidneys, heart muscle and resting skeletal muscle (913). Fatty acids undergoing oxidation in tissues of higher animals came either from extracellular fluid or from endogenous intracellular lipids (913). Vertebrate blood contains considerable amounts of triacylglycols but very small amounts of free fatty acids (913). The major endogenous source of fatty acids for fuel is storage fat, in the form of fat droplets in the cytoplasm, which consists largely of triacylglycerol (913). Before the fatty acids can undergo activation and oxidation, the triacylglycerols first must undergo hydrolysis by intracellular lipases to yield free fatty acids and glycerols (913). Free fatty acids are therefore derived either from the blood stream, in which they are transported bound to serum albumin, or from hydrolysis of intracellular lipids (913). They are first activated by esterification with CoA to form acyl CoA ester at the outer mitochondrial membrane (913). Then the fatty acyl group from CoA is carried across the inner membrane by the carrier molecule carnitine (913). Finally the fatty acyl group is transferred from carnitine to intramitochondrial CoA: through several enzymatic reactions, a two-carbon fragment of the fatty acid is removed as acetyl CoA and two pairs of hydrogen atoms are given to specific acceptors (913). Along chain fatty acid will go through several spirals (913). Thus the 16-carbon palmitic acid undergoes a total of 7 such cycles, to yield 8 molecules of acetyl CoA and 14 pairs of hydrogen atoms (913). The acetyl CoA formed as a product of the fatty acid oxidation will then enter the tricarboxylic acid cycle, which is the final common pathway of oxidative catabolism of all fuel molecules in aerobic cells. (913).

According to Florey (454), the glycerine derived from the hydrolysis of glycerides is converted directly into glucose while the fatty acids are broken down into 2-carbon units which contribute to the formation of citric acid. Thus fats are fed into the pathways of glycolysis and the citric acid cycle (454).

In an experiment in which rats were given D-palmitic acid in free form or as mono- or triglyceride, Buensod and Favarger (182) found that monopalmitin is digested more completely than the free acid or triglyceride form, although it is digested more slowly than tripalmitin. Measurement of radioactivity 24 hours after oral administration of a mixture of mono- and diglycerides of ^{14}C-1,4-diacetyl tartaric acid (0.5-0.8 g/kg) by Lang and Schmidt (880) revealed that 26-31% of the activity was absorbed, 12-21% was oxidized to $^{14}CO_2$, 8-13% was excreted in the urine and 2% was retained in the carcass. According to Koppanyi and Dardin (1802), TEM has a digestibility coefficient of roughly over 90%,

it is absorbed from the small intestine, and the tartaric acid released during its metabolism is slowly excreted by the kidneys. Sourkes and Koppanyi (1473) found that when 2.72 g of TEM per kg of body weight was administered per os to dogs, 88% of the TEM was absorbed within 12 hours and less than 20% of the theoretically available tartaric acid was excreted in the urine during the same time. The digestibility coefficient of TEM in the adult rat was reported to be 95% (1473).

Gil'miyarova (531) reported that the prolonged administration of glycerine (50 mg/kg/day for 5-6 months) to rabbits resulted in an increase of the glycerine level of the blood, liver, skeletal and cardiac muscles and intestines, while a single dose (100 mg/kg) did not produce any significant effects. A striking hyperglyceridemia, elevation of plasma phospholipid level and increased liver triglyceride content were observed by Nikkila and Ojala (1121) as a result of administration of glycerine to rats as 15% of their drinking water for 1-2 weeks. Macdonald (966) reported that the serum glycerides of men were raised 1-2.5 hours after the injestion of 1 ml of glycerine/kg, whereas in young women given the same amount of glycerine the serum glycerides were not raised. In men who were given a supplement of glycerine (1 ml/kg/day) to their free choice diet there was a rise in the concentration of fasting serum glycerides but no rise was found when a similar supplement was given to young women (966).

Wishnofsky (1752) reported that the ingestion of 80 g of glycerine produced an increase in the concentration of blood sugar in the majority of 13 normal and 10 diabetic persons. Martin and Stenzel (1005) reported that orally administered glycerine (4 g/kg) increases the glucuronic acid excretion of rats by as much as 100%. Orekhovich and Plotnikova (1163) found that the daily intragastric administration of 20ml of a 50% glycerine solution produced a 100% rise in the cholesterol content of the blood of rabbits within 2 weeks, causing the development in the aortic arch of formations similar in outward appearance to atherosclerotic plagues found in the initial stage of atherosclerosis. However, through in vitro and in vivo (rats) studies, Migicovsky (1056) found that glycerine inhibits the synthesis of cholesterol.

The hydrolysis of caseinogen by pancreatic proteinase was found by Farber and Wynne (419) to be inhibited by glycerine. Shapiro (1419) reported that glycerol, when fed in amounts demonstrated to be glycogenic exerted a definite ketolytic action in rats fed diacetic acid. Both the force and amplitude of contractions of in vitro rabbit intestine were noted by Pal and Prasad (1186) to be increased by the addition of 0.4-1% glycerine. A series of studies by Ayres and Isgrig (48) demonstrated that moderate intragastric doses of glycerol depress the voluntary running activity, as well as food intake, of rats.

A number of workers have reported that doses of 1-1.5 g of glycerine/kg are effective in reducing the intracranial (179, 203) and intraocular (1678, 1364, 353, 1581, 211) pressure in human subjects.

Bornmann et al. (137) reported that glycerine produces a strong laxative effect in rats, guinea pigs and dogs.

Farber and Wynne (419) found that triacetin, when properly emulsified, caused inhibition of the hydrolysis of caseinogen by pancreatic proteinase. Zuckerman et al. (1785) found that orally administered oleic acid (9 ml/day) stimulates the flow of liver bile in a cholecystectomized human subject. Administered with bile salts, oleic acid increases the choleretic effect of the bile salts beyond the additive effect of each of these stimulating agents (1785). Pinter (1229) reported that 25-50g of glyceryl monolinoleate caused an increase in the serum triglyceride level of human subjects while similar quantities of glyceryl monostearate had little or no effect.

Voegtlin et al. (1679) reported that the coma and convulsions occurring in albino rats starved for 18 hours and injected with a MLD of insulin s.c. are promptly removed by the i.p. or i.v. injection of about 1g of glycerine per kg of body weight. Noble and MacLeod (1130) reported that subcutaneously administered glycerine has no effect on insulin shock in rabbits.

In reproduction tests in which glycerine was fed to rats, no deleterious effects attributable to dietary treatment were observed until extremely high dietary levels were reached. Administration of 10 ml of a 20% aqueous solution of glycerine per kg of body weight to 10 male and 10 female rats for a period starting 8 weeks prior to mating and lasting until birth (5 rats) or weaning (5 rats) of the young was found by Wegener (1708) to produce no untoward effects. In an extensive series of studies on the reproduction of rats which were fed purified diets containing as much as 30% glycerine, Guerrant et al. (559) found that the glycerine produced no adverse effects. In a reproduction study by Johnson et al. (740) in which rats were maintained on diets containing 0, 41, and 61% glycerine, no untoward effects on reproduction were noted among those rats given 41% glycerol, but the animals receiving glycerol at the 61% dietary level failed to become pregnant.

Koppanyi and Dardin (1802) reported that the mating of female rats fed a diet containing 10% TEM with male rats maintained on a 20% TEM diet resulted in the production of normal healthy offspring which revealed no indication of damages attributable to the ingestion of TEM. Dietary levels of 0.5 and 5.0% oxystearin were found by Hodge et al. (1801) to have no effect on the reprodutive performance, nor the health of the offspring, of rats. In a test of reproduction by Ambrose et al. (26), at a dietary level of 10%, aceto-oleins AG-3 and AG-21, and acetostearin AG-26 supported reproduction and lactation through the test period of 3 generations, while acetostearins AG-194 and AG-31 failed to support reproduction beyond the first generation.

Solomides (1469) reported that injections of glycerine produce a temporary immunity in rabbits followed by sensitization to re-injection.

However, following 10 injections of glycerine in guinea pigs, Hine et al. (646) noted that a further injection of the compound gave no indication of sensitization.

Staples et al. (1485) reported that repeated oral administration of high concentrations of glycerine are irritating to the gastrointestinal mucosa of rats and dogs. Accelerated emptying of the intestinal contents was found by Loeser et al. (934) to result from application of glycerine to the rectum of rats and guinea pigs. However, Loeser et al. (934) found that the immersion of rats' tails in undiluted glycerine and the application of undiluted glycerine to the conjunctiva of rabbits, cats, and dogs caused no apparent changes. Hine et al. (646) reported that no measurable irritation was produced by the application of glycerine to the skin and eyes of rabbits. Subcutaneous injections of mono- and diacetin were noted by Li et al. (922) to occasionally cause local irritation in mice and rats. In the rabbit eye, 50% monoacetin caused only a slight degree of irritation while diacetin and triacetin in similar concentrations caused marked congestion and moderate edema (922). The daily application, for 45 days, of a 30% acetoglyceride emulsion to the skin of albino guinea pigs was reported by Ambrose and Robbins (24) to result in no local irritation or systemic reactions.

In a study in which weanling male and female Holtzman rats were fed diets with or without 50% saturated, partially acetylated monoglycerides, Herting and Crain (639) noted the appearance of a foreign-body type reaction in the body fat occurring within 8 weeks of initiation of the test.

In the only reported feeding study using chicks, 10 one-day old chicks were fed diets containing diacetyl tartaryl glycerol monostearate (TEM), glycerol lactopalmitate (GLP) or succinylated monoglyceride (SMG) at dosage levels of 570 mg/kg, 2.85 g/kg and 8.55 g/kg for a period of 90 days (1803). TEM caused growth depression at all levels at 7 weeks, slight hyperemia of duodenum and ileum at the lower levels and moderate to severe hyperemia at the highest level (1803). Gizzard erosion was observed at the high level, with only slight changes at the intermediate level (1803). GLP caused growth depression at 90 days (1803). Liver weight was slightly increased and there were instances of very slight hyperemia and gizzard erosion at the intermediate level at 90 days (1803). SMG caused very slight growth retardation at 90 days (1803). Cecal size was increased at each level, slight hyperemia, and one instance of gizzard erosion, occurred at the intermediate level (1803).

After a 6-hour fast, 91 male Swiss inbred white mice were given by Hine et al. (646) intragastric doses of 15-31.5 g of undiluted natural or synthetic glycerine per kg of body weight. Body tremors, erection of the tail in the typical Straub manner, and generalized clonic convulsions preceded all deaths (646). Hyperemia of the small intestine and lungs occurred in some mice at the higher dosage levels (646). Slight hyperemia of the kidneys and the mucosa of the small intestine were also noted (646).

Wrestlind (1760) found that injections of triacetin (LD_{50} = 1600 mg/kg) into the tail veins of mice produced almost immediate convulsions, failure of the righting reflexes and respiratory arrest. In some animals respiration did not return and death occurred in 1-3 minutes; other animals started to breath spontaneously within a few minutes and survived (1760). Li et al. (922) reported that subcutaneous injections of lethal doses of mono-, di, and triacetin produced, in white mice, marked depression, weakness, prostration and, in some animals, labored respiration prior to death. Gross observations of animals poisoned by monoacetin revealed dilation of the heart and diffuse congestion of the lungs (922). In animals dying as a result of the toxic effects of di- and triacetin, hemorrhagic areas in the lungs frequently appeared (922). Preliminary studies of tissue sections showed some cloudy swelling of the convoluted tubules of the kidney and in some cases the lumen was filled with casts (922). Hydropic degeneration and necrosis of the tubules was noted in some areas and the liver appeared to be congested (922).

Two long-term feeding studies in which glycerine was administered to rats have been reported in the literature. Hine et al. (646) conducted a 2-year study in which 22 rats (Long-Evans strain) of each sex were maintained on diets containing 5, 10, or 20% natural or synthetic glycerine and 26 animals were kept as controls. High incidences of albuminuria and glycosuria were observed in the test animals but, due to the random distribution throughout the test groups, no significance could be attached to these findings (646). Females receiving 20% synthetic glycerine exhibited liver/body weight ratios significantly higher than those of the controls and females receiving 5% synthetic glycerine had heart/body weight ratios significantly greater than those on 5% natural glycerine (646). In a 2-year feeding study, in which 336 weanling rats, equally divided as to sex, were assigned to diets containing 20, 10, or 5% synthetic or natural glycerine and a control diet, no deleterious or toxicological effects attributable to glycerine ingestion were noted (1805).

For a period of 20 weeks, Guerrant et al. (559) fed groups of 10 young, growing rats, equally divided as to sex, diets containing glycerine at levels of 0, 1, 3, 6, 10, 15, 20, 30, 40, 50, and 60%. Results showed no marked difference in the growth response when dietary levels of 30% or less were fed, but higher levels of glycerine produced subnormal growth rate, with the majority of the animals surviving the 20-week test period (559). Pathological examinations revealed no changes in the tissues from animals which had received diets containing less than 10% glycerine, occasional changes at the 10% level, and frequent and pronounced changes (marked hydropic and fatty degeneration of liver parenchymatous cells) at higher levels (559). In a study by Johnson et al. (740) in which 51 rats were maintained for 20 weeks on diets containing 0, 20, 41 or 61% glycerine, no effect on growth was observed at the lower 2 levels of treatment, but when glycerine was fed at 61% growth of the rats was greatly impaired, the rats were less active, and their coats lacked the smooth gloss of the controls. However, the addition of starch to the diet of these rats allowed the resumption of normal growth, indicating that the described effects were not resultant from any toxicity of glycerine (740). In a study by Anderson et al. (29)

in which 10 rats were given 5% glycerine as drinking water for six months the only untoward effect exhibited by the treated animals was the presence of calcified masses in the tubules near the junction of the kidney cortex and medulla in 6 of the 10 rats. Through a 96-day chronic toxicity test in which groups of 6 male and 6 female rats were given 0, 1, 2, 5, 10, or 20% aqueous glycerine solutions as drinking water, Bornmann (136) found that growth of the rats receiving 10% or less of glycerine was normal, while the animals receiving 20% glycerine exhibited a temporary impairment of growth.

In further short-term feeding studies in which glycerine was administered to rats at levels as high as 20% of the diet for 1 year, 41% of the diet for 25 weeks, or 20% of the drinking water for 3 months, no untoward effects attributable to glyceride ingestion were apparent (1805, 740, 934, 1503, 1504, 664, 447).

A table of acute toxicity is presented on page 25. In tests of acute toxicity, lethal doses of glycerine were found to produce restlessness, mild cyanosis, diminished arterial blood pressure, increased rate and magnitude of respiration, followed by decreased respiration, debility, diuresis, tremor, colapse, clonic convulsions, coma, and death in respiratory and circulatory failure (318, 646). Sublethal doses produced similar but less severe effects, except that the debility, diuresis, loss of appetite and the consequent loss of weight were more pronounced (318, 646). Pathological examinations by Hine et al. (646) revealed hyperemia of the pylorous and small intestine, paleness of the spleen, and congestion of the lungs.

For a period of 2 years, Fitzhugh et al. (448) maintained 24 rats on a basal diet with a 25% supplement of Myverol 18-00 (glyceryl monostearate). Compared to a similar number of control animals, growth and longevity of the test rats was normal and detailed microscopic pathological examinations of all major organs and tissues revealed only a single change - an increase in the number of calcified renal tubular casts - attributable to the treatment (448).

In a study by Mattson et al. (1027) in which 12 groups of 10 weanling, male Sprague-Dawley rats were fed diets containing various pure mono-, di-, and triglycerides at a level of 25% for a period of 10 weeks, growth of all groups was normal and autopsies revealed no peculiarities. Ames et al. (27) reported that the feeding of monoglycerides (derived from the fatty acids of cotton seed oil) to rats for 3 generations disclosed no untoward effects attributable to the ingestion of the compounds. The only deleterious effect noted by Braun and Shrewsbury (156) to result from the feeding of 8-24% monostearin or monolinolein in the diets of rats for 8 weeks was a somewhat slower growth of the monostearin-treated rats. On the basis of a feeding study in which rats were fed mono-, di-, and triglycerides (prepared from a mixture of partially hydrogenated soybean and cottonseed oils) at levels of 15 or 25% of the diet for 70 days, Harris and Sherman (620) stated that these compounds exhibit no differences in caloric efficiency nor produce any differences in body weight gain.

Groups of 5 male weanling albino rats were raised by Ambrose and Robbins (24) on diets containing 0, 0.25, 0.5, and 1% aceto-olein and 0.25, 0.5, 1, 2, and 4% acetostearin for a period of 57 weeks. The authors suggested that the testicular hypoplasia and suppression

of spermatogenesis, which was observed to varying degrees in all rats, may have been the result of insufficient vitamin E in the diet (24). For periods from 400 to 709 days, Ambrose et al. (26) maintained groups of 10 male and 10 female rats on diets containing acetostearin AG-194, AG-26, or AG-31 or aceto-olein AG-21 or AG-3 at dietary levels of 5, 10 and 20%. No significant differences in growth were noted between the control animals and those fed acetoglycerides except in the case of male rats on dietary levels of 20% acetostearins AG-194 and AG-31, and female rats receiving 20% AG-31 (26). Testes of rats receiving the three acetostearin diets were significantly smaller than those of the control rats or rats fed the two aceto-oleins (26). The livers of male rats fed 20% acetostearin AG-194 and of both male and female rats fed 20% acetostearin AG-31 were significantly larger than those of the control rats (26). Livers of female rats fed 20% of either aceto-olein were smaller than those of the control rats (26). Kidneys of female rats fed 20% acetostearin AG-31 were significantly larger than those of the controls (26). Acetostearins produced a foreign-body reaction in the fatty tissue of a number of the organs, presumably due to crystalline deposits of a high melting point fat (26). Acetostearins AG-194, AG-26, and AG-31 produced some scarring of kidney tissue, presumably due to deposited calcium (26).

In a study by Mattson et al. (1026) in which rats were maintained for 8-12 weeks on diets containing 15-50% of various acetin fats, it was found that acetin fats derived from the usual edible triglycerides are nutritious materials.

Growth, organ weights, and mortality were normal for all groups of 8 young male rats which were maintained for 608 days on diets containing 10% glyceryl lactopalmitate with low monoglyceride content, 10% glyceryl lacto-oleate with high or low monoglyceride content, 10% polyglycerol lacto-oleate with high monoglyceride content, or 10% acetylated tartaric acid ester of glycerol monostearate (1804).

Low growth rate, attributed to poor palatability of the test diets, was observed for groups of weanling rats which were given, for a period of 7 days, a basal diet containing 20% of either monoglyceride citrate or glyceryl lactopalmitate (1800).

In a study in which groups of 50 male and 50 female weanling albino rats were given diets containing 0, 0.5, 5 or 15% oxystearin for 2 years, the only apparent effect was an unusual number of Leydig cell adenomas in the testes of the rats in the 15% oxystearin diet (1801).

In a paired feeding study in which the effects of feeding a diet containing 20% triacetin for a period of 7 months were examined, McManus et al. (1045) observed no discrepancies between the treated animals and the controls. Li et al. (922) reported that animals receiving lethal doses of mono-, di-, or triacetin exhibit marked depression, weakness, prostration, and in some animals labored respiration prior to death (922). Gross observations of animals poisoned by monacetin revealed dilation of the heart, diffuse congestion of the lungs, and in 2 rats, a small amount of fluid in the pleural cavity (922). In animals dying as a result of the toxic effects of di- and triacetin hemorrhagic areas in the lungs frequently appeared

(922). Preliminary studies of sections of tissues showed some cloudy swelling of the convoluted tubules of the kidney and in some cases the lumen was filled with casts (922). Hydropic degeneration and necrosis of the tubules was noted in some areas (922). The liver appeared to be congested (922).

Koppanyi and Dardin (1802) found that the addition of 5, 10, or 20% TEM to the diet of Wistar rats for 2 years produced no gross or microscopic pathological changes. When 6 rats, each weighing about 100 g, were given by stomach tube 30 g of TEM, the animals showed no ill effects and all survived (1802).

In a study by Orten and Dajani (1167) in which male weanling hamsters were maintained for 28 weeks on diets containing 5-15% glyceryl monostearate, the animals of the 15% group showed a slight weight loss while the 5% hamsters exhibited a higher weight gain then the controls; no consistent pathological changes were observed.

In a test by Ostwald (1174), guinea pigs receiving more than 5 ml daily of a 50% glycerine solution by stomach tube died with acutely toxic symptoms. The red blood count of the guinea pigs receiving glycerine dropped during the experiment, indicating a probable anemic effect (1174). Solemides (1467) reported that i.v. or i.p. administration of 1 ml of glycerine to guinea pigs resulted in the death of the animals within 14-22 days whereas administration of the same amount s.c. caused only a temporary weight loss, the animals returning to normal within 15 days. Autopsy of the animals which died revealed extensive injury to the lungs, liver, and kidneys (1467). Hine et al. (646) reported that tremors of the head and body, initiated by auditory stimuli, occurred in guinea pigs immediately after administration of glycerine and that death was usually preceded by tremors. Histological examination revealed hyperemia of the pylorus and small intestine, the lungs were usually congested, and the spleen appeared pale (646).

Ostwald (1174) reported that doses of 10 ml of 50% glycerine solution daily for 3-40 days were well tolerated by rabbits. Deichmann (318) found that lethal doses of glycerine produced, in the rabbit, restlessness, mild cyanosis, diminished arterial blood pressure, increased rate and magnitude of respiration, followed by decreased respiration, debility, diuresis, tremor, collapse, clonic convulsions, coma, and death in respiratory and circulatory failure. Sublethal doses produced similar but less severe effects except that the debility, diuresis, loss of appetite and the consequent loss of weight were more pronounced (318).

Two-year feeding studies in which dogs were maintained on diets containing 0, 5, 10, or 20% natural or synthetic glycerine revealed no pathological or toxicological effects attributable to glycerine ingestion (1805). No abnormalities were observed by Johnson et al. (740) in 3 dogs maintained for 50 weeks on a diet containing 35% glycerine. Kopf et al. (822) reported 2 dogs to be unaffected by the administration, per os, of 2 ml of glycerine/kg twice weekly for 4 months.

No histopathological changes nor abnormalities in growth or mortality were observed in 8 dogs maintained for 1 year on diets

containing 0.25 or 2.5% oxystearin (1801). In a study by Koppanyi and Dardin (1801), after 25 months on diets containing 5, 10, or 20% TEM, all treated dogs were still alive and in excellent condition. Oral administration of 27 g of TEM/kg to 6 dogs was found by Sourkes and Koppanyi (1473) to produce no untoward effects.

In the treatment of 37 cases (human) of cerebral edema caused by acute cerebral infarction, Meyer et al. (1053) noted that daily administration of 1.2 g of glycerine/kg i.v. or 1.5 g of glycerine/kg p.o. produced neurological improvement in all cases during and at the end of 4 days of treatment. No toxic effects were observed which were attributable to glycerine administration (1053). When 14 graduate students were given, by Johnson et al. (740), 30 ml of a 95% glycerine solution daily for a period of 50 days, the only effect observed was a slight tendency toward an increase in body weight. D'Alena and Ferguson (290) reported that an 83-year-old woman who received 200 ml of 50% glycerine per os developed, approximately 1.5 hours later, a headache in the occipital region, shaking of the right arm, quivering of the eyes, and nausea. A second patient, a 68-year-old diabetic women, who received a total of 280 ml of a 50% glycerine solution within a period of 3 days developed severe diabetic acidosis (290).

GLYCERIN

Chemical Information

I. Nomenclature

A. Common Names

1. Glycerol
2. Glycerin
3. Glycerine
4. Glycyl alcohol
5. 1,2,3-Propanetriol
6. 1,2,3-Trihydroxypropane

B. Chemical Name

Glycerol

C. Trade Names

(none)

D. Chemical Abstracts Registry No.

000056815

II. Empirical Formula

$C_3H_8O_3$

III. Structural Formula

```
   H
   |
H-COH
   |
H-COH
   |
H-COH
   |
   H
```

IV. Molecular Weight

92.09

V. Specifications

Food Chemical Codex

Assay	Not less than 95.0% of $C_3H_8O_3$.
Specific gravity	Not less than 1.249.
Limits of Impurities	
Acrolein, glucose, and ammonium compounds	Passes test.
Arsenic (as As)	Not more than 3 ppm.
Butanetriols	Not more than 0.2%.
Chlorinated compounds (as Cl)	Not more than 30 ppm.
Color	Passes test.
Fatty acids and esters	Passes test (limit about 0.1%, calculated as butyric acid).
Heavy metals (as Pb)	Not more than 5 ppm.
Readily carbonizable substances	Passes test.
Residue on ignition	Not more than 0.01%.

VI. Description

A. General characteristics

Glycerin is a clear, colorless, syrupy liquid, having a sweet taste. It has not more than a slight characteristic odor, which is neither harsh nor disagreeable.

B. Physical Properties

Glycerin is hygroscopic and its solutions are neutral. Glycerin is miscible with water and with alcohol. It is insoluble in benzene, in chloroform, in ether, in carbon tetrachloride, in carbon disulfide and in fixed and volatile oils. It has a boiling point of 290°C, melting point at 18.6°C, a density of 1.2613 at 25°C, and a viscosity of 954 cps, at 25°C. Pure glycerin flashes at 177°C.

C. Stability

Store in tight containers.

VII. Analytical Methods

Glycerin occurs in many commercial products. The official method for separating glycerin from dry wines, beer, lemon, lime, and orange extracts involves evaporating the sample and treating the residue with milk of lime, then re-evaporating. The sample is washed with alcohol and ether, followed by evaporation. Loss on ignition of the residue equals the weight of the glycerin (42). Glycerin can also be determined by oxidation with dichromate using back titration with a ferrous ammonium sulfate solution (42). Glycerin can be detected in eggs and egg products by oxidation with periodate to yield formic acid. Then, it is titrated with sodium hydroxide to a light purple end point (42).

Glycerin can be qualitatively detected by heating with potassium bisulfate; pungent vapors of acrolein are evolved.

DeSimone and Vicedomini have separated glycerol from several other diols, triols, and tetrols using thin-layer chromatography on lead (II) impregnated Silica Gel H/plates. The liquid phase consisted of a mixture of ethanol, ammonia, and water. In an alkaline medium, a complex formation occurrs between lead (II) and polyols; this formation is affected by the number and position of the hydroxyl groups. The mobility of the polyols decreases with an increase in the number of adjacent hydroxyl groups. Glycerol was separated from 1,3-propanediol ethylene glycol, 1,2,5-trihydroxypentane, 1,2,4-butanetriol propylene glycol, erythritol, mannitol, dulcitol, and sorbitol (329).

VIII. Occurrence

A. Animal

Glycerin is obtained from natural oils and fats as a by-product in the manfacture of soaps and fatty acids. The methods commonly used involve saponification by alkali, hydrolysis with water, or alcoholysis.

In animals, glycerin may be synthesized from carbohydrates or glycogen by glycolysis (Embden-Meyerhof Pathway).

B. Synthetic

Glycerin can be synthesized from propylene by cracking petroleum.

C. Bacterial

Glycerin may be produced from sugars by microbial fermentation.

Diacetyl tartaric acid esters

Chemical Information

I. Nomenclature

A. Common Name

Diacetyl tartaric acid esters of mono- and diglycerides

B. Chemical Name

Tartaric acid esters, diacetylated, of mono- and diglycerides

C. Trade Name

None

D. Chemical Abstracts Registry No.

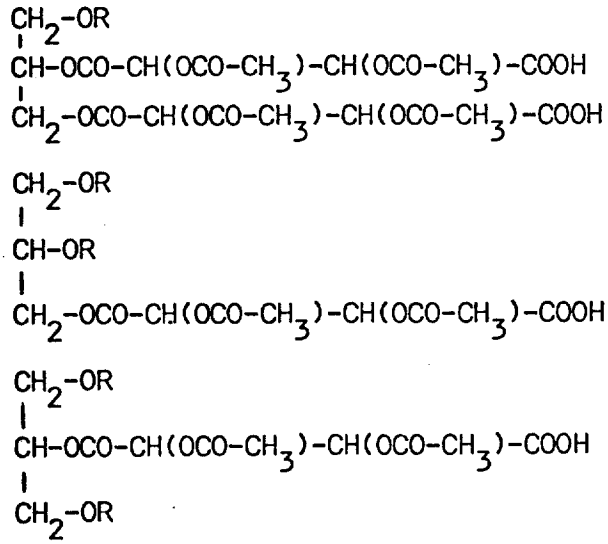
977051298

II. Empirical Formula

(not applicable)

III. Structural Formula

Major components are:



Where R represents various saturated and unsaturated fatty acid moiety or hydrogen in II or III.

The product consists of esters made by the interaction of diacetyl tartaric anhydride with monoglycerides, or with mono-diglyceride mixture made from edible oils, fats or fatty acids therefrom. The material appearing in commerce often consists of mixtures of the product described above with mono- and diglycerides.

V. Specifications

Food Chemicals Codex

Assay for tartaric acid	Between 17.0 and 20.0 grams of tartaric acid ($C_4H_6O_6$) per 100 grams after saponification.
Acetic acid	Between 14.0 and 17.0 grams of CH_3COOH per 100 grams after saponification.
Acid value	Between 62 and 76.
Fatty acids, Total	Not less than 56.0 grams of total fatty acids per 100 grams after saponification.
Glycerin	Not less than 12.0 grams of $C_3H_8O_3$ per 100 grams after saponification.
Saponification value	Between 380 and 425.
Limits of Impurities	
Arsenic (as As)	Not more than 3 ppm (0.0003%.
Heavy metals (as Pb)	Not more than 10 ppm (0.001%.
Residue on ignition	Not more than 0.01%.

VI. Description

A. General Characteristics

The esters range in appearance from sticky, viscous liquids through a fat-like consistency to a waxy solid, depending upon the iodine value of the oils or fats used in their manufacture; they have a faint acid odor.

B. Physical Properties

Diacetyl tartaroyl esters are miscible in all proportions with oils and fats. They are soluble in most common fat solvents, in methanol, in acetone, and in ethyl acetate, but insoluble in other alcohols, in acetic acid, and in water. They are dispersible in water and resistant to hydrolysis for moderate periods of time. The pH of a 3 percent dispersion in water is between 2 and 3.

C. Stability

Store in well closed containers.

VII. Analytical Methods

Diacetyl tartaric acid esters of mono- and diglycerides occur in many commercial products. They can be identified by dissolving in methanol and adding lead acetate T.S.; a white, flocculent, practically insoluble precipitate is formed.

Diacyltartaric acid was isolated from milk products and identified by thin-layer chromatography on cellulose powder. Ten grams or milliliters of the sample were triturated with annealed sodium sulfate, and mixed with dimethyl sulfoxide (DMSO), for the purpose

of albumin denaturation and to precipitate the fat-albumin complex. After several mixings with chloroform, the DMSO - chloroform extracts were filtered. The chloroform was brought into contact with a large water phase to purify the chloroform phase. This chloroform phase plus the phase between the chloroform and water phase was then evaporated; the residue was hydrolysed with saturated barium-hydroxyl solution. Barium citrate and barium tartrate precipitated, were filtered (separating them from lactic acid), and the acid released by a solution of potassium hydrogen sulfate. These acids were mixed in an acetone-ether solution and chromatographed on cellulose 300, using a pentanol-formic acid-water liquid phase. After spraying with a pyridine-acetic acid anhyhydride agent, the tartaric acid became a light blue and the citric acid was a greenish-yellow (flourescence) under ultra violet light (521). (See mono- and diglycerides for further analytical methods).

VIII. Occurrence

Synthetic

Diacetyl tartaric acid esters are made by the interaction of diacetyl tartaric anhydride with monoglycerides, or with mono-diglyceride mixture made from edible oils, fats, or fatty acids therefrom.

TRIACETIN

Chemical Information

I. Nomenclature

A. Common Names

1. Triacetin
2. Glyceryl triacetate
3. Glycerol triacetate

B. Chemical Name

Triacetin

C. Trade Name

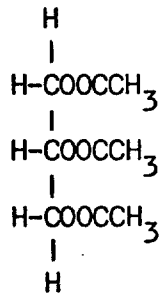
1. Enzactin
2. Vanay

D. Chemical Abstracts Registry No.
000102761

II. Empirical Formula

$C_9H_{14}O_6$

III. Structural Formula



IV. Molecular Weight

218.21

V. Specifications

Food Chemicals Codex

Assay	Not less than 98.5% of $C_9H_{14}O_6$.
Refractive index	Between 1.429 and 1.431 at 25°
Specific gravity	Between 1.154 and 1.158.
Limits of Impurities	
Acidity	Passes test.
Arsenic (as (As)	Not more than 3 ppm.
Heavy metals (as Pb)	Not more than 10 ppm.
Unsaturated compounds	Passes test.
Water	Not more than 0.2%.

VI. Description

A. General Characteristics

Triacetin is a colorless, somewhat oily liquid with a slight, fatty odor, and a bitter taste.

B. Physical Properties

Triacetin is soluble in water, and is miscible with alcohol, with ether, and with chloroform. It distills between 258° and 270°. The melting point is -78°C, boiling point 258-260°C, and has a refractive index of 1.4307.

C. Store in tight containers, and do not permit contact with metal.

VII. Analytical Methods

Triacetin is used in many commercial products. It can be identified by heating with potassium bisulfate; pungent vapors of acrolein are evolved.

Kogan and Strezlick have purified triacetin from liquid and dried egg whites using gas-liquid chromatography. First, the triacetin from the liquid egg white was extracted with diethyl ether (dried egg whites were reconstituted with water prior to extraction). This extract was purified by injecting into the gas chromatogram and subsequently collecting the eluted vapors. An infra-red spectra was determined for the eluted triacetin, and the spectra was compared to a triacetin standard for verification (814). (See mono-and diglycerides for further identification)

VIII. Occurrence

Synthetic

Triacetin is prepared by acetylation of glycerol or by the reaction of oxygen with a liquid phase mixture of allylacetate and acetic acid using bromide as a catalyst.

Mono- and Diglycerides

Chemical Information

I. Nomenclature

A. Common Names

Monoglycerides and Diglycerides

B. Chemical Names

Glycerol monostearate or Monostearin
Glycerol monopalmitate or Monopalmitin
Glycerol monooleate or Monoolein

C. Trade Name

none

D. Chemical Abstracts Registry No.

977051323

II. Empirical Formula

(Not applicable)

III. Structural Formula

α-mono-	β-mono-	αβ-di-	αα'-di-
CH_2OOCR	CH_2OH	CH_2OOCR	CH_2OOCR
$CHOH$	$CHOOCR$	$CHOOCR$	$CHOH$
CH_2OH	CH_2OH	CH_2OH	CH_2OOCR

where -OOCR represents the fatty acid moiety.

IV. Molecular Weight

Glyceryl monostearate 352
Glyceryl distearate 624

These are two major components of the commercial material.

V. Specifications

Food Chemicals Codex

The following specifications should conform to the representations of the vendor: 1-Monoglyceride content, Total monoglycerides. Hydroxyl value, Iodine value, and Saponification value.

Limits of Impurities

Acid value	Not more than 6.
Arsenic (as As)	Not more than 3 ppm.
Free glycerin	Not more than 7%.
Heavy metals (as Pb)	Not more than 10 ppm.
Residue on ignition	Not more than 0.5%.

VI. Description

A. General Characteristics

Mono- and diglycerides are white to cream colored fats of waxy appearance, plastic products, or viscous liquids.

B. Physical Properties

Mono- and diglycerides are insoluble in water, and soluble in ethanol, chloroform, and benzene. They have an infrared absorption curve characteristic of a partial ester of a polyol.

VII. Analytical Methods

Mono-, di-, and triglycerides occur in many commercial products. The official method for the separation of mono-, di-, and triglycerides from monoglyceride concentrates, is based on liquid chromatography using silica gel. The sample is dissolved in trichloro methane and applied to the column. First, benzene is applied to the column to elute the triglycerides. Ten percent ether elutes the diglycerides plus free fatty acids (FFA); ether elutes the monoglycerides. Finally, the tri-, di-plus FFA, and monoglycerides are evaporated and weighed. To determine the FFA content of the weighed diglyceride fraction, warm alcohol is added and then titrated with sodium hydroxide using phenolphthalein as a indicator (42).

Mono- and diglyceride can be separated from lard and bread by a series of extractions with petroleum ether, acetone, ethyl ether, and methanol, followed by crystallization (853).

Berner has separated mono-, di-, and triglycerides from soybean oil using thin layer chromatography. The separated zones were then extracted with dichloromethane, and the extracts were saponified with potassium hydroxide and filtered. The filtrate was analysed by an unspecified enzymatic method to obtain quantitative glycerol content of the glycerides (91).

Calderon and Baumann suggest using gel permeation chromatography on Sephadex to separate diglycerides of long-chain fatty acids from fatty alcohols. Separation is based on differences in molecular weights or molecular shapes (195).

Kuksis has identified diglycerides from cod liver, maize, and linseed oils according to carbon number and degree of unsaturation by gas chromatography. The diglycerides were chromatographed as the trimethylsilyl ethers and acetates. Among the diglycerides identified were palmitosterin, palmitolein, palmitolinolein, distearin, stearolein, oleolinolein, and dilinolein. Quantitative recoveries were achieved (854).

Blum and Koehler used gas liquid chromatography to quantitatively identify mono- and diglycerides in shortenings and margarines. He used a hydrogen flame detector with temperature programming. Glycerol came off the column at 90°C. Monoglycerides came off between 165-205°C; diglycerides came off between 240-255°C. Prior to injection an internal standard, cholestryl acetate, was added to the mixture, which was subsequently silylated. Identification was based on the comparison of retention times of pure standards to the samples (120).

Kresze et al. also used gas chromatography with temperature programming of the trimethylsilyl derivatives to separate monoglycerides (alpha-capron, alpha-caprylin, alpha-caprin, alpha-laurin, beta-laurin, alpha-myristin, alpha-palmitin, alpha-stearin, alpha-olein). Diglycerides were similarly separated. Then the nuclear magnetic resonance spectrum of the silylated derivitives were analyzed to distinguish between the alpha, -alpha' diglyceride and the alpha, -beta diglyceride of a particular fatty acid (835).

By equipping gas chromatograph with a micro reactor apparatus (MRA), Bitner et al. have developed a new method for determining linoleic acid, other conjugatable dienes and nonconjugatable dienes in triglyceride esters, simple esters, and fatty acid mixtures. A mixture of the sample fat and tetramethylammonium hydroxide is injected into a MRA reaction chamber simultaneously conjugating polyunsaturation and forming methyl esters. This subsequent reaction mixture is injected into the injection part of the gas chromatograph. The conjugatable dienes present can be calculated by integration of peak areas. The advantages of this method are those of ease, microscale capability, speed, and versatility (112).

Baur and Distler determined quantitatively, the 1-monoglyceride content of fat samples using the revised Miner procedure for periodic acid oxidation. Samples are dissolved in trichloro methane before oxidizing. A periodic acid-water solution extracts and oxidizes free glycerine; a periodic acid-methanol solution oxidizes monoglyceride plus glycerine. The amount of unused periodic acid is determined iodometrically. Monoglyceride content equals the difference in periodic acid consumption between the above two solutions (72).

In a later study Distler and Baur used the revised Miner procedure to separate the 1-monoglycerides from shortenings, a "commercial" concentrate, and one distilled concentrate, with

monoglyceride levels ranging from 2-90%. This method was more precise at the lower levels (344).

VIII. Occurrence

A. Synthetic

Mono- and diglycerides are usually manufactured by the glycerolysis of edible fats and oils. They may also be prepared by esterification of fatty acids with glycerol, with or without molecular distillation of the product.

B. Animal

Natural fats consist mainly of mixed triglycerides. Mono- and diglycerides do not occur in gross quantities in fats, except those that have undergone hydrolysis. Minute quantities are found as mixtures with triglycerides in practically all fats.

C. Plant

Vegetable oils consist of mono-, di-, and triglycerides.

Peanut Stearine

Chemical Information

I. Nomenclature

A. Common Names

1. Peanut oil
2. Arachis oil
3. Ground nut oil
4. Earth nut oil
5. Katchung oil

B. Chemical Name

Peanut stearine

C. Trade Name

None

D. Chemical Abstracts Registry No.

977051594

II. Empirical Formula

(Not applicable)

III. Structural Formula

Peanut stearine is a mixture of glycerides of the following fatty acids: palmitic (8.3%), stearic (3.1%), arachidic (2.4%), behenic (3.1%), lignoceric (1.1%), oleio (56.0%), and linoleic (26.0%). Traces of capric and lauric acids have been reported. There is an 0.8% unsaponifiable fraction which includes tocopherols, sterols, squalene, and other hydrocarbons.

IV. Molecular Weight

(Not applicable)

V. Specifications

(Not applicable)

VI. Description

A. General Characteristics

Peanut stearine is a greenish-yellow, almost colorless oil with a mild pleasant odor and bland taste.

B. Physical Properties

Peanut stearine is miscible with ether, petroleum ether, chloroform, and carbon disulfide. It is soluble in benzene, carbon tetrachloride, oils, and very slightly in alcohol. At 25° it has a density of 0.910-0.915; at low room temperature, it clouds.

Peanut stearine solidifies at about -5°, has an A.S.T.M. cloudpoint at +4.5° and an A.S.T.M. pour point at +1°. It has a flash point at 283°C and an ignition temperature at 443°C. The refractive index at 2.5°C is 1.466-1.470.

C. Stability

On prolonged exposure to air, peanut stearine very slowly thickens and becomes rancid.

VII. Analytical Methods

Peanut stearine occurs in many commercial products. Peanut stearine (oil) was extracted from freshly roasted peanuts by Boudreaux et al. After the skin-free cotyledons and germs were flaked, the oil was extracted with hexane (142).

Fryer et al. have identified peanut oil qualitatively using gas-liquid chromatography. While the product undergoes moderate degradation in the column, a reproducible "finger print" elution curve has been obtained (483).

VIII. Occurrence

Plant

Peanut stearine is prepared by pressing the shelled and skinned seeds of Arachis hypogaea L., Leguminosae.

GLYCERIN AND GLYCERIDES

Biological Data

1. Acute toxicity

Substance	Animal	Sex & No.	Route	Dosage g/kg Bd. Wt.	Measurement	Ref.
Glycerine	rats	-	p.o.	27.50	LD_{50}	1458
Glycerine	guinea pigs	-	p.o.	7.75	LD_{50}	1458
Glycerine	mice	-	p.o.	18.9-23.3	LD_0	891
Glycerine	mice	-	p.o.	47.3-56.7	LD_{100}	891
Glycerine	guinea pigs	-	s.c.	12.6	LD_0	891
Glycerine	guinea pigs	-	s.c.	15.8	LD_{100}	891
Glycerine	mice	80	i.v.	4.3	LD_{50}	29
Glycerine	mice	120	p.o.	20.7	LD_{50}	29
Glycerine	mice	91 M	p.o.	23.0	LD_{50}	646
Glycerine	rats	12 F	p.o.	27.2	LD_{50}	646
Glycerine (Nat)	guinea pigs	M	p.o.	10.0	LD_{50}	646
Glycerine (Syn)	guinea pigs	M	p.o.	11.5	LD_{50}	646
Glycerine	rats	20	i.m.	7.6	MLD	155
Glycerine	rats	19	s.c.	15.1	MLD	155
Glycerine	mice	-	p.o.	26.09	LD_{50}	447
Glycerine DAB VI*	mice	-	p.o.	30.21*	LD_{50}	447
Glycerine DAB VI*	mice	-	s.c.	10.78*	LD_{50}	447
Glycerine DAB VI*	rats	-	p.o.	46.72*	LD_{50}	447
Glycerine DAB VI*	rats	-	s.c.	16.69*	LD_{50}	447
Glycerine	mice	-	s.c.	10.1	LD_0	890
Glycerine	mice	-	s.c.	12.6	LD_{50}	890
Glycerine	mice	-	s.c.	15.1	LD_{100}	890
Glycerine	mice	-	s.c.	10.1	MSD**	890
Glycerine	mice	-	s.c.	8.8	MND***	890
Glycerine	mice	-	p.o.	25.2	LD_0	890
Glycerine	mice	-	p.o.	31.5	LD_{50}	890
Glycerine	mice	-	p.o.	40.3	LD_{100}	890
Glycerine	mice	-	p.o.	12.6	MSD**	890
Glycerine	mice	-	p.o.	10.1	MND***	890
Glycerine	mice	-	i.v.	6.3	LD_0	890
Glycerine	mice	-	i.v.	7.6	LD_{50}	890

Substance	Animal	Sex & No.	Route	Dosage g/kg Bd. Wt.	Measurement	Ref.
Glycerine	mice	-	i.v.	8.8	LD_{100}	890
Glycerine	mice	-	i.v.	3.8	MSD**	890
Glycerine	mice	-	i.v.	2.5	MND***	890
Triacetin	mice	60	i.v.	1.6	LD_{50}	1760
Triacetin	mice	40	s.c.	2.7	LD_{50}	922
Triacetin	mice	30	s.c.	3.2	LD_{50}	922
Triacetin	mice	M	p.o.	1.8	LD_{50}	508
Triacetin	mice	F	p.o.	1.1	LD_{50}	508
Triacetin	mice	M	i.p.	1.7	LD_{50}	508
Triacetin	mice	F	i.p.	1.4	LD_{50}	508
Triacetin	rats	-	s.c.	3.25	LD_{50}	508
Triacetin	rats	-	i.p.	2.1	LD_{50}	508

* Glycerine, DAB VI is 83-87% pure glycerine

** Minimum symptomatic dose

*** Maximum non-symptomatic dose

Mice

Undiluted synthetic and natural glycerine were administered intragastrically by Hine et al. to 91 male Swiss inbred white mice (20 ± 2 g after fasting 6 hours) in doses of 15 to 31.5 g/kg. Mortality, weight changes, and physiologic signs were recorded over a 10-day period. Examinations were made for gross lesions in all animals which died and in selected survivors which were killed for the purpose. Specimens of brain, heart, liver, spleen, stomach, intestine, and kidney were taken routinely for histological study. The chief pharmacologic effect was stimulation of the central nervous system; body tremors, erection of the tail in the typical Straub manner, and generalized clonic convulsions preceded all observed deaths. The approximate LD_{50} was calculated as 23.0 g/kg for both natural and synthetic glycerine. Hyperemia of the small intestine and lungs occurred in some mice of both groups at the higher dosage levels. Slight hyperemia of the kidneys and of the mucosa of the small intestine were also noted (646).

In a comparison of natural and synthetic glycerine, Anderson et al. were unable to show any statistical difference between the acute toxicities of the two products. LD_{50}'s of 4.3 and 20.7 g/kg, respectively, were established for intravenous and oral administration to mice (i.v. - 80 mice; p.o. - 120)(29).

Latven and Molitor investigated the toxicity of glycerol by i.v., s.c., and p.o. administration to white mice. The compound was tested at six dosage levels with at least four mice on each dose. The number of deaths during the first 24 hours was used to calculate the toxicity figures. The results of the test are presented in Table II (890).

TABLE II. Acute toxicity of glycerol in white mice (ml/kg body wt).

route	LD_0	LD_{50}	LD_{100}	MSD*	MND**
s.c.	8.0	10.0	12.0	8.0	7.0
p.o.	20.0	25.0	32.0	10.0	8.0
i.v.	5.0	6.0	7.0	3.0	2.0

* MSD - minimum symptomatic dose

** MND - maximum non-symptomatic dose

Wrestlind investigated the acute toxicity of triacetin by injecting 25% emulsions into tail veins of mice (60) weighing from 13 to 29 g. Injection of triacetin produced almost immediate convulsions, failure of the righting reflexes and respiratory arrest. In some animals respiration did not return and death occurred in 1-3 minutes; other animals started to breathe spontaneously within a few minutes and survived. An LD_{50} of 1600 mg/kg was calculated (1760).

Li et al. studied the acute toxicity of mono- and di-, and triacetin through subcutaneous injection of the compounds in 100 inbred white mice, weighing 17-21 g. The animals were observed for 5 days after injection. Animals dying during the period of observation were examined and the following organs were removed for microscopic study: lungs, heart, liver, spleen and kidney. Gross observations of animals poisoned by monacetin revealed dilatation of the heart and diffuse congestion of the lungs. In animals dying as a result of the toxic effects of diacetin and triacetin hemorrhagic areas in the lungs appeared frequently. Preliminary studies of sections of tissues showed some cloudy swelling of the convulted tubules of the kidney and in some cases the lumen was filled with casts. Hydropic degeneration and necrosis of the tubules was noted in some areas. The liver appeared to be congested. Animals receiving fatal doses usually died in from 20 minutes to 3-4 hours after injection, exhibiting symptoms of marked depression, weakness, prostration and in some animals, labored respiration just before death. Animals in which death was delayed for 24 hours or more generally showed no symptoms except slight transient depression soon after injection. LD_{50}'s of 3.5, 2.5, and 2.3 ml/kg, respectively, were calculated for mono-, di-, and triacetin (922).

Rats

undiluted synthetic and natural glycerine were adminstered intragastrically by Hine et al. to 12 female rats of the Long-Evans strain (114 ± 8 g after fasting 22-24 hours) in doses of 27.26 g/kg. Mortality, weight changes, and physiologic signs were recorded over a 10-day period. Examinations were made for gross lesions in all animals that died and in selected survivors that were killed for the purpose. Specimens of brain, heart, liver, spleen, stomach, intestine, and kidney were taken routinely for histological study. The chief pharmacologic effect was stimulation of the central nervous system. Deaths of the rats were preceded by muscle spasms and clonic convulsions; respiratory failure occurred 15-135 minutes after administration. Survivors appeared normal within 2.5 hours after administration. The LD50 was calculated to be 27.2 g/kg for both synthetic and natural glycerine. Hyperemia of the pylorous and of the small intestine was noted in all of the rats. The lungs were usually congested and the spleen appeared pale. Three rats showed hyperemia of the cerebral meninges (646).

While investigating the toxicity of glycerol in white rats Deichmann obtained the following results:

SUMMARY OF LETHAL EFFECTS OF GLYCEROL ON WHITE RATS

Number of Rats	Dose cc/kg	% Dead	Time Till Death
	Oral Administration		
5	13	0	
5	15	0	
5	17	20	3 hr.
5	19	20	10 hr.
5	21	40	2 hr.
5	23	60	2–10 hr.
	Subcutaneous Administration		
5	7	20	3 days
5	9	0	
10	11	20	3 hr., 4 days
10	13	30	1 to 3 days
10	15	60	3 hr. to 3 days
10	16	70	10 hr. to 4 days
10	17	90	1 to 9 hr.
	Subcutaneous Administration to Rats Starved for 3 Days		
5	13	80	1 to 3 hr.
5	15	80	3 to 4 hr.
5	16	100	2 to 3 hr.
5	17	80	1 to 3 hr.
	Intraperitoneal Administration		
5	1		
10	3	10	3 days
10	4	20	25 min. to 2 days
10	5	40	50 min. to 3 days
10	6	80	30 to 100 min.
5	7	100	20 to 50 min.
5	9	100	30 to 60 min.
5	13	100	15 to 30 min.

Repeated and extensive applications of glycerol, alone or in 50% aqueous solutions, upon the skin of rats did not induce definite or fatal intoxication. Regardless of the mode of administration, lethal doses of glycerol produced restlessness, mild cyanosis, diminished arterial blood pressure, increased rate and magnitude of respiration, followed by decreased respiration, debility, diuresis, tremor, collapse, clonic convulsions, coma, and death in respiratory and circulatory failure. Sublethal doses produced similar but less severe effects except that the debility, diuresis, loss of appetite and the consequent loss of weight were more pronounced. Several rats remained inactive for four days, but the rats recovered after having suffered moderately severe convulsions. The rats showed no changes in alimentary activity. Hemoglobinuria occurred regularly after subcutaneous injection, occasionally after intravenous and intraperitoneal injection, but never after cutaneous or oral administration (318).

The acute toxicity of glycerine was examined by Braun and Cartland by intramuscular and subcutaneous injections in rats (i.m.-20; s.c.-19). An MLD of 7.6 g/kg was established for intramuscular glycerine and 15.1 g/kg was set as the MLD for subcutaneous glycerine (155).

Twenty-seven acute experiments were performed by Johnson et al. in which, under light ether anesthesia, doses of glycerol ranging from 7 to 18 g/kg were administered by stomach tube to 11 rats which had been starved for 24 hours. In no case were ill effects observed in the ensuing 24 hours (740).

Li et al. studied the acute toxicity of mono-, di-, and triacetin through subcutaneous injection of the compounds in 105 inbred albino rats weighing from 200 to 350 g. The animals were observed for 15 days after injection. Animals dying during the period of observation were examined and the following organs were removed for microscopic study: lungs, heart, liver, spleen and kidney. Gross observations of animals poisoned by monacetin revealed dilatation of the heart, diffuse congestion of the lungs, and in 2 rats, a small amount of fluid in the pleural cavity. In animals dying as a result of the toxic effects of diacetin and triacetin hemorrhagic areas in the lungs appeared frequently. Preliminary studies of sections of tissues showed some cloudy swelling of the convoluted tubules of the kidney and in some cases the lumen was filled with casts. Hydropic degeneration and necrosis of the tubules was noted in some areas. The liver appeared to be congested. Animals receiving fatal doses usually died in from 20 minutes to 3-4 hours after injection, exhibiting symptoms of marked depression, weakness, prostration and in some animals, labored respiration just before death. Animals in which death was delayed for 24 hours or more generally showed no symptoms except slight transient depression soon after injection. LD_{50}'s of 5.5, 4.0, and 2.8 ml/kg, respectively, were determined for mono-, di-, and triacetin (922).

Ambrose and Robbins investigated the acute toxicity of a mixture of acetostearin and acetoolein by administration of the mixture, by gastric intubation, to mature male (10) and female (10) rats. A 40% emulsion containing 20 g of acetostearin, 20 g of acetoolein, 2 g of Span 20, and 1 g of Tween 60 was employed. No obvious toxic symptoms or ill effects were observed within 4 weeks following administration of 4 g/kg of this emulsion (24).

Thirteen adult female rats were given by stomach tube 10 ml each of a suspension of oxystearin containing 288 mg/ml. This corresponded to a dose of approximately 16000 mg/kg. All rats survived. In another study 13 adult female rats were given 10 cc of a similar suspension of oxystearin intraperitoneally. This dose corresponded to approximately 14000 mg/kg. All animals survived 24 hours. After allowing these rats to survive about 5 months masses of material were found in the abdominal cavities. The pathologist determined that the rats had treated the injected oxystearin as a typical foreign body (1801).

Koppanyi and Dardin reported that when 6 rats, each weighing about 100 g, were given by stomach tube 30 g of TEM (the diacetyl tartaric acid esters of mixed mono- and diglycerides obtained from natural fats), the animals showed no ill effects and all survived (1802).

Guinea pigs

In a study of acute toxicity, undiluted synthetic and natural glycerine were administered intragastrically by Hine et al. to 29 male guinea pigs (325 ± 50 g). Mortality, weight changes, and physiologic signs were recorded over a 10-day period. Examinations were made for gross lesions in all animals that died and in selected survivors that were killed for the purpose. Specimens of brain, heart, liver, spleen, stomach, intestine and kidney were taken routinely for histological study. The chief pharmacologic effect was stimulation of the central nervous system. Tremors of the head and body, initiated by auditory stimuli, occurred in the guinea pigs immediately after injection. Death was usually preceded by tremors, but animals in which tremors developed did not invariably die. Histological examination revealed hyperemia of the pylorus and small intestine, the lungs were usually congested, and the spleen appeared pale. The LD_{50}'s were calculated as 10.0 ± 1.3 g/kg for natural glycerine and 11.5 ± 2.8 g/kg for synthetic glycerine (646).

Solomides reported that i.v. or i.p. administration of 1 ml of glycerine to guinea pigs resulted in death of the animals within 14-22 days whereas administration of the same amount subcutaneously caused only a temporary weight loss, the animals returning to normal within 15 days. Autopsy of the animals which died revealed extensive injury to the lungs, liver, and kidneys. The lungs showed extensive hemorrhagic edema of alveoli with diffuse infiltration of the stroma. Venous and capillary ectasia, perivasculary edema, hemorrhagic areas and steatosis of hepatic cells were the more striking histological alterations of the hepatic tissue. Granulary necrosis was observed in the kidneys. Dilution of the glycerine dose with water drastically reduced its toxicity. An i.p. injection of 1 ml of physiological serum immediately following the i.p. injection of 1 ml of glycerine reduced the toxicity of the glycerine to such an extent that no untoward effects were observed (1467).

Rabbits

While investigating the toxicity of glycerol in albino rabbits, Deichmann obtained the following results:

SUMMARY OF EFFECTS OF GLYCEROL ON ALBINO RABBITS

Number of Rabbits Employed	Dose cc/kg	Fate
	Single Oral Administration	
4	6 to 12	inactivity, tremor, recovered
3	14 to 18	convulsions, death in 100 to 120 min.
	Single Subcutaneous Administration	
6	4 to 8	slight diarrhea, recovered
5	10 to 18	inactivity, tremor, death in from 2 hours to 3 days
	Single Intraperitoneal Administration	
4	4 to 6.5	inactivity, convulsions, recovered
3	7 to 9	convulsions, death in 60 to 110 min.

Repeated and extensive applications of glycerol, alone or in 50% aqueous solutions, upon the skin of rabbits did not induce definite or fatal intoxication. Regardless of the mode of administration, lethal doses of glycerol produced restlessness, mild cyanosis, diminished arterial blood pressure, increased rate and magnitude of respiration, followed by decreased respiration, debility, diuresis, tremor, collapse, clonic convulsions, coma, and death in respiratory and circulatory failure. Sublethal doses produced similar but less severe effects except that the debility, diuresis, loss of appetite and the consequent loss of weight were more pronounced. The rabbits, particularly those treated orally, occasionally showed a mild diarrhea. Lethal subcutaneous doses of glycerol caused great reduction in the number of erythrocytes and leucocytes and in the hemoglobin content of the blood of rabbits, while repeated sublethal doses produced corresponding but less severe effects upon the erythrocytes and hemoglobin, and a wide fluctuation in the numbers of leucocytes. Glycerol injected into the heart produced a decrease in the number of erythrocytes and in the hemoglobin content of the blood, together with a slight increase in the number of leucocytes. Intraperitoneal injections resulted in considerable increases in leucocytes, but no other significant changes in the formed elements of the blood. Hemoglobinuria occurred regularly after subcutaneous injection, occasionally after intravenous and intraperitoneal injection, but never after cutaneous or oral administration (318).

Dogs

Johnson et al. performed 71 experiments on 22 dogs in which glycerol was given per os by stomach tube in doses from 2 to 4 g/kg. Eight of these experiments were on 6 dogs which had been starved 18-24 hours. In none of the tests were albumin or hemoglobin detected in the urine (740).

After giving 6 dogs 5 mg of morphine sulfate/kg intramuscularly to prevent vomiting, Koppanyi and Dardin administered 27 g of TEM/kg to the animals by means of a stomach tube. None of the dogs vomited

or showed diarrhea. No pathological symptoms were observed and urinalysis revealed no sugar, albumin, or casts. Gross and microscopic examination of the liver and kidneys of two of the dogs 10 days after administration of TEM, revealed no evidence of liver or kidney damage (1801).

Sourkes and Koppanyi studied the acute toxicity of the diacetyl tartaric acid esters of mixed mono- and diglycerides obtained from natural fats (TEM) by administering, by stomach tube, 27 g/kg of TEM to 6 dogs. No untoward effects were observed. Microscopic study of the kidneys of 2 dogs sacrificed 10 days after the administration of TEM revealed no evidence of nephrotic changes characteristic of tartaric acid damage (1473).

Man

D'Alena and Ferguson reported cases of two patients who had adverse effects after oral administration of glycerol. The first patient, an 82-year-old hypertensive, mentally senile woman, received 200 ml of 50% glycerol orally for primary angle-closure glaucoma. Approximately 1.5 hours later the woman developed a headache in the occipital region, shaking of the right arm, quivering of the eyes, and nausea. With the second patient, a 68-year-old diabetic woman, orally administered glycerol (a total of 280 ml of a 50% solution within a period of 3 days) was felt to be responsible for the ensuing severe diabetic acidosis (290).

II. Short-term Studies

Chicks

Groups of 10 one-day old chicks were fed diets containing dosage levels of 570 mg/kg, 2.85 g/kg and 8.55 g/kg of each of 3 emulsifiers, diaretyl tartaryl glycerol monostearate (TEM), glycerol lactopalmitate (GLP) and succinylated monoglyoride (SMG). A similar group on basal diets served as controls. At 7 weeks in the experiment, all chicks fed the highest level of each emulsifier and 2 chicks from each of the other groups were sacrificed. The remaining chicks were continued until termination of 90 days of feeding. The birds were observed for effects on growth, liver weight, cecal size and any intestinal tract change. Deleterious effects, largely limited to the high levels of feeding were: TEM caused growth depression in all groups at 7 weeks, slight hyperemia of duodenum and ileum at the 2 lower levels and moderate to severe hyperemia at the highest level. Gizzard erosion was observed in the high level and only slight changes at the intermediate level. GLP caused growth deppression at 90 days but only questionable effect at 7 weeks. Live weight was slightly increased at 7 weeks and 90 days. At 90 days there were instances of very slight hyperemia and gizzard erosion at the intermediate level. SMG caused very slight growth retardation at 90 days. Cecal size was increased at each level. Slight hyperemia and one instance of gizzard erosion occurred at the intermediate level (1803).

Rats

Guerrant et al. fed groups of 10 young growing rats, equally divided as to sex, diets containing glycerine at levels of 0, 1, 3, 6, 10, 15. 20, 30, 40, 50, and 60% for a period of 20 weeks. In groups in which high mortality was experienced during the early stage of the feeding trial, additional animals were subsequently added. Food and water were provided ad libitum and its consumption was recorded. The animals were observed daily and their body weights were recorded weekly. Hemoglobin determinations were made at regular intervals. Selected animals from each group were further observed for their inclination to exercise and other representative animals were tested for the effects of glycerine consumption on water intake and urinary output. Animals which succumbed during the test period as well as representatives from each dietary group were subjected to pathological examination (list of tissues examined was not given). Other animals were sacrificed for the purpose of determining the effect of diet on dry matter, fat and liver glycogen. The results show no marked difference in the growth response when dietary levels of 30% or less were fed, but higher levels of glycerine produced subnormal growth rate, with the majority of the animals surviving the 20-week test period. Pathological examinations revealed no changes in the tissues for animals which had received diets containing less than 10% glycerine, occasional changes at the 10% level, and frequent and pronounced changes at higher levels. In general, the most marked pathological changes found in the glycerine-fed animals were found in the liver and consisted of marked hydropic and fatty degeneration of parenchymatous cells (559).

For a period of 40 weeks, Johnson et al. maintained 51 rats on diets containing 0, 20, 41, or 61% glycerol (glycerol replaced starch). No effect on growth was observed at the lower 2 levels of treatment but when glycerol was fed at 61%, growth of the rats was greatly impaired. These rats were also less active and their coats lacked the smooth gloss of the controls. However, these effects were not resultant from any toxicity of glycerol, for at the 7th week when starch was added to the diet of 8 of the rats in the proportion of 5 g of starch for every 10 g of the diet, they immediately resumed normal growth despite an unchanged daily glycerol intake (740).

In a comparison of natural and synthetic glycerine, Anderson et al. were unable to demonstrate any statistical difference between the short-term toxicities of the two products. Fifteen female rats, 6-8 weeks old, were divided into groups of five and were given as drinking water either tap water or a 5% solution of natural or synthetic glycerine for 6 months. All rats were weighed at weekly intervals. Hemoglobin was determined, and erythrocytes, leucocytes, and differentials were counted monthly for a period of six months. The animals were then sacrificed and submitted to necropsy. Both gross and microscopic studies were made of the heart, lungs, liver, spleen, stomach, intestines, kidneys, thymus, thyroid, and adrenals. The only untoward effect exhibited by the glycerine-treated rats was the presence of calcified masses in the tubules near the junction of the kidney cortex and medulla in 6 of the 10 rats (29).

Bornmann conducted a 96-day chronic toxicity test in which groups of 6 male and 6 female rats (average weight 100 g) were given 0, 1, 2, 5, 10, or 20% aqueous solutions of glycerine as drinking water. Growth of the rats receiving 10% or less of glycerine was normal. The animals receiving 20% glycerine exhibited normal growth for the first few weeks, followed by a temporary impairment. Growth of these rats then returned to normal (136).

In a one year study 126 weanling rats (63 male and 63 female) were assigned equally to 6 diets containing 20%, 10%, or 5% Atlas synthetic glycerol (not exceeding 0.1% triols), or 20%, 10% or 5% natural glycerol and a control diet. Clinical tests on urine and blood were made. Data were collected on growth, food and caloric efficiency. Ratio of organ weights to body weights were analyzed by the analysis of variance. All animals were examined at necropsy and major tissues were saved for microscopic examination. A total of 21 tissues were examined microscopically from each of the rats in the 20% glycerol groups and the control. Five tissues (liver, kidneys, spleen, gonads and adrenals) were examined from each of the other rats. No significant differences occurred among the groups of rats when fed (for 50 weeks) diets containing 5, 10, or 20% of either Atlas synthetic glycerol or natural glycerol or among either of these experimental groups and controls (1805).

Johnson et al. reported that 30 rats maintained on diets containing 0, 20, or 41% glycerol for 25 weeks exhibited no differences in growth rate. At the close of the experiment, the rats were sacrificed and autopsied. Histological examinations of the kidneys, liver, and intestine were performed. No abnormalities were found (740).

In an experiment by Johnson et al., 10 rats were fed the same control diet for 5 weeks, after which the quantity fed was reduced to just below the minimum required to maintain their weight. A supplement of 12 g of glycerol for every 10 g of the control diet was added to the diet of 5 of the rats. The glycerol-treated rats exhibited normal growth while the control rats showed a gradual loss of weight. After 8 weeks the diets of the two groups were switched. The animals now receiving glycerol showed an immediate return to normal growth while the other rats began to lose weight (740).

Johnson et al. reported that 10 rats maintained on diets containing either 0 or 41% glycerol for 21 weeks exhibited no discrepancies in growth rate (740).

In a study by Loeser et al., 30 male and 30 female rats (120-130 g) received 1.0 ml of a 50% aqueous glycerine solution per 100 g body weight for approximately one half a year. The same number of rats received distilled water and served as controls. All of the rats developed normally and the mortality of all groups was comparable. After 6, 12, and 18 weeks, 8-20 rats from each group were sacrificed, dissected and their organs removed for histological examination. No pathological changes were disclosed and determination of liver glycogen revealed normal levels. X-ray examinations carried out on 6 animals of each group at the beginning as well as in intervals of 6 weeks, remained without abnormal findings (934).

Loeser et al. conducted a 3-month study in which young male and female rats (average weight 115 g) received as their only drinking water a 1, 2, 5, 10, or 20% solution of glycerine. Control rats received distilled water to drink. Neither the longevity nor the growth of the animals was influenced by the administration of glycerine. Two of the 12 rats receiving 20% glycerine died during the 6th week of the test; the others showed, at this point, disturbance of the development and growth but recovered and grew normally in the further course of the experiment such that at the end of the test period no indication of injury attributable to glycerine ingestion was perceivable. Post mortem examination, including histological investigation of the internal organs (spleen, stomach, kidneys, heart, adrenal, intestines, lungs, endocrine glands), revealed no peculiarities. Weight of the pituitary, thyroid, adrenals, and ovaries was normal (934).

Loeser et al. followed the growth and general behavior of young male and female rats (average weight 50 g) which, during a period of some 50 days, received by stomach tube on weekdays, 1.0 ml of a 20% aqueous glycerine solution or 1.0 ml of distilled water per 100 g of body weight. Weight gain of the treated animals was normal and no indication of any damage attributable to glycerine ingestion was noted through observation of general behavior. Mortality of the rats was unaffected and post mortem examination of the heart, liver, kidneys, stomach, intestines, bladder, spleen, and endocrine glands revealed no histological changes. Liver glycogen was normal and the pituitary, thyroid, adrenals, and ovaries were of normal weight. X-ray examinations of the teeth and legs of the rats, carried out on 4 animals of each group on test day 49, gave no support for injury of the skeletal system (934).

In a study by Stoewsand and Dymsza, male weanling Carworth strain albino rats were fed diets containing 30% fat with or without 20% supplement of glycerol for a period of 4 weeks. At the end of 4 weeks, the rats were killed and both epididymal fat pads were quantitatively excised, dried, and weighed. Liver total lipids and cholesterol were determined. These criteria, as well as weight gain, food intake, and calorie intake, revealed no adverse effects attributable to glycerol consumption (1503).

In an 11-week experiment by Holck, in which 4.5-month-old female rats (unspecified number) were given glycerine as 20% of their diet, the growth rate of the treated rats was comparable to that of the controls (664).

In a study by Stoewsand et al., male weanling albino rats (CFE Carworth strain, 40-60 g) were given 30% fat diets with or without a 20% supplement of glycerol as follows: 10 rats per each dietary treatment maintained for 4 weeks at 25°; 10 rats per each dietary treatment maintained for 4 weeks at 5°; 18 rats per each dietary treatment maintained for 5 weeks at 25°, followed by exposure to -20° until each group had 9 surviving rats. After 4 weeks, 5 rats in each of the dietary treatments at 25° and 5° were killed, liver total lipids and cholesterol were determined, and the epididymal fat pads were excised and weighed. Consideration of weight

gain, food intake, food efficiency, resistance to cold, liver total lipids and cholesterol, and epididymal adipose tissue revealed no untoward effects attributable to glycerol ingestion (1504).

To 6 groups of 20 rats each (male, 75-110 g) Fischer et al. administered, by stomach tube, 1 ml of a 0, 1, 5, 10, or 20% aqueous solution of glycerine per day for 44 days. Growth curves, lethality, and histological examination of the kidneys, livers, and bladders of the rats disclosed no untoward effects (447).

In a study by Mattson et al., 12 groups of 10 weanling, male Sprague-Dawley rats were fed diets containing various pure mono-, di-, or triglycerides at a level of 25% for a period of 10 weeks. The following results were obtained:

Average Cumulative Values and Standard Error of the Means[a] for Gain in Body Weight, Food Consumption, and Caloric Efficiency

	Third Week			Sixth Week			Tenth Week		
	Body weight gain	Food consumed	Caloric efficiency[b]	Body weight gain	Food consumed	Caloric efficiency[b]	Body weight gain	Food consumed	Caloric efficiency[b]
Soybean oil/cottonseed oil (partially hydrogenated)	g.	g.		g.	g.		g.	g.	
Triglyceride	132.9 ±2.7	225.7 ±4.7	11.1 ±0.15	240.0 ±4.9	538.1 ±12.6	8.51 ±0.11	305.0 ±7.7	938.1 ±25.0	6.20 ±0.11
Mono + Di + Tri	133.3 ±3.4	227.0 ±5.4	11.3 ±0.12	242.0 ±6.7	542.8 ±12.4	8.65 ±0.09	314.3 ±8.8	957.3 ±21.7	6.37 ±0.08
Diglyceride	129.3 ±5.9	223.3 ±7.71	11.1 ±0.19	236.2 ±7.4	534.7 ±14.7	8.49 ±0.05	311.8 ±14.8	930.8 ±31.9	6.41 ±0.12
Monoglyceride	122.7 ±3.1	212.8 ±3.2	11.5 ±0.16	207.2 ±9.4	493.6 ±12.6	8.34 ±0.20	275.8 ±9.1	866.5 ±20.3	6.30 ±0.08
Olein									
Triglyceride	115.9 ±2.7	200.4 ±5.0	10.9 ±0.15	220.1 ±3.9	491.8 ±8.0	8.52 ±0.09	287.9 ±6.1	867.2 ±15.4	6.32 ±0.07
Monoglyceride	112.5 ±2.8	198.1 ±3.6	11.1 ±0.20	210.6 ±5.0	475.7 ±8.4	8.77 ±0.13	270.0 ±4.9	850.0 ±11.7	6.29 ±0.08
Stearin									
Triglyceride	108.6 ±1.5	319.4 ±2.6	6.40 ±0.10	206.0 ±1.2	773.7 ±7.3	5.02 ±0.09	274.0 ±4.4	1417.0 ±16.2	3.64 ±0.05
Monoglyceride	95.5 ±1.6	281.9 ±3.6	6.68 ±0.10	202.6 ±2.8	716.6 ±10.0	5.50 ±0.06	271.0 ±3.1	1314.0 ±14.8	4.01 ±0.06
Laurin									
Triglyceride	113.2 ±3.3	225.3 ±4.7	9.76 ±0.18	203.9 ±7.6	518.3 ±10.2	7.60 ±0.17	276.6 ±10.1	931.7 ±10.3	5.77 ±0.09
Monoglyceride	75.0 ±2.0	171.1 ±5.4	8.09 ±0.26	155.4 ±3.3	418.7 ±10.5	7.61 ±0.12	214.8 ±5.0	779.9 ±18.4	5.65 ±0.06
Coconut oil									
Triglyceride	140.8 ±2.2	239.1 ±4.7	11.4 ±0.11	255.5 ±4.4	505.4 ±10.6	8.90 ±0.14	333.8 ±6.4	901.0 ±18.6	6.60 ±0.08
Mono + Di + Tri	130.0 ±3.0	222.5 ±4.4	11.6 ±0.11	237.1 ±5.1	524.8 ±6.8	8.83 ±0.07	304.9 ±7.3	923.9 ±12.9	6.54 ±0.11

[a] Standard error of the mean calculated by the formula $\sqrt{\frac{\Sigma(x-\bar{x})^2}{n(n-1)}}$ where x is the individual value, $\bar{x}$ is the group mean, and n is the number of observations.

[b] Body weight gain/calories consumed × 100 = caloric efficiency.

The mono-, di-, and triglycerides of corresponding fatty acid composition were found to be of equivalent caloric efficiency. The caloric efficiencies of the glycerides of pure lauric or stearic acid were found to be low, possibly as a result of poor absorption. Throughout the test all animals appeared normal and autopsies performed at the end of the experiment and histological examination of the liver, kidney, spleen, lung, heart, stomach, and small and large intestines revealed no abnormalities. The body fat of the animals was the same regardless of the type of glyceride structure fed, however, the type of body fat deposited reflected, in part, the fatty acid component of the dietary glyceride (1026).

Ames, et al. compared the utilization of monoglycerides derived from the fatty acids of cottonseed oil to cottonseed oil itself in the rat. Eighty rats were observed for growth during the initial 8-week period (27).

Level of Fat	Total No. Animals	Total No. Males	Total No. Females	Type of Fat
15%	20	10	10	Monoglycerides
25%	20	10	10	Monoglycerides
15%	20	10	10	Cottonseed Oil
25%	20	10	10	Cottonseed Oil

Then 6 females and 2 males from each group were bred; the females remained on the diet on which they started. The second generation was maintained on the same diet as were their parents for 5 weeks. Then they were bred to produce the 3rd generation. The three generations showed no significant difference in growth between monoglycerides and cottonseed oil for either the 15% or 25% level. Reproduction was essentially the same for all four groups and for every generation as regards the mean size of the litters and the mean weight of the young at birth. Lactation performance appeared the same for all 4 groups of each generation, based on weanling weights (27).

After the initial 8 week growth study, 8 male rats of the two 25% fat diets were segregated. The feces were collected and food consumption determined daily for seven days. Then the rats were placed on a fat free diet. After a seven day equilibration period, feces were collected for another seven days. There was no significant difference between either diet as to the mean daily food consumption or the mean digestive coefficient. The author concluded that the monoglycerides derived from cottonseed oil are nutritionally equivalent to cottonseed oil itself (27).

To examine the nutritive properties of certain monoglycerides, Braun and Shrewsbury incorporated monostearin (8 and 24%), monolinolein (8 and 24%, the monolinolein contained about 20% monoolein), and lard (8%) into the diets of 5 groups of rats (2 male and 2 female rats per group). Due to the slow growth of the rats given the diets containing 24% monoglycerides during the first 4 weeks, these diets were altered to contain only 8% monoglycerides for the remainder of the 8-week test period. Records of growth and food consumption indicate that synthetic monoglycerides are utilized practically as well for the growth of rats as natural triglycerides (lard) when fed at a level of 8%. Growth of the rats given monostearin, however, was somewhat slower than those on the lard or monolinolein rations. It was found that stearic acid from monostearin was not stored to any appreciable degree in the body fat of rats while linoleic acid from monolinolein was apparently stored directly in the body fat. The fatty acids fed as monoglycerides appeared in the depot fat of rats as triglycerides (156).

In a feeding study by Harris and Sherman, in which 180 male weanling rats were fed mono-, di-, and triglycerides (prepared from a mixture of partially hydrogenated soybean and cottonseed oils) and a mixture of these fats as 15% and 25% of a synthetic type diet and as 15% of a diet of natural foods for 70 days on an equi-caloric, restricted, pair-feeding basis, no significant difference in body weight gain or

caloric efficiency was observed, the fats appearing to be nutritionally equivalent (639).

Mattson et al. conducted a series of experiments in which groups of 10 weanling male rats (Holtzman strain) were maintained for 8 or 12 weeks on diets of the following composition:

Percentage composition of diets

CONSTITUENT	SERIES 1				SERIES 2			SERIES 3	
	I.V. 80 fat	I.V. 80 diacetin fat	I.V. 1 fat	I.V. 1 diacetin fat	I.V. 80 fat	I.V. 80 fat	I.V. 80 diacetin fat	I.V. 80 fat	I.V. 80 monoacetin fat
	%	%	%	%	%	%	%	%	%
Vitamin mixture[1]	5.9	5.3	5.9	5.3	5.0	5.0	5.0	5.0	5.0
Soybean oil	2.0	2.0	2.0	2.0	..	..	..	..	..
Casein	32.1	28.4	32.1	28.4	35.0	35.0	35.0	27.0	27.0
Sucrose	28.7	33.1	28.7	33.1	4.0	..	..	47.0	47.0
Salt mix[2]	3.3	3.2	3.3	3.2	3.0	3.0	3.0	3.0	3.0
Cellu flour	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Ethyl cellulose	..	..	..	..	..	4.0	4.0	..	..
Fat	25.0	25.0	25.0	25.0	50.0	50.0	50.0	15.0	15.0

[1] Each 5 gm contains: vitamin A, 1250 I.U.; vitamin D, 125 I.U.; α-tocopherol, 10 mg; menadione, 0.3 mg; thiamine, 0.4 mg; riboflavin, 0.5 mg; niacin, 2.0 mg; calcium pantothenate, 2.0 mg; choline, 300 mg; para-aminobenzoic acid, 10 mg; biotin, 0.03 mg; folic acid, 0.025 mg; inositol, 200 mg; ascorbic acid, 10 mg; pyridoxine, 0.4 mg; sucrose to make 5 gm.

At the conclusion of the growth study, urine samples were taken from 5 animals of each group and were analyzed for volume, pH, titratable acidity, total nitrogen, ammonia nitrogen, urea nitrogen, creatine, creatinine, glucose, albumin, and acetone. The animals were then sacrificed and blood samples taken. Red, white, and differential counts were made and total nitrogen, urea nitrogen, non-protein nitrogen, and plasma CO_2-combining power were determined. The body weight gains and food consumption of these animals fed the acetin fats were essentially the same as those of animals fed the corresponding conventional triglycerides. The levels of a number of blood and urine constituents of the rats fed the experimental and control fats were also of the same order of magnitude. It was concluded that acetin fats derived from the usual edible triglycerides are nutritious materials (1026).

In a preliminary study by Ambrose and Robbins, groups of 5 weanling female albino rats were placed on diets containing 0, 5, 10 and 20% acetostearin for a period of 14 weeks. Differences in growth between the controls and test animals were not significant. Autopsies performed at termination of the experiment revealed no abnormalities (24).

Groups of 8 young male rats were fed purified diets containing 10% glyceryl lacto-palmitate with low monoglyceride content; or 10% glycerol lacto-oleate with low monoglyceride content or with high monoglyceride control; 10% polyglycerol lacto-oleate with high monoglyceride content; a 10% acetylated tartaric acid ester of glycerol monostearate, for 5 weeks. Rats fed 20% lard served as controls. Growth was normal for all groups and there was no difference noted in organ weights at sacrifice (1804).

Three groups (unspecified number per group) of male rats, 45 days old were fed diets containing 0, 2 or 10% lactopalmitate. The

20% group was discontinued at about 4 weeks and the other were sacrificed at 15 weeks. Growth and organ weights were normal and no toxicity effects were attributable to treatment (1804).

A group of 10 weanling rats (5 males and 5 females) was fed a basal diet containing 20% monoglyceride citrate for 7 days. Two similar groups were fed either the basal diet or 20% glyceryl lactopalmitate. The growth rates of the rats on both monoglyceride citrate and glyceryl lactopalmitate were less than that of the control rats, however, the food efficiencies of the two groups were of the same magnitude as that of the control. The author attributed the lagging growth rates to the poor palatability of the test diets. No symptoms of toxicity were attributable to the additives (1800).

In a pilot study, groups of 5 male rats were placed at weaning on diets containing 0 (controls), 0.5, 2 and 20% oxystearin. During the experimental period of one month, the rats ate well and with the exception of the 20% group grew equally as well as the controls. Organ weights obtained at autopsy were within normal values (1801).

Groups of 10 male and 10 female weanling rats were maintained for one month in diets containing 0 (control group), 0.1, 5, 10 or 15% of oxystearin. Animals were observed for effects on growth, food consumption, mortality, hematology, organ weights and gross and miscroscopic pathology. The following tissues were examined microscopically from 5 male and 5 female rats from each group: heart, lungs, spleen, stomach, small and large intestine, liver, kidney, adrenal, testes, bone marrow and brain. No gross or microscopic changes were produced by feeding diets containing as much as 15% oxystearin(1801).

In a paired feeding study using 20 pairs of rats, McManus et al. examined the effects of feeding a diet containing 20% triacetin for a period of 7 months. Observations on growth, rate of metabolism, and urinary excretion of volatile acids revealed no discrepancies between the treated animals and the controls fed 15% glucose as an equi-caloric amount.

Hamsters

Orten and Dajani fed 15 weanling male hamsters (Golden Syrian) 5% glyceryl-monostearate for 8 weeks. Fifteen animals ate control (standard) diets. Five animals continued on the 5% feed while 10 were switched to a 15% glyceryl-monostearate diet for 28 weeks total. The 15% group lost weight (very little) after the 20% week. The 5% group had a higher increase in weight gain per 100 gram food and per gram protein than the control. No consistent pathologic changes were observed in the liver, kidney, bladder, or testis (1167).

Guinea pigs

In a test by Ostwald, 10 guinea pigs were given either a 50% aqueous solution in saline or saline alone either by stomach tube or

from a drinking cup daily for 30 to 40 days. The plasma cholesterol levels and red blood counts were determined repeatedly through the experiment. Guinea pigs receiving more than 5 ml of a 50% glycerol solution daily by stomach tube died with acutely toxic symptoms. Autopsies performed on animals at termination of the experiment revealed no pathological changes. The plasma cholesterol level showed no consistent changes attributable to the intake of glycerol while the red blood count of the guinea pigs receiving glycerol dropped during the experiment indicating a probable anemic effect of glycerol in the guinea pig (1174).

Rabbits

In a test by Ostwald, 4 rabbits were given either a 50% glycerol solution in saline or saline alone either by stomach tube or from a drinking cup daily for 30 to 40 days. The plasma red blood cell cholesterol levels were determined repeatedly throughout the experiment. Doses of 10 ml of the glycerol solution daily were well tolerated by the rabbits. Those animals which were autopsied at the end of the experiment showed no gross pathological changes. Neither the plasma nor the red blood cell cholesterol levels showed any consistent changes which could be attributed to the intake of glycerol (1174).

Dogs

Two-year feeding studies were conducted in dogs to investigate the oral toxicity of Atlas synthetic glycerine compared to natural glycerine. Six groups of eight young dogs (4 males and 4 females), approximately balanced with respect to initial weight and age, were placed on balanced diets contained 5, 10 & 20% synthetic glycerine or 5, 10 or 20% natural glycerine. Another untreated group served as controls. Observations were made at intervals on appearance, behavior, body weight, food consumption, water intake, urine output, hematological and blood chemical determinations, and live function tests. One male and one female per group were sacrificed at 12 months. At 2 years, all survivors were sacrificed. After gross examinations at necropsy, the liver, kidneys, spleen, heart, and gonads were weighed and the organ to body weight ratios determined. In addition sections of major organs and tissues to an overall total of 23 were examined microscopically. The observations failed to reveal any treatment effects from either the Atlas synthetic or the natural glycerine (1805).

Johnson et al. placed three 5-week-old dogs on a diet containing 35% glycerol and maintained another 3 dogs as controls. Growth of the animals was unaffected for 31 weeks. At age 36 weeks, the food consumption of the dogs was reduced to see whether at a caloric intake of bare maintanence level, some difference in the two diets might appear. In the ensuing 14 weeks the controls maintained their weight while the glycerol-fed dogs lost an average of 16% of their previously attained weight. The urine of the dogs was examined repeatedly for hemoglobin and albumin from the 13th to the 45th week and in no instance was even a trace of either found. Diuresis was noted among the test dogs, the treated animals excreting approximately 5 times as much urine as the controls. Careful autopsies at the end of the experiment revealed no gross differences in the 2 groups. Microscopic studies on the stomach, intestine, lung,

spleen, kidney, and liver revealed normal organs. Formalin fixation and fat stains of the liver revealed no degeneration (740).

Kopf et al. administered 2.0 ml of glycerine/kg of body weight to 1 male and 1 female dog per os twice weekly for 4 months (there was a 2-week interruption of treatment after the first month). One male and 1 female dog were maintained as controls. Growth of the test animals was normal and weekly urine tests revealed no abnormalities. At the termination of the test, histological examination of the liver, kidneys, bladder, stomach, intestines, spleen, adrenals, heart muscle, and lungs revealed no abnormalities (822).

Two groups of 4 dogs each were given diets containing 0.25 or 2.5% oxystearin for a period of one year. There were 2 male and 2 female dogs in a control group, and 1 male and 3 females in each of the 0.25 and 2.5% groups. Observations were made on body weights, urinalysis, hematology, mortality and organ weights. Histological examination were conducted on tissue from the heart, kidneys, lung, spleen, stomach, small and large intestine, pancreas, liver, adrenal, urinary bladder, gonads, skin, thyroid, bone marrow and brain. No gross or histopathological changes were attributed to treatment of either dosage level (1801).

A female dog was given a diet containing 25% oxystearin for a period of one month. Body weight was maintained. Normal values were found on hematological examination. Urine analysis was negative. Organ weights were normal. An examination of 13 major tissues revealed no evidence of histological changes as a result of treatment (1801).

In a test by Koppanyi and Dardin, 4 groups of 2 dogs (about 3 years old; weighing between 4.7 and 8.9 kg) were maintained on diets containing 0, 5, 10, or 20% TEM. After 3 months, one dog from each group was sacrificed and subjected to extensive examinations which revealed no evidence of any damage attributable to TEM ingestion. After a period of 25 months of feeding, all four remaining dogs were still alive and in excellent condition (1802).

Hartwig, et al. studied the effect of intravenous infusion of diacetyl tartaric acid esters of mixed mono- and diglyceride (TEM). He injected thirteen mongrel dogs with between 150-295 mg/kg TEM daily, five times a week for six weeks. Four dogs died, eight were sacrificed, and one was held over for observation. A bilateral pleural effusion occured in 6 dogs; all but one of these 6 dogs had a pericardial effusion, in addition. These dogs showed no unusual distress until 3 or 4 days prior to death or sacrifice; the signs were dyspnea and downward displacement of the liver. Four of the six dogs had atelectasis, two had pulmonary edema, and two had congested livers. Six of the infused dogs had a chronic hepatitis. Hemoglobin content fell in eleven dogs. The differential white cell count, bromosulfalein test, and blood urea nitrogen, all were normal. Intravenous fat pigment was demonstrated in the lung, liver, and spleen of 2 dogs and in the spleen of a third dog (609).

Man

In the treatment of 37 cases (human) of cerebral edema caused by acute cerebral infarction, Meyer et al. administered daily

1.2 g/kg of glycerol i.v. or 1.5 g/kg orally. Seventeen other patients with cases of edema of the central nervous system were similarly treated. During treatment, mortality of the group with cerebral infarction was 11%, however these deaths were not related to glycerol administration. Neurological improvement occurred in all other cases during and at the end of 4 days of treatment. No toxic effects were observed which were attributable to glycerol administration (1053).

Fourteen graduate students (10 men, 4 women) served as subjects for an experiment by Johnson et al. For a period of 50 days, each subject drank 30 ml of 95% glycerol with orange juice after each of three daily meals. Preceding and following this period were 10-day control periods. Both red and white cell counts and hemoglobin estimations were made twice during each control period and 6 times during the glycerol period, on each subject. Analyses of urine samples were made on each subject twice during each control period and from 5 to 7 times during the glycerol period. Observations were also made on body weight, excretion of urine, body temperature, basal metabolic rate, and activity of the large intestine. The only effect of glycerol ingestion noted was a slight tendency toward an increase in body weight (740).

III. Long-term Studies

Rats

Hine et al. conducted a 2-year feeding study using 158 (72 males, 96-109 g; 86 females, 92-108 g) young healthy rats of the Long-Evans strain. Groups of 22 animals of each sex were maintained on diets containing 5, 10, or 20% glycerine, while 26 animals were kept as controls. The following criteria were used for evaluating the results of the experiment: hemoglobin determination and urinalysis; lipid and glycogen content of the livers of the groups receiving 20% of glycerine in the diet (these animals were sacrificed after one year); mean weekly food consumption; mean organ/body weight ratios (liver, kidney, heart, spleen, lung); growth curves; mortality; general appearance of the animals throughout the experiment; and gross and microscopic lesions (sections of liver, spleen, adrenal, kidney, small intestine, bladder, and reproductive organs were examined). High incidences of albuminuria and glycosuria were observed in the test animals but, due to the random distribution throughout the test groups, no significance could be attached to these findings. Females receiving 20% synthetic glycerine exhibited liver/body weight ratios significantly higher than those of the controls and females receiving 5% synthetic glycerine had heart/body weight ratios significantly greater than those on 5% natural glycerine. On the basis of these findings, it was concluded that neither synthetic nor natural glycerine, when fed at levels up to 20% of the diet, cause any untoward effects in rats (646).

In a 2-year feeding study 336 weanling rats equally divided as to sex, were assigned to 6 diets containing 20%, 10% or 5% Atlas synthetic glycerol, or 20%, 10% or 5% natural glycerol, and a control. All rats were observed for the following: appearance, laxation, body weight, survival, gross examination at necropsy, organ weights of adrenals, gonads, heart, kidneys, liver, and spleen, and histological

examination of major tissues (liver and kidneys in all controls and 20% glycerol and other organs in representative animals). Selected rats were observed at specific intervals throughout the study for water consumption, urine output, food consumption and caloric efficiency, urine analysis and blood chemistry and hemalology. No deliterious or toxicological effects were noted among the groups of rats fed for two years on diets containing 5, 10 or 20% of either Atlas synthetic glycerol or natural glycerol or among either of these experimental groups and the controls (1805).

For a period of two years, Fitzhugh et al allotted two groups of 24 rats each a basal diet with or without a 25% supplement of Myverol 18-00 (glyceryl monostearate). The rats were housed in individual cages and were allowed food and water ad libitum. Myverol was well consumed and found to be nutritionally adequate in a 25% diet, providing normal growth of both sexes throughout the experiment. Longevity of the animals was unaffected by the supplement and hematology studies showed normal values for the test rats. The weight of the liver, however, was increased 27% by the inclusion of Myverol 18-00 in the diet. Detailed microscopic pathological examinations of all major organs and tissues revealed only a single change attributable to treatment, an increase in the number or calcified renal tubular casts or tubular segments in the test animals, compared to the controls.

In a 57-week study by Ambrose and Robbins, groups of 5 male weanling albino rats were raised on diets containing 0, 0.25, 0.5, and 1% aceto-olein and 0.25, 0.5, 1, 2, and 4% acetostearin. Growth of the rats on the experimental diets did not vary significantly from that of the controls. Determination of organ weights (liver, kidney, testes, spleen, heart, adrenals) revealed no discrepancies between the test and control rats except in the testes. The testes of rats on acetoglyceride diets were much smaller both in terms of absolute and relative body weight than were those of the controls. Hypoplasia of the testes was observed with rats on the 0.25 and 0.5% acetostearin diets and on the 0.25, 0.5 and 1% aceto-olein diets. The only pathological effects disclosed by histolopathological examination of the organs (liver, kidney, testes, spleen, heart, adrenals, thyroid, intestine, stomach, pancreas, bladder) occurred in the testes. Testicular hypoplasia and suppression of spermatogenesis of varying degrees were observed in all rats. The authors suggest that the effect on the testes may possibly be the result of insufficient vitamin E in the diet. Hematological studies made at 4-month intervals showed no toxic effect on the blood at any dosage level (24).

Using weanling albino rats of the Slonaker strain, weighing 43 to 51 g, Ambrose et al. investigated the effects of long term feeding of various acetoglycerides (see Table ·).

TABLE

Description, physical and chemical characteristics of acetoglycerides (AG)[1]

Sample No.	Description	Hydroxyl value (free)	Saponi. value	Iodine value	Acetyl %	Linoleic acid %	FFA as oleic acid %	Unsaponifiable matter %	M.P. °C
AG-194	Fully acetylated acetostearin from hydrogenated lard	0.00	389	0.69	16.2	0.04	0.56	0.54	30.7
AG-26	Fully acetylated acetostearin from hydrogenated lard	0.8	380	0.96	18.5	0.13	1.14	0.30	32.5
AG-31	Partially acetylated aceto-stearin from hydrogenated lard	118.4	297	1.08	11.5	0.15	0.61	0.32	57.0
AG-21	Fully acetylated aceto-olein from prime steamed edible lard	0.4	401	42.0	19.8	6.01	0.92	0.23	5.0
AG-3	Fully acetylated aceto-olein from cottonseed oil	0.4	391	73.2	17.9	32.92	0.72	0.59	−2.0

[1] Data supplied by the Southern Utilization Research and Development Division, U. S. Department of Agriculture.

These compounds were fed to groups of 10 male and 10 female rats at dietary levels of 5, 10, and 20% for periods from 400 to 709 days. Triacetin (3.3%) and sodium acetate (6.1%) were fed to groups of 5 female rats. Thirty rats of each sex were maintained on the basal diet as a control group. Food consumption and body weight of the animals were recorded weekly during the first 300 days, only body weights being recorded thereafter at monthly or bimonthly intervals. All rats on diets containing 20% of an acetoglyceride and an appropriate number of control rats were sacrificed at the end of 400 days, while rats on diets containing 5 and 10% acetoglyceride sample AG-194 were sacrificed at 600 days; all surviving rats on the 5 and 10% levels of the other acetoglycerides were sacrificed at the end of approximately 709 days. At time of sacrifice all organs were examined for gross evidence of injury. Livers, kidneys, spleens, hearts, and testes were removed and weighed. These organs, in addition to the bladder, stomach, intestines, pancreas, adrenals, thyroid, brain, hypophysis, parathyroid, ovaries, and uterus, were subjected to histopathological examination. No significant differences in growth were noted between the control animals and those fed acetoglycerides except in the case of male rats on dietary levels of 20% acetostearins AG-194 and AG-31, and female rats receiving 20% AG-31. Testes of rats receiving the three acetostearin diets were significantly smaller than those of the control rats or rats fed the two acetooleins. The livers of male rats fed 20% acetostearin AG-194 and of both male and female rats fed 20% acetostearin AG-31 were significantly larger than those of the control rats. Livers of female rats fed 20% of either acetoolein were smaller than those of the control rats. Kidneys of female rats fed 20% acetostearin AG-31 were significantly larger than those of the controls. The storage of liver glycogen by rats on the various acetoglyceride-containing diets was not enhanced. Excess fat observed in rats fed the various acetoglycerides appeared to be a storage phenomenon since it was unaccompanied by evidence of cellular damage. Acetostearins produced a foreign body reaction in the fatty tissue of a number of the organs, presumably due to crystalline deposits of a

high melting point fat. Acetostearins AG-194, AG-26, and AG-31 produced some scarring of kidney tissue, presumably due to deposited calcium (26).

Groups of 8 young male rats between 39 and 45 days old were fed purified diets containing 10% glyceryl lactopalmitate, with low monoglyceride content; or 10% glyceryl lactooleale with low monoglyceride content or with high monoglyceride content; 10% polyglycerol lactooleate with high monoglyceride content and 10% acetylated tartaric acid ester of glycerol monostearate. Rats fed 20% lard served as controls. Surviving rats were sacrificed after 608 days on the diets. Growth, organ weights, and mortality were normal for all groups (1804).

Groups of 50 male and 50 female weanling albino rats were given diets containing 0 (control group), 0.5, 5 or 15% oxystearin for 2 years. Observations were made of body weights, food consumption, clinical condition, hematology, urine analysis, mortality and organ weights. Microscopic examinations were made on tissues from the brain, lungs, heart, liver, spleen, kidneys, adrenals, gonads, stomach, small and large intestine, bladder, bone marrow and muscle. Although there were an unusual number of Leydig cell adenomas of the testes of rats on 15% oxystearin, the author concluded that there were no gross or histological changes caused by the administration of oxystearin in amounts up to 15% of the diet over a period of 2 years (1801).

To determine the effects of chronic feeding of TEM, Koppanyi and Dardin maintained 6 groups of 8 Wistar rats on various diets for a period of 2 years. The control group (males) "normal control" was fed standard laying mash (poultry feed); the second group (females) was fed 95% laying mash and 5% TEM; the third group (females) 90% laying mash and 10% TEM; the fourth group (males) 80% laying mash and 20% TEM; the fifth group (males) 50% laying mash and 50% ground white bread, the "bread control" group, and the last group (females) 50% ground bread which was baked with 10% TEM in terms of flour weight and 50% laying mash ("bread test"). Growth curves and survival rates were comparable for all of the groups. No differences in external appearance were exhibited by any of the rats and gross and microscopic examination of the organs and tissues (unspecified) revealed no pathological changes (1802).

IV. Special Studies

Reproduction

In a reproduction test by Wegener, 10 male and 10 female rats were treated daily with 10 ml of a 20% aqueous solution of glycerine per kg of body weight per os for 8 weeks. When the females reached a weight of 170-215 g they were mated, with daily rotations of the males. For 5 of the females, treatment was continued until birth of the offspring and for the other 5, treatment was continued until weaning. To serve as controls, a similar number of animals received a corresponding quantity

of distilled water and were handled in the same way. All of the test animals became pregnant and produced an average of 9 young, (average control litter was 8.1). Growth of the young animals (F_1-generation) and attainment of maturity (onset of estrus cycle) were normal. Histological examination of the inner-secretory organs of a part of the F_1-generation sacrificed at approximately 100 days of age revealed no untoward effects attributable to glycerine. Ten male and 10 female rats from both the F_1 test and control groups, at an average weight of the females of 200 g, were mated. These animals remained untreated. Nine of the animals whose parents had been treated with glycerine became pregnant and produced an average litter of 8.7, while all of the selected offspring of the control animals became pregnant and produced an average litter of 8.1. Growth and maturity of these animals (F_1-generation) was also normal and histological examination of the inner-secretory organs again showed no peculiarities (1708).

In an extensive series of studies on the reproduction of rats which were fed purified diets containing as much as 30% glycerine, Guerrant et al. found that the glycerine produced no untoward effects. On the contrary, the glycerine-treated rats fared better than those maintained on the control diet, which was found to be unsatisfactory (559).

In a reproductive study by Johnson et al., in which rats (unspecified number) were maintained on diets containing 0, 41, and 61% glycerol, no untoward effects on reproduction were noted among those rats given 41% glycerol, but the animals receiving glycerol at the dietary level of 61% failed to become pregnant (740).

To study the effect of feeding of TEM on reproduction, Koppanyi and Dardin utilized rats which were maintained as part of a long-term feeding study (see Long-term Studies, Koppanyi and Dardin, ref. 1802). Three female rats fed a diet containing 10% TEM were mated with male rats maintained on a 20% TEM diet. The offspring of these rats were used for the second generation TEM experiments. Three females of the bread test group were mated with the control males to provide the second generation control and bread control rats. All females became pregnant and gestation and lactation were normal. Upon weaning, the rats were divided into 4 groups of 8 animals each; (a) controls (males); (b) 20% group (females); (c) bread control (males); (d) bread test (males). These regimens were maintained for 22 months, during which all of the animals grew normally and mortality was comparable for all groups. Autopsy and microscopic examination of the organs (unspecified) revealed no deleterious effects attributable to the ingestion of TEM (1802).

Sixteen female and 8 male rats from groups receiving 0, 0.5 and 5% oxystearin in the chronic study were selected as the parent stock for the reproduction study. These animals produced 2 litters (F_{1a} and F_{1b}. From the F_{1b} litter, 16 female and 8 male rats were selected at 100 days of age. As before, each female produced 2 litters (F_{2a} and F_{2b}). From the F_{2b} litter, 16 females and 8males were selected at the age of 100 days for breeding. These rats produced the F_{3a} and F_{3b} litter. When the F_3 litter was 21 days old, the rats were sacrificed, autopsy examinations were made, and samples of tissues were taken for histological study. There was no indication of differences in

reproductive performance due to dietary treatment. There was no effect upon the growth of the rats. Average organ weight-body weight ratios were within normal range. There was no indication of any abnormalities in the treated rats and a thorough histopathological study of the tissues (not listed) from the F_{3b} generation showed no difference among the rats attributable to dietary treatment (1801).

Using weanling albino rats of the Slonaker strain, weighing 43 to 51 g, Ambrose et al. investigated the effect of feeding various acetoglycerides (see table) on reproduction.

TABLE

Description, physical and chemical characteristics of acetoglycerides (AG)[1]

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[1] Data supplied by the Southern Utilization Research and Development Division, U. S. Department of Agriculture.

For each acetoglyceride, ten female and 5 male weanling rats were placed on a diet containing 10% of the compound. A similar number of rats were maintained on the basal diet. After approximately 3 months on the respective diets, all rats were mated and the number of siblings cast and weaned was recorded. Those acetoglycerides permitting successful reproduction in the first generation were studied on several successive generations. All rats receiving other acetoglycerides were remated several times before discontinuance. Rats on diets containing 10% acetoolein (AG-3 and AG-21) supported reproduction and lactation through the test period of three generations. Each generation of rats was remated several times, and the rats remained fertile throughout. The total litters cast, the number of siblings per litter and the average body weights at weaning, and at 50 and 100 days for each generation were similar in the control and experimental groups. With the acetostearins, AG-194, AG-26, and AG-31, only AG-26 supported reproduction through 3 generations comparable to the controls. Both AG-194 and AG-31 failed to support reproduction beyond the first generation (26).

Sensitization

Solomides reported that injections of glycerine produce a temporary immunity followed by sensitization to re-injections. After receiving injections of 1.2 ml of glycerine G_2 (70% glycerine, 10%

water), rabbits exhibited increased tolerance for subsequent injections (which were fatal to previously untreated animals) for 3-27 days after the initial injection. Injections (which were not fatal to previously untreated animals) given more than 27 days after the initial injection caused an anaphylactic reaction and death within several hours (1469).

Following 10 injections of 0.1 ml of a 0.1% solution of natural or synthetic glycerine, Hine et al. noted that a further injection of 0.05 ml of the solutions gave no indication of sensitization to glycerine in any of the 24 adult white male guinea pigs (350-429 g) (646).

Local irritation

Studies by Staples et al. indicate that repeated oral administrations of high concentrations of glycerine are irritating to the gastrointestinal mucosa of rats and dogs. Eight doses of up to 3 g of glycerine given 3 times daily produced hyperemia, petechial hemorrhage and erosions in the test animals (1485).

Using a variety of methods, Loeser et al. conducted a series of experiments to determine the effects of local application of glycerine. The immersion of rats' tails in undiluted glycerine (4 days, 4 hrs daily; 6 days, 1 hr daily; 6 days, 2 hrs daily) produced no changes in the skin. Application of undiluted glycerine to the conjunctiva of rabbits, cats, and dogs caused no visible changes; corneal reflex and pupil reaction remained normal. No visible changes were noted following administration of glycerine to the mucous membranes of the mouth (rat, rabbit, dog) or of the stomach (rabbit, dog). However, when applied in the rectum of rats and guinea pigs, glycerine caused an accelerated emptying of the intestinal contents. It was not determined whether this effect was based on a local stimulation or was the result of a filling effect (934).

Twenty-four female albino rabbits (2.0 to 3.8 kg) were used by Hine et al. for the determination of the skin irritating properties or of natural and synthetic glycerine. The glycerines were applied to a shaven dorsal area (approximately 30% of the body surface) of the rabbits in doses of 0.5-4.0 ml/kg for periods of 8 hours 5 times weekly until 90 applications had been made. The urine of the rabbits on the 2 highest dosages was examined microscopically and analyzed for pH, sugar, and albumin at 30 and 60 days after the start of the experiment. The blood was examined for total hemoglobin and red and white blood cell counts at 45 and 90 days. At termination of the tests, the animals were sacrified and autopsied. After gross examination, specimens of thyroid, heart, lung, spleen, stomach, liver, adrenal, kidney, small intestine, ovary, uterus, bladder, and treated skin were examined microscopically. Results of the tests indicate that neither skin irritation nor any other abnormalities are effected by topical application of either synthetic or natural glycerine (646).

Hine et al. applied glycerine, in amounts of 6.25-18.7 g/kg for a period of eight hours, to the shaven trunks of 6 male albino

rabbits (3.0 ± 0.5 kg). No untoward effects were observed. The authors suggest that the glycerine was absorbed in quantities insufficient to produce pharmacologic effect (646).

By application of 0.1 ml of natural or synthetic glycerine to the eyes of 4 albino rabbits, Hine et al. found that glycerine produces no measurable eye irritation (646).

Li et al. noted that, occasionally, rats and mice receiving subcutaneous injections of monoacetin and diacetin exhibited local irritation of the skin at the site of injection. In the eye of the rabbit, 50% monoacetin caused only a slight degree of transient injection of conjunctival vessels, but diacetin and triacetin in similar concentrations caused marked congestion and moderate edema (922).

The daily application, for 45 days, of 4 ml of a 30% acetoglyceride emulsion (20% acetostearin, 10% acetoolein, 2% Span 20, and 1% Tween 60) to the skin of albino guinea pigs were reported by Ambrose and Robbins to result in no local irritation or systemic reactions. No sensitization or allergic response was observed in the test animals (24).

Foreign-body

In a study in which weanling male and female Holtzman rats were fed diets with or without 50% saturated partially acetylated monoglyceride, Herting and Crain noted the appearance of a foreign-body type reaction in the body fats occurring within 8 weeks of initiation of the test (639).

GLYCERIN AND GLYCERIDES

Biochemical Aspects

I. Breakdown

The heating of fats can lead to oxidation, polymerization and decomposition which may result in a vast mixture of reaction products, including polymors, peroxides, aldehydes, ketones, epoxides, lactones and free fatty acids. The possibility of heated fats having carcinogenic properties has been extensively researched without producing a definite conclusion (34).

Kieckebusch et al. reported that the blowing of oils at high temperatures (180°C) greatly increases their toxicity, probably due to the formation of oxypolymers. The preparation of food products containing glyceride additives could possibly result in formation of similar compounds (788).

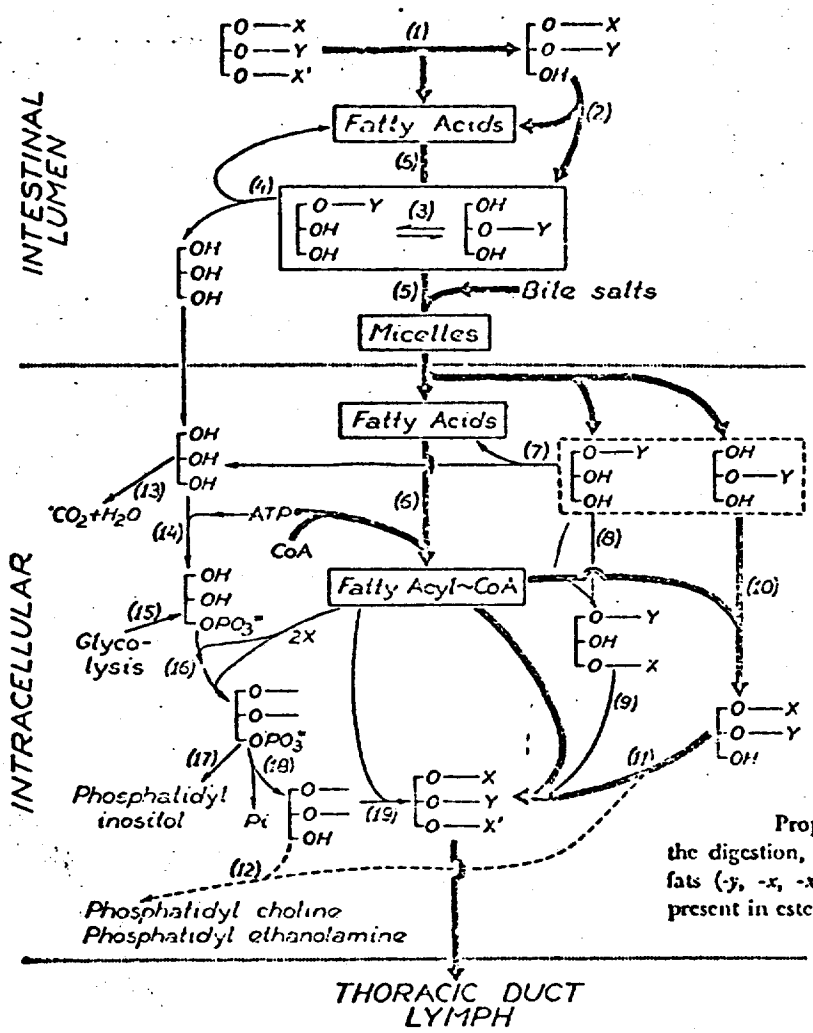
II. Absorption - Distribution

Hueber and Hueber reported that approximately 70-87% of glycerol (4 ml of 0.1 m solution) is absorbed from the isolated loops of rat intestine within 25 minutes (656).

In experiments on anesthetized dogs, Johnson et al. showed that although little glycerol was absorbed from the stomach, about 50% of the administered glycerol was absorbed from intestinal loops in 2-3 hours (740).

According to Kayden et al. (775) analysis of intestinal lumen contents of man after fat feeding has shown a separation of glycerides into two phases: an oil phase which contains mainly diglycerides and triglycerides and some fatty acid, and a micellar phase which is a combination of bile salts, free fatty acids, and monoglycerides. From the micellar solutions monoglycerides and free fatty acids are absorbed through the intestinal wall, leaving the bile salts within the lumen to form additional micelles. The fate of the free fatty acids within the mucosal cell is determined by their chain length and saturation; those of less than 12 carbons are preferentially abosrbed by rats directly into the portal blood, whereas longer chain fatty acids are incorporated mainly into triglycerides, and appear in lymph chylomicrons. Monoglycerides within epithelial cells may be esterified to di- and triglycerides or cleaved to glycerol and free fatty acids. The 2-monoglycerides are esterified especially rapidly to triglycerides, but esterification of 1-monoglycerides lags and these are more likely to undergo hydrolysis particularly if the fatty acids are of short chain length.

In a review of the literature of fat absorption, Johnston stated that the mechanism of fat absorption can be summarized as shown in the following figure.



Proposed mechanism involved in the digestion, absorption, and biosynthesis of fats (-y, -x, -x', and —— denote fatty acids present in ester linkages).

The heavy arrows denote the major pathway. The emulsified triglycerides are hydrolyzed by pancreatic lipase, at the 1 and 3 positions, forming monoglycerides and fatty acids which in combination with bile salts form a micellar dispersion (reaction 5). The monoglycerides and fatty acids form micelles which penetrate the lumen membrane by an energy-independent mechanism, presumably by diffusion (reaction 5). The monoglycerides and fatty acids migrate to the endoplasmic reticulum, where the fatty acids are activated to their CoA derivatives (reaction 6) and are resynthesized into triglycerides by the acylation of monoglycerides in a multienzyme complex (reactions 10 and 11).

By administering oleic acid, monoolein and diolein (9 g of acid radical/kg) to groups of albino rats, Piretti determined that free oleic acid is poorly absorbed (1.1 g/kg) while mono- and diolein are more readily absorbed (4.55 g/kg and 3.64 g/kg, respectively) (1215).

Tidwell reported that the rate of absorption of glyceryl monooleate, when fed alone (to rats), is much slower than that of other glycerides

due to its formation into a thick gel which causes prolonged retention of the compound in the stomach, thereby rendering it unavailable for absorption (1590).

Four rats were used to determine the approximate caloric value for succinylated monoglycerides (SMG). Similar groups were assigned to levels of prime steam lard varying from 0.5 to 3.0 g for comparison to the group on 1.0 g SMG per rat per day. From the data it was found that 1.0 g SMG has the metabolizable caloric value of approximately 0.25 g of lard or approximately 2.25 cal per gram. The coefficient of digestibility of SMG when fed at a level of 1 g per rat per day was calculated to be of the order of 0.75 to 0.80 (1803).

In an attempt to determine if the feeding of the 2 components of SMG Myverol (a mixture of monoglycerides) and succinic acid would support similar weight gains in rats and yield an apparent coefficient of digestibility of the same magnitude, the results gave an apparent coefficient of digestibility of 62.5 for SMG and a coefficient of 43.3 for Myverol (1803).

By feeding acetostearins and acetooleins to rats, Ambrose and Robbins found that these compounds are well absorbed and have digestibility coefficients of 80-85% and 99%, respectively (24).

III. Metabolism and Excretion

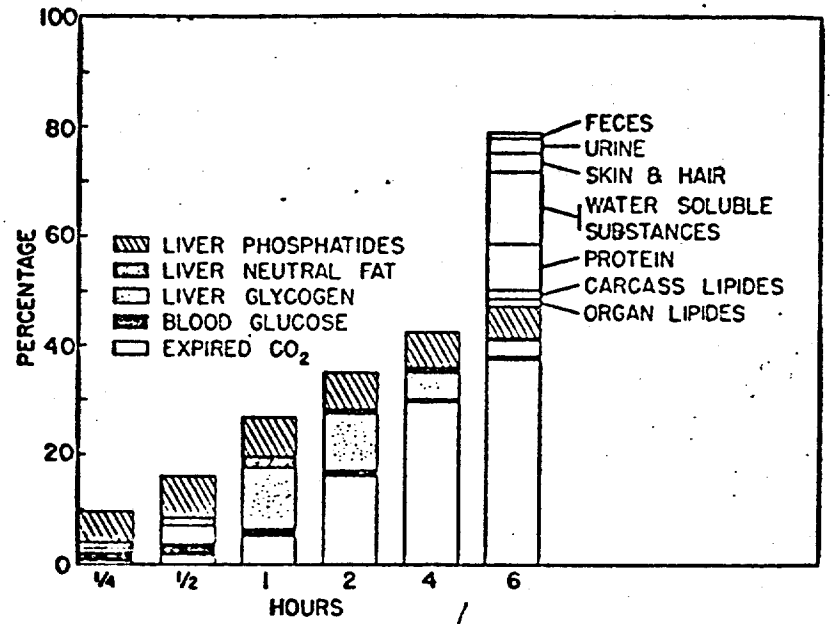
Determination of liver glycogen of rats 1-6 hours after oral administration of 1 ml of glycerol led Catron and Lewis to the conclusion that glycerol is readily metabolized and deposited as glycogen in the livers of young white rats (214).

In a series of experiments using cats and human subjects, Holst found that the rate of metabolism of ingested glycerol is constant and independent of the blood concentration. The tests revealed that the metabolism of the glycerol occurs almost exclusively in the liver and that the oxidation in the liver is incomplete (669).

Voegtlin et al. reported that glycerol (4 g/kg, i.p. or p.o.) given to fasted rabbits produces a hyper-glycemia of considerable extent and duration, indicating a conversion of glycerol into glucose (1681).

Chambers and Deuel found that feeding glycerine to phlorhizinized dogs led to excretion of extra glucose in the urine, amounting to 96 to 98% of the glycerol fed. The conclusion that glycerine is converted to glucose in the phlorhizinized dog is confirmed by the observation that the respiratory quotient of these animals was decreased by glycerol feeding. The conversion of glycerine to glucose consumes O_2 without producing CO_2 and should therefore decrease the respiratory quotient (228).

The fate of ^{14}C-glycerol in the rat, following administration of up to 238 mg, was determined by Gidez and Karnovsky to be as follows (526):



The percentage of administered counts in different metabolites at various time intervals. The CO_2 values and the counts of organ lipides (other than liver), urine, feces, protein, "water-soluble substances," skin, and hair, and carcass lipides are from one typical animal. All other data are the means of two to six animals.

Fatty acids play an important role as an energy source. Their oxidation is prominent in higher animals which can store large amounts of neutral fat as a fuel reserve. It is estimated that fatty acid oxidation provides at least half of the oxidative energy in the liver, kidneys, heart muscle and resting skeletal muscle. Fatty acids undergoing oxidation in tissues of higher animals come either from extracellular fluid or from endogenous intracellular lipids. Vertebrate blood contains considerable amounts of triacylglycols but very small amounts of free fatty acids. The major endogenous source of fatty acids for fuel is storage fat, in the form of fat droplets in the cytoplasm, which consists largely of triacylglycerol. Before the fatty acids can undergo activation and oxidation, the triacylglycerols first must undergo hydrolysis by intracellular lipases to yield free fatty acids and glycerols. Free fatty acids are therefore derived either from the blood stream, in which they are transported bound to serum albumin, or prior hydrolyses of intracellular lipids. They are first activated by esterification with CoA to form acyl CoA esters of the outer mitochondrial membrane. Then the fatty acyl group from CoA is carried across the inner membrane by the carrier molecule carnitine. Finally the fatty acyl group is transferred from carnitine to intramitochondrial CoA: through several enzymatic reactions. A two-carbon fragment of the fatty acid is removed as acetyl CoA and two pairs of hydrogen atoms are given to specific acceptors. A long chain fatty acid will go through several spirals. Thus the 16-carbon palmitic acid undergoes a total of 7 such cycles, to yield 8 molecules of acetyl CoA and 14 pairs of hydrogen atoms. The acetyl CoA formed as a product of the fatty acid oxidation will then enter the tricarboxylic acid cycle, which is

the final common pathway of oxidative catabolism of all fuel molecules in aerobic cells (913).

According to Florey, the glycerine derived from the hydrolysis of glycerides is converted directly into glucose. While the fatty acids are broken down into 2-carbon units (acetate) that combine with coenzyme A to form active acetate which, with oxalacetate, contributes to the formation of citric acid. Thus fats are fed into the pathways of glycolysis and the citric acid cycles. This is shown diagrammatically in Figure 2 (454).

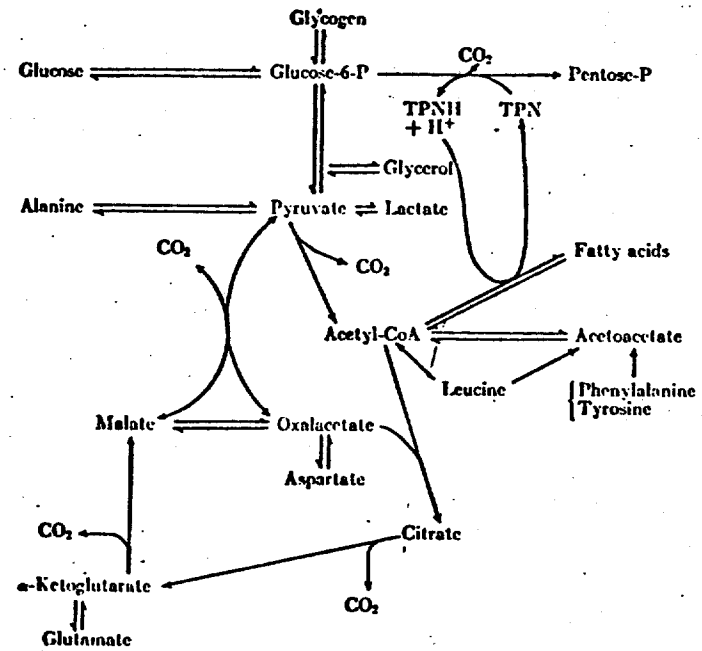


Figure 2. Metabolic interrelations of carbohydrates with fatty acids and amino acids. Citrate, alpha-ketoglutarate, and malate are part of the tricarboxylic acid cycle.

In an experiment in which rats were given D-palmitic acid in free form or as mono- or triglycerides (15% of the diet for 3-5 days), Buensod and Favarger found that monopalmitin is digested more completely than the free acid or triglyceride form although it is digested more slowly than tripalmitin (182).

Lang and Schmidt administered per os to male Sprague-Dawley rats, which had been starved for 24 hours, a mixture (0.5-0.8 g/kg) of mono- and diglycerides of ^{14}C-1,4-diacetyl tartaric acid. Measurement of the radioactivity 24 hours later revealed that 26-31% of the activity was absorbed, 12-21% was oxidized to $^{14}Co_2$, 8-13% excreted in the urine and 2% retained in the carcass (880).

According to Koppanyi and Dardin, TEM has a rough digestibility coefficient of over 90%. It is absorbed from the small intestine and its tartaric acid is slowly excreted by the kidneys (1802).

Of 2.72 g of TEM (diacetyl tartaric esters of mono- and diglycerides) /kg administered by duodenal tube to 4 dogs, Sourkes and Koppanyi recovered in the urine less than 20% of the available tartaric acid in the 12 hours following administration. Analysis of the intestinal contents of 2 of the dogs 12 hours after administration of TEM indicated that 88% of the TEM had been absorbed. The digestibility coefficient of TEM in the adult rat was reported to be 95% (1473).

IV. Effects on Enzymes and Other Biochemical Parameters

The prolonged administration of glycerine (50 mg/kg/day) to rabbits as a part of their food over a course of 5-6 months, resulted in an increase of the glycerine level of the blood, liver, skeletal and cardiac muscles, and intestines. A single dose of 100 mg of glycerine/kg did not produce any significant change in the blood glycerine level (531).

Two groups of 8 white male rats weighing 250-300 g were given, as drinking fluid, 15% glycerol or water. Nikkila and Ojala maintained the animals on these regimens for periods of 1-2 weeks, after which they were sacrificed and the plasma and liver triglyceride and phospholipid, and plasma cholesterol were determined. The administration of glycerol produced a striking hyperglyceridemia and an elevation of plasma phospholipid level, whereas plasma cholesterol remained unchanged. The liver triglyceride content was increased in the treated animals but the change was less marked than that of plasma triglyceride (1121).

Macdonald reported that the serum glycerides of men were raised 1-2.5 hours after the ingestion of glycerol (1 ml/kg), whereas in young women given the same level of glycerol the serum glycerides were not raised. In men who were given a supplement of glycerol (1 ml/kg/day) to their free choice diet there was a rise in the concentration of fasting serum glycerides but no rise was found when a similar supplementation was given to young women (966).

Wishnofsky et al. reported that the ingestion of 80 g of glycerine produced an increase in the concentration of blood sugar in the majority of 13 normal and 10 diabetic persons (1752).

Martin and Stenzel reported that orally administered glycerine (4 g/kg) increases the glucuronic acid excretion of rats by as much as 100% (1005).

Orekhovich and Plotnikova found that the daily intragastric administration of 20 ml of a 50% glycerine solution produced a 100% rise in the cholesterol content of the blood of rabbits, within 2 weeks. In approximately 2 weeks, formations similar in outward appearance to atherosclerotic plaques found in the initial stage f atherosclerosis, appeared in the aortic arch (1163).

Through in vitro and in vivo (rats) studies, Migicovsky found that glycerine inhibits the synthesis of cholesterol (1056).

Jacobs et al. investigated the hemolytic effect of glycerol by adding one part of blood to 500 parts of an isosmotic glycerol solution.

They observed rapid hemolysis of the erythrocytes of man, rat, mouse, rabbit and guinea pig, and found that this could be retarded by increasing the CO_2 tension of the solution. The erythrocytes of the ox, sheep, horse, pig, dog and cat hemolyzed slowly and this hemolysis could not be retarded by increasing the CO_2. They also found that a striking inhibition of the effect of glycerol on the erythrocytes could be produced by traces of copper or of copper salts in the water (715).

While investigating the influence of various chemical compounds on the initial rate of hydrolysis of caseinogen at pH 8.7 by pancreatic proteinase, Farber and Wynne found that the reaction was inhibited by glycerol, the degree of inhibition being in direct proportion to the concentration (419).

Shapiro reported that glycerol, when fed in amounts demonstrated to be glycogenic, exerted a definite ketolytic action in rats fed diacetic acid (1419).

Both the force and amplitude of contractions of in vitro rabbit intestine were noted by Pal and Prasad to be increased by the addition of 0.4-1% glycerine (1186).

A series of studies by Ayres and Isgrig demonstrated that moderate intragastric doses of glycerol depress the voluntary running activity, as well as food intake, of rats. These effects were found to increase monotonically with increasing dose level (48).

Buckell and Walsh reported that the oral administration of 75 ml of glycerine was effective in lowering the intracranial pressure of human patients suffering with raised intracranial pressure from many causes. The glycerine produced an increase in plasma osmolality and caused mild diuresis. Very little glycerol was found in the cerebrospinal fluid even after several days of repeated oral administration (179).

Cantori et al., found that oral administration of glycerol to nuerosurgical patients reduced cerebral edema and cerebrospinal-fluid pressure (203).

Sarda et al. reported that oral administration of 3.0 g of 50% glycerol in 0.9% saline/kg of body weight produced a 47% decrease in intraocular pressure one hour after administration to normal rabbits, normal pressure returning after 4 hours. When the intraocular pressure was artificially raised (x1.5) before administration, the glycerol produced a 52.4% drop in pressure after 1.5 hours, followed by a gradual return to normal. The ingested glycerol also produced a mild reduction in the level of Na and K of the serum (Cl remained unaltered), presumably through hemodilution. No significant changes were observed in the electrolyte levels of aqueous humor (1364).

Drance examined the effects of oral administration of 50% glycerine (1.5 g/kg) on the intraocular pressure of 10 nonglaucomatous and 14 glaucomatous eyes (human). Glycerine effectively lowered the intraocular pressure of both normal and glaucomatous eyes, the effect being greater where the initial pressure was higher. The maximum reduction of pressure occurred 2 hours after treatment with normal pressure returning in about 6 hours. Nausea and headache were common side effects of the treatment (353).

Orally administered glycerine (1-1.5 ml/kg) was reported by Thomas to cause a profound reduction in intraocular pressure in the majority of human eyes tested. The effect was always apparent within 30 minutes after administration, frequently appearing within 10 minutes, and lasting from two to several hours (1581).

Casey and Trevor-Roper reported the use of glycerol (1.5 g/kg orally) in the treatment of 22 patients with various types of glaucoma. In 16 cases of chronic, simple (open-angle) glaucoma, there was an average fall in pressure of half of its initial level, occurring 1 hour after treatment. In 5 cases of acute glaucoma, the fall in tension was dramatic (as much as 88%) (211).

Virno et al. reported their observations on the treatment of 26 glaucomatous and 20 normal patients with glycerol (orally, 50% solution in 0.9% saline) in doses ranging from 1.0 to 1.5 g/kg of body weight. They found that glycerol is most effective in cases of acute angle-closure glaucoma; in such cases, intraocular pressure was reduced to about normal levels within 30-60 minutes (1678).

In a study using 10 normal human subjects, Iuglio reported that the administration of a 50% solution of glycerine at a dose of 1 ml/kg of body weight produced a slight reduction in the mean corneal thickness, both central and peripheral, of the normal human eyeball. This result was not shown to be statistically significant (703).

Bornmann et al. reported that glycerine produces a strong laxative effect in rats, guinea pigs, and dogs (137).

Farber and Wynne found that triacetin, when property emulsified, caused inhibition of the hydrolysis of caseinogen by pancreatic proteinase (419).

In tests employing a young, healthy, cholecystectomized female human, Zuckerman et al. found that the oral administration of oleic acid (9 ml/day) with an average mixed diet stimulated the flow of liver bile. When administered together with bile salts, oleic acid potentiated the choleretic effect of the bile salts beyond the additive effects of each of these stimulating agents. Glycerine (3 or 9 ml/day) administered in the same way exhibited a slight choleretic effect but did not potentiate the choleretic effect of added bile salts. Olive oil (15 ml/day) neither stimulated the flow of liver bile nor potentiated the choleretic effect of bile salts (1785).

Pinter et al. administered 25 g of glyceryl monolinoleate (GML) or 25 g of glyceryl monostearate (GMS) to 6 fasting volunteers while another 4 subjects received either 50 g of GML or 50 g of GMS. Fasting, 2-, 4-, 6, and 8- hour blood samples were collected and analyzed for serum triglycerides. GML caused an increase in the serum triglyceride level, the increase being more pronounced at the higher GML level, while GMS had little or no effect at either level (1229).

V. Drug Interaction

Voegtlin et al. reported that the coma and convulsions occurring in albino rats starved for 18 hours and injected with a MLD of insulin s.c. are promptly removed by the i.p. injection of about 1 g/kg of body weight of a 20% solution of pure glycerol. The same result was obtained by a slow i.v. injection of the same amount of glycerol (1679).

Noble and MacLeod reported that subcutaneously administered glycerine has no effect on insulin shock in rabbits (1130).

VI. Consumer Exposure Information

Glycerol is one of the polyhydric alcohols used as a food additive. It is used in foods as a crystillization modifier, a humectant, a plasticizer and a rehydration agent. Glycerol and other polyols contribute to improved quality and shelf life of many substances particularly confectionery. Glycerine is also used in place of alcohol as a solvent for flavoring concentrates.

Mono and diglycerides, acetyl tartaric acid esters of mono and diglycerides, triacetin and glycerol monostearate are surface active agents and are used as emulsifiers in many foods particularly baked goods. They are also used in carbonated beverages as a flavor dispersant, in confections for their anti-staling and anti-sticking properties and also in jam, jellies, dairy products, coffee whiteners and whipped toppings.

The following tables were compiled from data submitted by user firms. Food consumption values for each food category were derived from the Market Research Corporation of America (MRCA) data on frequency of eating and from the USDA data on mean portion size of foods in each food category. The food consumption values thus derived were coupled with the usage level data obtained in the surveys to calculate the daily intake of each substance.

Table 2 reports the usage of glycerin and glycerides and Table 3 their use in infant formulas or baby foods. Table 11 reports the annual poundage data for the various alginates. Table 13 reports the possible daily intake per food category and total dietary based on food consumption by total sample. Table 14 reports potential daily intakes in mg of NAS Appendix A substances (Groups 1 and 11) per food category reported, based on food consumption by eaters only.

TABLE 2 -- 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, %
GLYCERIN	01	BAKED GOODS(R)	23	1.78417	3.40268
NAS 0039 FEMA 2525	04	FATS OILS(R)	*	.01996	.02096
	05	MILK PRODS(R)	*	8.35941	8.35941
	06	CHEESE(R)	*	.00037	.00044
	07	FROZN DAIRY(R)	12	.03296	.04633
	08	PROCSD FRUT(R)	*	.00174	.00696
	10	MEAT PRODS(R)	4	.11120	.17619
	16	SOFT CANDY(R)	26	.17012	.37488
	17	CONF FROST(R)	*	6.97170	6.97381
	18	JAM JELLY(R)	*	4.20000	5.40000
	19	SWEET SAUCE(R)	*	7.40167	7.40167
	20	GELATIN PUD(R)	6	2.56401	4.27479
	22	SNACK FOODS(R)	*	2.15000	2.35000
	23	BEV TYPE I(R)	17	.07195	.13862
	24	BEV TYPE II(R)	7	.04948	.09153
	27	GRAVIES(R)	*	.01660	.02000
	30	HARD CANDY(R)	8	2.17060	2.24858
	31	CHEWING GUM(R)	7	1.36320	1.85483
	48	SEAS FLAVRS(R)	*	.00520	.00520
	49	MISC UNCLAS(R)	*	.04706	.62501
GLYCEROL LACTOPALMITATE	01	BAKED GOODS(R)	*	.16100	.16100
NAS 0281	04	FATS OILS(R)	*	6.00000	10.00000
GLYCERYL MONOSTEARATE	01	BAKED GOODS(R)	8	.31973	.51818
NAS 0090 FEMA 2527	02	BREAK CERLS(R)	*	.23163	.36800
	03	OTHER GRAIN(R)	*	.22565	.50586
	04	FATS OILS(R)	5	.12895	.14232
	06	CHEESE(R)	*	.50000	1.00000
	07	FROZN DAIRY(R)	4	.04840	.08184
	10	MEAT PRODS(R)	*	.01000	.03000
	16	SOFT CANDY(R)	6	.36550	.38879
	17	CONF FROST(R)	*	.02500	.08000
	19	SWEET SAUCE(R)	*	.39000	.39000
	20	GELATIN PUD(R)	*	.50000	.50000
	21	SOUPS(R)	*	.03000	.03000
	22	SNACK FOODS(R)	*	.10559	.20083
	23	BEV TYPE I(R)	*	.00200	.00250
	25	NUT PRODS(R)	*	2.00000	2.50000
	28	IMIT DAIRY(R)	*	.19227	.29151
	31	CHEWING GUM(R)	6	********	********

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TABLE 2 -- 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, %
MONO- AND DIGLYCERIDES NAS 0130 FEMA 3511	01	BAKED GOODS(R)	24	.61436	1.27934
	02	BREAK CERLS(R)	*	.24980	.49960
	03	OTHER GRAIN(R)	*	.25000	.25000
	04	FATS OILS(R)	15	3.33450	8.24346
	05	MILK PRODS(R)	4	.14973	.16249
	06	CHEESE(R)	*	.00250	.01000
	07	FROZN DAIRY(R)	9	.10351	.19350
	08	PROCSD FRUT(R)	*	3.80000	3.80000
	10	MEAT PRODS(R)	*	.14337	.19047
	11	POULTRY(R)	*	.00605	.01254
	13	FISH PRODS(R)	*	.03500	.03600
	14	PROCSD VEGS(R)	7	.03004	.03214
	15	CONDM RELSH(R)	*	.02000	.02000
	16	SOFT CANDY(R)	7	.24977	.49977
	17	CONF FROST(R)	*	.70000	1.40000
	18	JAM JELLY(R)	*	1.00000	3.00000
	19	SWEET SAUCE(R)	*	4.00000	4.00000
	20	GELATIN PUD(R)	5	1.66956	2.29107
	21	SOUPS(R)	*	.04656	.04688
	22	SNACK FOODS(R)	*	.20000	.50000
	23	BEV TYPE I(R)	*	.00151	.01580
	24	BEV TYPE II(R)	*	.10000	.10000
	25	NUT PRODS(R)	5	1.09936	1.92247
MONO- AND DIGLYCERIDES NAS 0130 FEMA 3511	26	RECONST VEG(R)	*	.35841	.38142
	27	GRAVIES(R)	*	.01660	.07194
	28	IMIT DAIRY(R)	9	.63713	.84600
	30	HARD CANDY(R)	*	.03313	.03435
	31	CHEWING GUM(R)	8	********	********
	48	SEAS FLAVRS(R)	*	.15000	.20000
MONOGLYCERIDE CITRATE NAS 0131	02	BREAK CERLS(R)	*	.00010	.00010
	04	FATS OILS(R)	*	.00200	.00350
	22	SNACK FOODS(R)	*	.01030	.01030
MONOSODIUM PHOSMONODIGLY NAS 0135	28	IMIT DAIRY(R)	*	.52500	.52500

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TABLE 3 -- 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, %
0090	GLYCERYL MONOSTEARATE	82	CEREALS(B)	*	.40000	.42000
		83	FORMULAS(B)	*	.20000	.20000
0130	MONO- AND DIGLYCERIDES	83	FORMULAS(B)	*	.04091	.11218

TABLE 11. 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
GLYCERIN NAS 0089 FEMA 2525	34/ 41	1,786,759	4,006,003	5,830,336	38	650,360	6,480,696
GLYCERYL MONOSTEARATE NAS 0090 FEMA 2527	25/ 34	1,007,131	5,704,479	6,109,793	5	33,022	6,142,815
GLYCEROL LACTOPALMITATE NAS 0281	*/ *	0	20,374	60,374			60,374
MONO- AND DIGLYCERIDES NAS 0130 FEMA 3511	67/ 73	44,082,329	70,082,664	72,736,594	*	4,711	72,741,305
MONOGLYCERIDE CITRATE NAS 0131	*/ *	0	4,000	4,000			4,000
MONO-DIGLY NA SULFOAC DER NAS 0294	*/ *	4,000	40,000	40,000			40,000
MONOSODIUM PHOSMONODIGLY NAS 0135	/ *			27,000			27,000
TRIACETIN NAS 0233 FEMA 2007	*/ *	2,552	8,940	13,940	19	23,284	37,224

COMPREHENSIVE GRAS SURVEY -- NAS/NRC 1972 10/02/72

TABLE 13, 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 EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	POSSIBLE DAILY INTAKE, MG. (AGE)	AVERAGE	HIGH A	HIGH B
GLYCERIN NAS 0089	01 BAKED GOODS(R)	23	0-5 MO.	60.661780	80.287650	115.725120
			6-11 MO.	453.179180	924.200060	864.534720
			12-23 MO.	972.372650	1602.194660	1855.005600
			2-65+ YR.	2447.881240	3636.138460	4669.848960
GLYCERIN NAS 0089	04 FATS OILS(R)	*	0-5 MO.	.099800	.099800	.104000
			6-11 MO.	.568880	1.497000	.568880
			12-23 MO.	1.297480	2.395200	1.320480
			2-65+ YR.	3.493000	6.307360	3.680000
GLYCERIN NAS 0089	05 MILK PRODS(R)	*	0-5 MO.	451.408140	334.376400	451.408140
			6-11 MO.	5216.271840	25086.589410	5216.271840
			12-23 MO.	4555.878450	14578.811040	4555.878450
			2-65+ YR.	3301.966950	10081.449460	3301.966950
GLYCERIN NAS 0089	06 CHEESE(R)	*	0-5 MO.	**********	.000370	**********
			6-11 MO.	.009990	.035890	.011680
			12-23 MO.	.028860	.082140	.034320
			2-65+ YR.	.034780	.087320	.041360
GLYCERIN NAS 0089	07 FROZN DAIRY(R)	12	0-5 MO.	.329600	1.351360	.463300
			6-11 MO.	3.131200	8.701440	4.401350
			12-23 MO.	4.746240	11.140480	6.671520
			2-65+ YR.	8.437760	20.336320	11.860480
GLYCERIN NAS 0089	08 PROCSD FRUT(R)	*	0-5 MO.	.081780	.219240	.327120
			6-11 MO.	.901320	2.244600	3.605280
			12-23 MO.	1.750440	3.474780	7.001760
			2-65+ YR.	2.058420	4.360440	8.233680
GLYCERIN NAS 0089	10 MEAT PRODS(R)	4	0-5 MO.	1.223200	3.224800	1.938090
			6-11 MO.	23.018400	62.049600	36.471330
			12-23 MO.	33.582400	57.712800	53.209380
			2-65+ YR.	87.180000	144.671200	138.132960
GLYCERIN NAS 0089	16 SOFT CANDY(R)	26	0-5 MO.	.356240	3.562400	.749760
			6-11 MO.	2.918640	12.112160	8.247360
			12-23 MO.	6.234200	16.565160	13.120800
			2-65+ YR.	10.330960	31.349120	21.743040
GLYCERIN NAS 0089	17 CONF FROST(R)	*	0-5 MO.	**********	6.971700	**********
			6-11 MO.	6.971700	13.943400	6.973810
			12-23 MO.	13.943400	48.801900	13.947620
			2-65+ YR.	20.915100	55.773600	20.921430
GLYCERIN NAS 0089	18 JAM JELLY(R)	*	0-5 MO.	**********	12.600000	**********
			6-11 MO.	281.400000	936.600000	361.800000
			12-23 MO.	126.000000	470.400000	162.000000
			2-65+ YR.	239.400000	743.400000	307.800000

TABLE 13, 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 EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO.	NAME	# OF FIRMS	POSSIBLE DAILY INTAKE, MG. (AGE)	AVERAGE	HIGH A	HIGH B
GLYCERIN NAS 0089	19	SWEET SAUCE(R)	*	0-5 MO.	22.205010	29.606680	22.205010
				6-11 MO.	66.615030	229.451770	66.615030
				12-23 MO.	192.443420	562.526920	192.443420
				2-65+ YR.	503.313560	1324.898930	503.313560
GLYCERIN NAS 0089	20	GELATIN PUD(R)	6	0-5 MO.	51.280200	69.228270	85.499000
				6-11 MO.	326.193280	994.835800	547.173120
				12-23 MO.	353.833380	861.507360	589.921020
				2-65+ YR.	523.058040	1346.105250	872.057160
GLYCERIN NAS 0089	22	SNACK FOODS(R)	*	0-5 MO.	**********	2.150000	**********
				6-11 MO.	8.600000	23.650000	9.400000
				12-23 MO.	23.650000	66.650000	25.850000
				2-65+ YR.	27.950000	79.550000	30.550000
GLYCERIN NAS 0089	23	BEV TYPE I(R)	17	0-5 MO.	1.726800	2.590200	3.326880
				6-11 MO.	16.332650	55.905150	31.466740
				12-23 MO.	38.996900	116.918750	75.132040
				2-65+ YR.	74.828000	199.005150	144.164800
GLYCERIN NAS 0089	24	BEV TYPE II(R)	7	0-5 MO.	.000000	.000000	.000000
				6-11 MO.	**********	.049450	**********
				12-23 MO.	**********	.098960	**********
				2-65+ YR.	16.081000	46.709120	29.747250
GLYCERIN NAS 0089	27	GRAVIES(P)	*	0-5 MO.	.016600	.049800	.020000
				6-11 MO.	.232400	.647400	.280000
				12-23 MO.	.597600	1.693200	.720000
				2-65+ YR.	1.377800	3.535800	1.660000
GLYCERIN NAS 0089	30	HARD CANDY(R)	8	0-5 MO.	.000000	.000000	.000000
				6-11 MO.	2.170600	6.511800	2.248580
				12-23 MO.	6.511800	19.535400	6.745740
				2-65+ YR.	13.023600	36.900200	13.491480
GLYCERIN NAS 0089	31	CHEWING GUM(R)	7	0-5 MO.	**********	**********	**********
				6-11 MO.	1.363200	1.363200	1.854930
				12-23 MO.	1.363200	4.089600	1.854930
				2-65+ YR.	2.726400	5.452800	3.709860
GLYCERIN NAS 0089	48	SEAS FLAVRS(R)	*	0-5 MO.	**********	**********	**********
				6-11 MO.	**********	.000520	**********
				12-23 MO.	**********	.001040	**********
				2-65+ YR.	.000520	.002600	.000520
GLYCERIN NAS 0089	ALL CATEGORIES		82	0-5 MO.	589.385150	546.318670	681.764020
	**********			6-11 MO.	6412.866310	2836.388760	7161.942750
	**********			12-23 MO.	6333.190420	18424.589390	7560.856980
	**********			2-65+ YR.	7264.057930	17766.832130	10082.911290

TABLE 13, 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 EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	********** POSSIBLE DAILY INTAKE, MG. ********** (AGE)	AVERAGE	HIGH A	HIGH B
GLYCEROL LACTOPALMITATE NAS 0281	01 BAKED GOODS(R)	*	0-5 MO.	5.474000	7.245000	5.474000
			6-11 MO.	40.894000	83.393000	40.894000
			12-23 MO.	87.745000	144.578000	87.745000
			2-65+ YR.	220.892000	328.118000	220.892000
GLYCEROL LACTOPALMITATE NAS 0281	04 FATS OILS(R)	*	0-5 MO.	30.000000	30.000000	50.000000
			6-11 MO.	168.000000	450.000000	200.000000
			12-23 MO.	378.000000	720.000000	630.000000
			2-65+ YR.	1050.000000	1896.000000	1750.000000
GLYCEROL LACTOPALMITATE NAS 0281	ALL CATEGORIES ********** ********** **********	*	0-5 MO.	35.474000	37.245000	55.474000
			6-11 MO.	208.894000	533.393000	320.894000
			12-23 MO.	465.745000	864.578000	717.745000
			2-65+ YR.	1270.892000	2224.118000	1970.892000
GLYCERYL MONOSTEARATE NAS 0090	01 BAKED GOODS(R)	8	0-5 MO.	10.870020	14.387850	17.618120
			6-11 MO.	81.211420	165.620140	131.617720
			12-23 MO.	174.252850	287.117540	282.408100
			2-65+ YR.	438.669560	651.609740	710.942960
GLYCERYL MONOSTEARATE NAS 0090	02 BREAK CEREALS(R)	*	0-5 MO.	1.389780	3.937710	2.208000
			6-11 MO.	51.653490	138.514740	82.094000
			12-23 MO.	60.455430	117.899670	96.048000
			2-65+ YR.	46.326000	119.984340	73.600000

TABLE 13, 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 EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	N OF FIRMS	POSSIBLE DAILY INTAKE, MG. (AGE)	AVERAGE	HIGH A	HIGH B
GLYCERYL MONOSTEARATE NAS 0090	03 OTHER GRAIN(R)	*	0-5 MO.	1.128250	3.836050	2.529300
			6-11 MO.	21.888050	64.535900	49.068420
			12-23 MO.	37.006600	85.521350	82.961040
			2-65+ YR.	62.730700	138.549100	140.629080
GLYCERYL MONOSTEARATE NAS 0090	04 FATS OILS(R)	5	0-5 MO.	.644750	.644750	.711600
			6-11 MO.	3.610000	9.671250	3.984960
			12-23 MO.	8.123850	15.474000	8.966160
			2-65+ YR.	22.566250	40.748200	24.906000
GLYCERYL MONOSTEARATE NAS 0090	06 CHEESE(R)	*	0-5 MO.	**********	.500000	**********
			6-11 MO.	13.500000	48.500000	27.000000
			12-23 MO.	39.000000	111.000000	78.000000
			2-65+ YR.	47.000000	118.000000	94.000000
GLYCERYL MONOSTEARATE NAS 0090	07 FROZN DAIRY(R)	4	0-5 MO.	.484000	1.984400	.818400
			6-11 MO.	4.598000	12.777600	7.774400
			12-23 MO.	6.986000	16.359200	11.784960
			2-65+ YR.	12.390400	29.862000	20.951040
GLYCERYL MONOSTEARATE NAS 0090	10 MEAT PRODS(R)	*	0-5 MO.	.110000	.290000	.330000
			6-11 MO.	2.070000	5.580000	6.210000
			12-23 MO.	3.020000	5.190000	9.060000
			2-65+ YR.	7.840000	13.010000	23.520000
GLYCERYL MONOSTEARATE NAS 0090	16 SOFT CANDY(R)	6	0-5 MO.	.731000	7.310000	.777580
			6-11 MO.	8.041000	24.854000	8.552380
			12-23 MO.	12.792500	33.991500	13.607650
			2-65+ YR.	21.195000	64.320000	22.549820
GLYCERYL MONOSTEARATE NAS 0090	17 CONF FROST(R)	*	0-5 MO.	**********	.025000	**********
			6-11 MO.	.025000	.050000	.080000
			12-23 MO.	.050000	.175000	.160000
			2-65+ YR.	.075000	.200000	.240000
GLYCERYL MONOSTEARATE NAS 0090	19 SHEET SAUCE(R)	*	0-5 MO.	1.170000	1.560000	1.170000
			6-11 MO.	3.510000	12.090000	3.510000
			12-23 MO.	10.140000	29.640000	10.140000
			2-65+ YR.	26.520000	69.810000	26.520000
GLYCERYL MONOSTEARATE NAS 0090	20 GELATIN PUD(R)	*	0-5 MO.	10.000000	13.500000	10.000000
			6-11 MO.	64.000000	194.000000	64.000000
			12-23 MO.	69.000000	168.000000	69.000000
			2-65+ YR.	102.000000	262.500000	102.000000

TABLE 13, 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 EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	POSSIBLE DAILY INTAKE, MG. (AGE)	AVERAGE	HIGH A	HIGH B
GLYCERYL MONOSTEARATE NAS 0090	21 SOUPS(R)	*	0-5 MO.	.060000	.450000	.060000
			6-11 MO.	6.990000	21.810000	6.990000
			12-23 MO.	10.440000	28.830000	10.440000
			2-65+ YR.	9.510000	25.350000	9.510000
GLYCERYL MONOSTEARATE NAS 0090	22 SNACK FOODS(R)	*	0-5 MO.	**********	.105590	**********
			6-11 MO.	.422360	1.161490	.803320
			12-23 MO.	1.161490	3.273290	2.209130
			2-65+ YR.	1.372670	3.906830	2.610790
GLYCERYL MONOSTEARATE NAS 0090	23 BEV TYPE I(R)	*	0-5 MO.	.040000	.072000	.060000
			6-11 MO.	.454000	1.554000	.567500
			12-23 MO.	1.084000	3.250000	1.355000
			2-65+ YR.	2.080000	5.554000	2.600000
GLYCERYL MONOSTEARATE NAS 0090	25 NUT PRODS(R)	*	0-5 MO.	**********	4.000000	**********
			6-11 MO.	74.000000	268.000000	92.500000
			12-23 MO.	54.000000	180.000000	67.500000
			2-65+ YR.	104.000000	310.000000	130.000000
GLYCERYL MONOSTEARATE NAS 0090	28 IMIT DAIRY(R)	*	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	2.691780	4.422210	4.001140
			12-23 MO.	1.538160	6.537180	2.332080
			2-65+ YR.	1.730430	2.804050	2.623590
GLYCERYL MONOSTEARATE NAS 0090	31 CHEWING GUM(R)	6	0-5 MO.	**********	**********	**********
			6-11 MO.	**********	**********	**********
			12-23 MO.	**********	**********	**********
			2-65+ YR.	**********	**********	**********
GLYCERYL MONOSTEARATE NAS 0090	82 CEREALS(B)	*	0-5 MO.	54.000000	93.200000	56.700000
			6-11 MO.	36.800000	96.400000	38.640000
			12-23 MO.	8.000000	36.000000	8.400000
GLYCERYL MONOSTEARATE NAS 0090	83 FORMULAS(B)	*	0-5 MO.	671.400000	1230.000000	671.400000
			6-11 MO.	136.800000	651.800000	136.800000
			12-23 MO.	44.000000	12.400000	44.000000
GLYCERYL MONOSTEARATE NAS 0090	ALL CATEGORIES ********** ********** **********	39	0-5 MO.	752.036600	1375.803350	764.383000
			6-11 MO.	512.265700	1721.341330	664.245240
			12-23 MO.	541.034400	1140.658730	792.372120
			2-65+ YR.	906.010010	1856.297060	1387.203260

TABLE 13, 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 EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO.	NAME	# OF FIRMS	(AGE)	POSSIBLE DAILY INTAKE, MG. AVERAGE	HIGH A	HIGH B
MONO- AND DIGLYCERIDES NAS 0130	04	FATS OILS(R)	15	0-5 MO.	16.672500	16.672500	41.217300
				6-11 MO.	93.366000	250.007500	230.816880
				12-23 MO.	210.073500	400.140000	519.337950
				2-65+ YR.	583.537500	1053.702000	1442.605500
MONO- AND DIGLYCERIDES NAS 0130	05	MILK PRODS(R)	4	0-5 MO.	8.085420	5.989200	8.774460
				6-11 MO.	93.431520	446.339730	101.393760
				12-23 MO.	81.602850	261.129120	88.557050
				2-65+ YR.	59.143350	180.574380	64.183550
MONO- AND DIGLYCERIDES NAS 0130	06	CHEESE(R)	*	0-5 MO.	**********	.002500	**********
				6-11 MO.	.067500	.242500	.270000
				12-23 MO.	.195000	.555000	.780000
				2-65+ YR.	.235000	.990000	.940000
MONO- AND DIGLYCERIDES NAS 0130	07	FROZN DAIRY(R)	9	0-5 MO.	1.035100	4.243910	1.935000
				6-11 MO.	9.833450	27.323640	18.382500
				12-23 MO.	14.905440	34.986380	27.864000
				2-65+ YR.	26.498560	63.865670	49.536000
MONO- AND DIGLYCERIDES NAS 0130	08	PROCSD FRUT(R)	*	0-5 MO.	178.600000	478.800000	178.600000
				6-11 MO.	1968.400000	4902.000000	1968.400000
				12-23 MO.	3622.800000	7588.800000	3622.800000
				2-65+ YR.	4495.400000	9522.800000	4495.400000
MONO- AND DIGLYCERIDES NAS 0130	10	MEAT PRODS(R)	*	0-5 MO.	1.577070	4.157730	2.095170
				6-11 MO.	29.677590	80.000460	39.427290
				12-23 MO.	43.297740	74.409030	57.521940
				2-65+ YR.	112.402080	186.524370	149.328430
MONO- AND DIGLYCERIDES NAS 0130	11	POULTRY(R)	*	0-5 MO.	.030250	.139150	.062700
				6-11 MO.	.235950	.798600	.439060
				12-23 MO.	.399300	1.113200	.827640
				2-65+ YR.	.780480	1.984480	1.617560
MONO- AND DIGLYCERIDES NAS 0130	13	FISH PRODS(R)	*	0-5 MO.	.035000	.105000	.036000
				6-11 MO.	.455000	1.715000	.468000
				12-23 MO.	1.890000	4.725000	1.944000
				2-65+ YR.	4.340000	10.815000	4.464000
MONO- AND DIGLYCERIDES NAS 0130	14	PROCSD VEGS(R)	7	0-5 MO.	.420560	1.261620	.469960
				6-11 MO.	7.209000	16.822600	7.713600
				12-23 MO.	11.715600	19.616120	12.534600
				2-65+ YR.	25.534000	43.017200	27.319000

TABLE 13, PART A — POSSIBLE DAILY INTAKES OF GRAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO.	NAME	# OF FIRMS	POSSIBLE DAILY INTAKE, MG. (AGE)	AVERAGE	HIGH A	HIGH B
MONO- AND DIGLYCERIDES NAS 0130	15	CONDM RELSH(R)	*	0-5 MO.	**********	.020000	**********
				6-11 MO.	.160000	.440000	.160000
				12-23 MO.	.560000	1.520000	.560000
				2-65+ YR.	1.700000	4.240000	1.700000
MONO- AND DIGLYCERIDES NAS 0130	16	SOFT CANDY(R)	7	0-5 MO.	.499540	4.955400	.999540
				6-11 MO.	5.494940	16.934360	10.994940
				12-23 MO.	8.741950	23.220610	17.401950
				2-65+ YR.	14.492660	43.959520	28.926660
MONO- AND DIGLYCERIDES NAS 0130	17	CONF FROST(R)	*	0-5 MO.	**********	.700000	**********
				6-11 MO.	.700000	1.400000	1.400000
				12-23 MO.	1.400000	4.900000	2.800000
				2-65+ YR.	2.100000	5.600000	4.200000
MONO- AND DIGLYCERIDES NAS 0130	18	JAM JELLY(R)	*	0-5 MO.	**********	3.000000	**********
				6-11 MO.	67.000000	223.000000	201.000000
				12-23 MO.	30.000000	112.000000	90.000000
				2-65+ YR.	57.000000	177.000000	171.000000
MONO- AND DIGLYCERIDES NAS 0130	19	SWEET SAUCE(R)	*	0-5 MO.	12.000000	16.000000	12.000000
				6-11 MO.	36.000000	124.000000	36.000000
				12-23 MO.	104.000000	304.000000	104.000000
				2-65+ YR.	272.000000	716.000000	272.000000
MONO- AND DIGLYCERIDES NAS 0130	20	GELATIN PUD(R)	5	0-5 MO.	33.391200	45.078120	45.821400
				6-11 MO.	213.703680	647.789280	253.256960
				12-23 MO.	230.399280	560.978160	316.107660
				2-65+ YR.	340.590240	876.519000	467.375280
MONO- AND DIGLYCERIDES NAS 0130	21	SOUPS(R)	*	0-5 MO.	.093120	.598400	.093760
				6-11 MO.	10.848480	33.849120	10.922040
				12-23 MO.	16.202880	44.744160	16.314240
				2-65+ YR.	14.759520	39.343200	14.860960
MONO- AND DIGLYCERIDES NAS 0130	22	SNACK FOODS(R)	*	0-5 MO.	**********	.000000	**********
				6-11 MO.	.800000	2.200000	2.000000
				12-23 MO.	2.200000	6.200000	5.500000
				2-65+ YR.	2.600000	7.400000	6.500000
MONO- AND DIGLYCERIDES NAS 0130	23	BEV TYPE I(R)	*	0-5 MO.	.036240	.054360	.079200
				6-11 MO.	.342770	1.173270	3.000000
				12-23 MO.	.810420	2.453750	2.583000
				2-65+ YR.	1.570400	4.193270	16.432000

TABLE 13, 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 EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO.	NAME	# OF FIRMS	POSSIBLE DAILY INTAKE, MG. (AGE)	AVERAGE	HIGH A	HIGH B
MONO- AND DIGLYCERIDES NAS 0130	24	BEV TYPE II(R)	*	0-5 MO.	.000000	.000000	.000000
				6-11 MO.	**********	.100000	**********
				12-23 MO.	**********	.200000	**********
				2-65+ YR.	32.500000	94.400000	32.500000
MONO- AND DIGLYCERIDES NAS 0130	25	NUT PRODS(R)	5	0-5 MO.	**********	2.190720	**********
				6-11 MO.	40.676320	147.314240	71.131350
				12-23 MO.	29.682720	98.942400	51.906600
				2-65+ YR.	57.160720	170.400800	99.9[illegible]
MONO- AND DIGLYCERIDES NAS 0130	26	BROCAST VEG(R)	*	0-5 MO.	.000000	.000000	.000000
				6-11 MO.	.000000	.000000	.000000
				12-23 MO.	.000000	.000000	.000000
				2-65+ YR.	.716820	2.150460	.782340
MONO- AND DIGLYCERIDES NAS 0130	27	GRAVIES(R)	*	0-5 MO.	.016800	.063000	.031010
				6-11 MO.	.282400	.647400	1.007160
				12-23 MO.	.597600	1.693200	2.[illegible]
				2-65+ YR.	1.377800	3.535000	5.971020
MONO- AND DIGLYCERIDES NAS 0130	28	INST DAIRY(R)	9	0-5 MO.	.000000	.000000	.000000
				6-11 MO.	8.919720	14.653900	11.344000
				12-23 MO.	5.097040	21.042420	6.700000
				2-65+ YR.	5.734170	9.550970	7.634000
MONO- AND DIGLYCERIDES NAS 0130	30	HARD CANDY(R)	*	0-5 MO.	.000000	.000000	.000000
				6-11 MO.	.033130	.090300	.034370
				12-23 MO.	.091300	.294170	.113050
				2-65+ YR.	.190780	.965210	.266100
MONO- AND DIGLYCERIDES NAS 0130	31	CHEWING GUM(R)	8	0-5 MO.	**********	**********	**********
				6-11 MO.	**********	**********	**********
				12-23 MO.	**********	**********	**********
				2-65+ YR.	**********	**********	**********
MONO- AND DIGLYCERIDES NAS 0130	49	SEAS FLAVRS(R)	*	0-5 MO.	**********	**********	**********
				6-11 MO.	**********	.015000	**********
				12-23 MO.	**********	.030000	**********
				2-65+ YR.	.015000	.075000	.020000
MONO- AND DIGLYCERIDES NAS 0130	33	FORMULAS(R)	*	0-5 MO.	137.344670	251.5[illegible]	375.[illegible]
				6-11 MO.	27.[illegible]	133.[illegible]	76.[illegible]
				12-23 MO.	9.000200	2.536420	24.679000

TABLE 13, 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 EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	POSSIBLE DAILY INTAKE, MG. (AGE)	AVERAGE	HIGH A	HIGH B
MONO- AND DIGLYCERIDES NAS 0130	ALL CATEGORIES	74	0-5 MO.	413.464510	872.105770	716.369850
	**********		6-11 MO.	2651.573430	7614.443450	3548.043310
	**********		12-23 MO.	5066.702910	10344.243620	6048.247740
	**********		2-65+ YR.	7074.008970	14753.772390	9200.220970
MONOGLYCERIDE CITRATE NAS 0131	02 BREAK CERLS(R)	*	0-5 MO.	.000000	.001700	.000000
			6-11 MO.	.022300	.059800	.022300
			12-23 MO.	.026100	.050900	.026100
			2-65+ YR.	.020000	.051900	.000000
MONOGLYCERIDE CITRATE NAS 0131	04 FATS OILS(R)	*	0-5 MO.	.010000	.010000	.010000
			6-11 MO.	.056000	.190000	.056000
			12-23 MO.	.120000	.240000	.220000
			2-65+ YR.	.350000	.632000	.612000
MONOGLYCERIDE CITRATE NAS 0131	22 SNACK FOODS(R)	*	0-5 MO.	**********	.010300	**********
			6-11 MO.	.041200	.113300	.041200
			12-23 MO.	.113300	.319400	.113200
			2-65+ YR.	.133900	.381100	.133900
MONOGLYCERIDE CITRATE NAS 0131	ALL CATEGORIES	*	0-5 MO.	.010000	.022000	.018100
	**********		6-11 MO.	.119900	.323100	.161500
	**********		12-23 MO.	.265400	.610200	.339900
	**********		2-65+ YR.	.503900	1.064900	.788400
MONOSODIUM PHOSPHOMONOGLY. NAS 0135	20 INST. DAIRY(R)	*	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	7.350000	12.075000	7.350000
			12-23 MO.	4.200000	17.850000	4.200000
			2-65+ YR.	4.725000	7.875000	4.725000
MONOSODIUM PHOSPHOMONOGLY NAS 0135	ALL CATEGORIES	*	0-5 MO.	.000000	.000000	.000000
	**********		6-11 MO.	7.350000	12.075000	7.350000
	**********		12-23 MO.	4.200000	17.850000	4.200000
	**********		2-65+ YR.	4.725000	7.875000	4.725000

TABLE 13, 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 EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO.	NAME	# OF FIRMS	(AGE)	POSSIBLE DAILY INTAKE, MG. AVERAGE	HIGH A	HIGH B
TRIACETIN NAS 0233	01	BAKED GOODS(R)	11	0-5 MO.	1.376660	1.822050	2.021640
				6-11 MO.	10.284460	20.973820	15.102840
				12-23 MO.	22.067050	36.360020	32.405700
				2-65+ YR.	55.552220	82.510620	81.979120
TRIACETIN NAS 0233	07	FROZN DAIRY(R)	7	0-5 MO.	.200800	.823280	.636300
				6-11 MO.	1.907800	5.301120	6.044550
				12-23 MO.	2.891520	6.787040	9.162720
				2-65+ YR.	5.140480	12.389360	16.299280
TRIACETIN NAS 0233	16	SOFT CANDY(R)	11	0-5 MO.	.040360	.403600	.152720
				6-11 MO.	.443560	1.372240	1.679520
				12-23 MO.	.702200	1.876740	2.672600
				2-65+ YR.	1.170440	3.551680	4.428880
TRIACETIN NAS 0233	17	CONF FROST(R)	*	0-5 MO.	**********	.001000	**********
				6-11 MO.	.001000	.002000	.002000
				12-23 MO.	.002000	.007000	.004000
				2-65+ YR.	.003000	.008000	.006000
TRIACETIN NAS 0233	20	GELATIN PUD(R)	5	0-5 MO.	.199200	.268920	.584000
				6-11 MO.	1.274880	3.664480	3.741440
				12-23 MO.	1.374480	3.246560	4.023740
				2-65+ YR.	2.031840	5.220000	5.962520
TRIACETIN NAS 0233	23	BEV TYPE I(R)	11	0-5 MO.	.275040	.412560	.682080
				6-11 MO.	2.601420	8.904420	6.451340
				12-23 MO.	6.211320	18.622500	15.403540
				2-65+ YR.	11.918400	31.824420	29.556800
TRIACETIN NAS 0233	24	BEV TYPE II(R)	*	0-5 MO.	.000000	.000000	.000000
				6-11 MO.	**********	.026130	**********
				12-23 MO.	**********	.052260	**********
				2-65+ YR.	8.492250	24.666720	23.273250
TRIACETIN NAS 0233	30	HARD CANDY(R)	*	0-5 MO.	.000000	.000000	.000000
				6-11 MO.	.003970	.011910	.004580
				12-23 MO.	.011910	.035730	.014040
				2-65+ YR.	.022820	.067490	.028080
TRIACETIN NAS 0233	31	CHEWING GUM(R)	*	0-5 MO.	**********	**********	**********
				6-11 MO.	.000600	.000600	.000600
				12-23 MO.	.000600	.001800	.000600
				2-65+ YR.	.001200	.002400	.001200
TRIACETIN NAS 0233	ALL CATEGORIES ********** ********** **********		21	0-5 MO.	2.092080	3.731410	4.077340
				6-11 MO.	16.517390	40.456720	33.027670
				12-23 MO.	33.265150	67.089650	63.697040
				2-65+ YR.	84.332710	160.257600	161.125530

TABLE 14

Food Category	Substance	# of Firms	Age	Average	High A	High B	Very High
Baked Goods	Glycerin	23	0-5 mo	276.546	915.279	527.570	1746.088
			6-11 mo	512.057	972.373	976.856	1855.006
			12-23 mo	979.509	1602.185	1868.620	3056.505
			2-65 yr	2449.665	3637.923	4673.253	6940.104
Fats, Oils	Glycerin	*	0-5 mo	.978	2.774	1.027	2.913
			6-11 mo	.818	1.657	.859	1.740
			12-23 mo	1.297	2.415	1.362	2.536
			2-65 yr	3.553	6.347	3.731	6.665
Milk Products	Glycerin	*	0-5 mo	4363.612	9471.212	4363.612	9471.212
			6-11 mo	10975.905	33404.202	10975.905	33404.202
			12-23 mo	7055.342	25705.186	7055.342	25705.186
			2-65 yr	4748.145	12990.523	4748.145	12990.523
Cheese	Glycerin	*	0-5 mo	.003	.004	.003	.005
			6-11 mo	.032	.060	.038	.072
			12-23 mo	.043	.092	.051	.110
			2-65 yr	.048	.100	.057	.119
Frozen Dairy	Glycerin	*	0-5 mo	2.307	5.340	3.243	7.505
			6-11 mo	5.043	10.152	7.088	14.270
			12-23 mo	6.065	12.096	8.525	17.003
			2-65 yr	11.108	22.611	15.613	31.782
Procsd Fruit	Glycerin	*	0-5 mo	.440	1.128	1.760	4.510
			6-11 mo	1.166	2.579	4.663	10.315
			12-23 mo	2.043	3.710	8.171	14.839
			2-65 yr	2.241	4.470	8.964	17.880
Meat Prods	Glycerin	4	0-5 mo	12.121	30.358	19.205	48.100
			6-11 mo	33.471	68.054	53.033	107.828
			12-23 mo	35.250	58.380	55.852	92.500
			2-65 yr	88.070	145.005	139.542	229.752
Soft Candy	Glycerin	26	0-5 mo	9.975	16.743	20.993	35.239
			6-11 mo	13.359	30.280	28.116	63.730
			12-23 mo	13.181	28.499	27.741	59.981
			2-65 yr	20.306	43.996	42.736	92.595

TABLE 14

Food Category	Substance	# of Firms	Age	Average	High A	High B	Very High
Conf Frost	Glycerin	*	0-5 mo	13.943	13.943	13.948	13.948
			6-11 mo	55.774	153.377	55.790	153.424
			12-23 mo	181.264	390.415	181.319	390.533
			2-65 yr	237.037	432.245	237.109	432.376
Jam, Jelly	Glycerin	*	0-5 mo	29.400	42.000	37.800	54.000
			6-11 mo	768.600	1722.000	988.200	3214.000
			12-23 mo	243.600	541.800	313.200	696.600
			2-65 yr	462.000	1062.600	594.000	1366.200
Sweet Sauce	Glycerin	*	0-5 mo	606.937	1554.351	606.937	1554.351
			6-11 mo	185.042	362.682	185.042	362.682
			12-23 mo	355.280	636.544	355.280	636.544
			2-65 yr	910.405	1806.007	910.405	1806.007
Gelatin, Pudding	Glycerin	6	0-5 mo	602.542	1851.215	1004.576	3086.398
			6-11 mo	587.158	1225.597	978.927	2043.350
			12-23 mo	515.366	1064.064	859.233	1774.038
			2-65 yr	792.279	1605.070	1320.910	2676.019
Snack Foods	Glycerin	*	0-5 mo	6.450	10.750	7.050	11.750
			6-11 mo	21.500	38.700	23.500	42.300
			12-23 mo	36.550	75.250	39.950	82.250
			2-65 yr	45.150	98.900	49.350	108.100
Bev Type I	Glycerin	*	0-5 mo	23.456	97.420	45.190	187.691
			6-11 mo	33.888	75.835	65.290	146.105
			12-23 mo	58.423	134.331	112.559	258.804
			2-65 yr	102.385	229.449	197.256	442.059
Bev Type II	Glycerin	*	0-5 mo	0.000	0.000	0.000	0.000
			6-11 mo	.198	.297	.366	.549
			12-23 mo	.346	.594	.641	1.098
			2-65 yr	66.402	163.284	122.833	302.049
Gravies	Glycerin	*	0-5 mo	.183	.349	.220	.420
			6-11 mo	.515	.979	.620	1.180
			12-23 mo	1.162	2.324	1.400	2.800
			2-65 yr	2.208	4.382	2.660	5.280

TABLE 14

Food Category	Substance	# of Firms	Age	Average	High A	High B	Very High
Hard Candy	Glycerin	8	0-5 mo	.000	.000	.000	.000
			6-11 mo	17.365	26.047	17.989	26.983
			12-23 mo	43.412	91.165	44.972	94.440
			2-65 yr	106.359	221.401	110.180	229.355
Chewing Gum	Glycerin	7	0-5 mo	---	---	---	---
			6-11 mo	10.906	19.085	14.839	25.968
			12-23 mo	8.179	17.722	11.129	24.113
			2-65 yr	9.542	20.448	12.984	27.823
Seas, Flavors	Glycerin	*	0-5 mo	---	---	---	---
			6-11 mo	.001	.002	.001	.002
			12-23 mo	.002	.003	.002	.003
			2-65 yr	.005	.010	.005	.010

TABLE 14

Food Category	Substance	# of Firms	Age	Average	High A	High B	Very High
Conf Frost	Diacetyl tartaric acid esters of mono and diglycerides	*	0-5 mo	.003	.003	.004	.004
			6-11 mo	.013	.037	.018	.048
			12-23 mo	.043	.093	.058	.124
			2-65 yr	.057	.104	.075	.137
Imit Dairy	Diacetyl tartaric acid esters of mono and diglycerides	*	0-5 mo	.000	.000	.000	.000
			6-11 mo	13.944	17.556	13.944	17.556
			12-23 mo	6.972	12.600	6.972	12.600
			2-65 yr	4.872	14.280	4.872	14.280

TABLE 14

Food Category	Substance	# of Firms	Age	Average	High A	High B	Very High
Break Cerls	Monoglyceride citrate	*	0-5 mo	.005	.013	.005	.013
			6-11 mo	.033	.065	.033	.065
			12-23 mo	.028	.051	.028	.051
			2-65 yr	.027	.056	.027	.056
Fats, Oils	Monoglyceride citrate	*	0-5 mo	.098	.278	.172	.487
			6-11 mo	.082	.166	.144	.291
			12-23 mo	.130	.242	.228	.424
			2-65 yr	.356	.636	.623	1.113
Snack Foods	Monoglyceride citrate	*	0-5 mo	.031	.052	.031	.052
			6-11 mo	.103	.185	.103	.185
			12-23 mo	.175	.361	.175	.361
			2-65 yr	.216	.474	.216	.474

TABLE 14

Food Category	Substance	# of Firms	Age	Average	High A	High B	Very High
Imit Dairy	Monosodium phosmono and diglycerides	*	0-5 mo	.000	.000	.000	.000
			6-11 mo	87.150	109.725	87.150	109.725
			12-23 mo	43.575	78.750	43.575	78.750
			2-65 yr	30.450	89.250	30.450	89.250

TABLE 14

Food Category	Substance	# of Firms	Age	Average	High A	High B	Very High
Soft Candy	Monoglycerides acetylated	*	0-5 mo	.560	.940	.560	.940
			6-11 mo	.750	1.700	.750	1.700
			12-23 mo	.740	1.600	.740	1.600
			2-65 yr	1.140	2.470	1.140	2.470

TABLE 14

Food Category	Substance	# of Firms	Age	Average	High A	High B	Very High
Nut Prod	Peanut Oil	*	0-5 mo	15.402	19.253	20.349	25.436
			6-11 mo	427.420	904.899	564.678	1195.490
			12-23 mo	233.337	519.835	295.057	686.771
			2-65 yr	419.719	912.600	554.504	1205.665

TABLE 14

Food Category	Substance	# of Firms	Age	Average	High A	High B	Very High
Baked Goods	Triacetin	11	0-5 mo	6.276	20.771	9.216	30.503
			6-11 mo	11.621	22.067	17.065	32.406
			12-23 mo	22.229	36.360	32.644	53.395
			2-65 yr	55.593	82.559	81.639	121.239
Soft Candy	Triacetin	11	0-5 mo	1.130	1.897	4.276	7.178
			6-11 mo	1.514	3.431	5.727	12.981
			12-23 mo	1.493	3.229	5.651	12.218
			2-65 yr	2.301	4.984	8.705	18.861
Conf Frost	Triacetin	*	0-5 mo	.002	.002	.004	.004
			6-11 mo	.008	.022	.016	.044
			12-23 mo	.026	.056	.052	.112
			2-65 yr	.034	.062	.068	.124
Gelatin, Pud	Triacetin	5	0-5 mo	2.341	7.191	6.869	21.104
			6-11 mo	2.281	4.761	6.694	13.972
			12-23 mo	2.002	4.133	5.875	12.130
			2-65 yr	3.078	6.235	9.032	18.298
Bev Type I	Triacetin	11	0-5 mo	3.736	15.517	9.265	38.481
			6-11 mo	5.398	12.079	13.386	29.955
			12-23 mo	9.306	21.396	23.077	53.060
			2-65 yr	16.308	36.546	40.442	90.631
Bev Type II	Triacetin	*	0-5 mo	.000	.000	.000	.000
			6-11 mo	.105	.157	.286	.430
			12-23 mo	.183	.314	.501	.859
			2-65 yr	35.066	86.229	96.101	236.313
Hard Candy	Triacetin	*	0-5 mo	.000	.000	.000	.000
			6-11 mo	.032	.048	.037	.056
			12-23 mo	.079	.167	.094	.197
			2-65 yr	.195	.405	.229	.477

TABLE 14

Food Category	Substance	# of Firms	Age	Average	High A	High B	Very High
Baked Goods	Glyceryl monostearate	8	0-5 mo	49.558	164.021	80.318	265.826
			6-11 mo	91.763	174.253	148.718	282.408
			12-23 mo	175.532	287.118	284.481	465.326
			2-65 yr	438.989	651.929	711.461	1056.569
Break Cerls	Glyceryl monostearate	*	0-5 mo	11.813	30.807	18.768	48.944
			6-11 mo	76.670	150.791	121.808	239.568
			12-23 mo	65.783	119.058	104.512	189.152
			2-65 yr	62.077	128.555	98.624	204.240
Other Grain	Glyceryl monostearate	*	0-5 mo	20.083	41.971	45.022	94.090
			6-11 mo	40.843	93.645	91.561	209.932
			12-23 mo	44.904	89.583	100.666	200.826
			2-65 yr	77.172	151.862	172.004	340.444
Fats, Oils	Glyceryl monostearate	5	0-5 mo	6.319	17.924	6.974	19.782
			6-11 mo	5.287	10.703	5.835	11.813
			12-23 mo	8.382	15.603	9.251	17.221
			2-65 yr	22.953	41.006	25.333	45.258
Cheese	Glyceryl monostearate	*	0-5 mo	3.500	6.000	7.000	12.000
			6-11 mo	43.500	81.500	87.000	163.000
			12-23 mo	57.500	124.500	115.000	249.000
			2-65 yr	65.000	135.500	130.000	271.000
Frozen Dairy	Glyceryl monostearate	4	0-5 mo	3.388	7.841	5.729	13.258
			6-11 mo	7.405	14.907	12.522	25.207
			12-23 mo	8.906	17.763	15.059	30.035
			2-65 yr	16.311	33.202	27.580	56.142
Meat Prods	Glyceryl monostearate	*	0-5 mo	1.090	2.730	3.270	8.190
			6-11 mo	3.010	6.120	9.030	18.360
			12-23 mo	3.170	5.250	9.510	15.750
			2-65 yr	7.920	13.040	23.760	39.120
Soft Candy	Glyceryl monostearate	6	0-5 mo	20.468	34.357	21.772	36.546
			6-11 mo	27.413	62.135	29.159	66.094
			12-23 mo	27.047	58.480	28.770	62.206
			2-65 yr	41.667	90.279	44.322	96.031

TABLE 14

Food Category	Substance	# of Firms	Age	Average	High A	High B	Very High
Nut Products	Glyceryl monostearate	*	0-5 mo	8.000	10.000	10.000	12.500
			6-11 mo	222.000	470.000	277.500	587.500
			12-23 mo	116.000	270.000	145.000	337.500
			2-65 yr	218.000	474.000	272.500	592.500
Imit Dairy	Glyceryl monostearate	*	0-5 mo	.000	.000	.000	.000
			6-11 mo	31.917	40.184	48.391	60.926
			12-23 mo	15.958	28.841	24.195	43.727
			2-65 yr	11.152	32.686	16.908	49.557
Cereals	Glyceryl monostearate	*	0-5 mo	61.200	94.000	64.260	98.700
			6-11 mo	60.000	122.000	63.000	128.100
			12-23 mo	48.000	72.800	54.400	76.440
Formulas	Glyceryl monostearate	*	0-5 mo	805.800	1305.600	805.800	1305.600
			6-11 mo	584.200	1219.400	584.200	1219.400
			12-23 mo	717.400	1487.400	717.400	1487.400
Conf Frost	Glyceryl monostearate	*	0-5 mo	.050	.050	.160	.160
			6-11 mo	.200	.550	.640	1.760
			12-23 mo	.650	1.400	2.080	4.480
			2-65 yr	.850	1.550	2.720	4.960
Sweet Sauce	Glyceryl monostearate	*	0-5 mo	31.980	81.900	31.980	81.900
			6-11 mo	9.750	19.110	9.750	19.110
			12-23 mo	18.720	33.540	18.720	33.540
			2-65 yr	47.970	95.160	47.970	95.160
Gelatin Pudding	Glyceryl monostearate	*	0-5 mo	117.500	361.000	117.500	361.000
			6-11 mo	114.500	239.000	114.500	239.000
			12-23 mo	100.500	207.500	100.500	207.500
			2-65 yr	154.500	313.000	154.500	313.000
Soups	Glyceryl monostearate	*	0-5 mo	1.260	2.100	1.260	2.100
			6-11 mo	14.490	33.600	14.490	33.600
			12-23 mo	14.880	32.370	14.880	32.370
			2-65 yr	15.180	32.010	15.180	32.010

TABLE 14

Food Category	Substance	# of Firms	Age	Average	High A	High B	Very High
Snack Foods	Glyceryl monostearate	*	0-5 mo	.317	.528	.602	1.004
			6-11 mo	1.056	1.901	2.008	3.615
			12-23 mo	1.795	3.696	3.414	7.029
			2-65 yr	2.217	4.857	4.217	9.238
Bev Type I	Glyceryl monostearate	*	0-5 mo	.652	2.708	.815	3.385
			6-11 mo	.942	2.108	1.178	2.635
			12-23 mo	1.624	3.734	2.030	4.668
			2-65 yr	2.846	6.378	3.558	7.973

TABLE 14

Food Category	Substance	# of Firms	Age	Average	High A	High B	Very High
Baked Goods	Mono and diglycerides	24	0-5 mo	95.226	315.167	198.298	656.301
			6-11 mo	176.321	334.826	367.171	697.240
			12-23 mo	337.284	551.695	702.358	1148.847
			2-65 yr	843.516	1252.680	1756.534	2608.574
Break Cerls	Mono and diglycerides	*	0-5 mo	12.740	33.223	25.480	66.447
			6-11 mo	82.684	162.620	165.368	325.240
			12-23 mo	70.943	128.397	141.886	256.794
			2-65 yr	66.946	138.639	133.893	277.278
Other Grain	Mono and diglycerides	*	0-5 mo	22.250	46.500	22.250	46.500
			6-11 mo	45.250	103.750	45.250	103.750
			12-23 mo	49.750	99.250	49.750	99.250
			2-65 yr	85.500	168.250	85.500	168.250
Fats, Oils	Mono and diglycerides	15	0-5 mo	163.391	463.496	403.930	1145.841
			6-11 mo	136.715	276.764	337.982	684.207
			12-23 mo	216.743	403.475	535.825	997.459
			2-65 yr	593.541	1060.371	1467.336	2621.420
Milk Prods	Mono and diglycerides	4	0-5 mo	78.159	169.644	84.820	184.101
			6-11 mo	196.595	598.321	213.349	649.310
			12-23 mo	126.372	460.420	137.142	499.657
			2-65 yr	85.047	232.680	92.294	252.509
Cheese	Mono and diglycerides	*	0-5 mo	.018	.030	.070	.120
			6-11 mo	.218	.408	.870	1.630
			12-23 mo	.288	.623	1.150	2.490
			2-65 yr	.325	.678	1.300	2.710
Frozen Dairy	Mono and diglycerides	9	0-5 mo	7.246	16.769	13.545	31.347
			6-11 mo	15.837	31.881	29.606	59.598
			12-23 mo	19.046	37.988	35.604	71.015
			2-65 yr	34.883	71.008	65.210	132.741
Procsd Fruit	Mono and diglycerides	*	0-5 mo	961.400	2462.400	961.400	2462.400
			6-11 mo	2546.000	5631.600	2546.000	5631.600
			12-23 mo	4461.200	8101.600	4461.200	8101.600
			2-65 yr	4894.400	9762.200	4894.400	9762.200

TABLE 14

Food Category	Substance	# of Firms	Age	Average	High A	High B	Very High
Meat Products	Mono and diglycerides	*	0-5 mo	15.627	39.140	20.761	51.998
			6-11 mo	43.154	87.742	57.331	116.568
			12-23 mo	45.448	75.269	60.379	99.997
			2-65 yr	113.549	186.954	150.852	248.373
Poultry	Mono and diglycerides	*	0-5 mo	.605	1.513	1.254	3.135
			6-11 mo	.659	1.246	1.367	2.583
			12-23 mo	.774	1.319	1.605	2.734
			2-65 yr	1.234	2.372	2.558	4.916
Fish Products	Mono and diglycerides	*	0-5 mo	1.120	2.065	1.152	2.124
			6-11 mo	1.435	2.695	1.476	2.772
			12-23 mo	3.080	5.495	3.168	5.652
			2-65 yr	6.615	12.565	6.804	12.924
Procsd Vegs	Mono and diglycerides	7	0-5 mo	2.463	4.716	2.635	5.046
			6-11 mo	9.072	17.633	9.706	18.866
			12-23 mo	12.136	19.736	12.985	21.116
			2-65 yr	26.864	43.077	27.480	46.089
Condm Relsh	Mono and diglycerides	*	0-5 mo	.080	.120	.080	.120
			6-11 mo	.360	.680	.360	.680
			12-23 mo	.840	1.940	.840	1.940
			2-65 yr	2.180	4.580	2.180	4.580
Soft Candy	Mono and diglycerides	7	0-5 mo	13.987	23.478	27.987	46.978
			6-11 mo	18.733	42.461	37.483	84.961
			12-23 mo	18.483	39.963	36.983	79.963
			2-65 yr	28.474	61.693	56.974	123.443
Hard Candy	Mono and diglycerides	*	0-5 mo	.000	.000	.000	.000
			6-11 mo	.265	.398	.275	.412
			12-23 mo	.663	1.391	.687	1.443
			2-65 yr	1.623	3.379	1.683	3.504
Seas, Flavors	Mono and diglycerides	*	0-5 mo	---	---	---	---
			6-11 mo	.030	.060	.040	.080
			12-23 mo	.045	.075	.060	.100
			2-65 yr	.150	.300	.200	.400

TABLE 14

Food Category	Substance	# of Firms	Age	Average	High A	High B	Very High
Formulas	Mono and diglycerides	*	0-5 mo	164.826	267.060	451.973	732.311
			6-11 mo	119.498	249.428	327.678	683.962
			12-23 mo	146.744	304.248	402.390	834.283
Bev Type I	Mono and diglycerides	*	0-5 mo	.492	2.045	5.151	21.393
			6-11 mo	.711	1.592	7.442	16.653
			12-23 mo	1.226	2.819	12.830	29.499
			2-65 yr	2.149	4.815	22.483	50.386
Bev Type II	Mono and diglycerides	*	0-5 mo	.000	.000	.000	.000
			6-11 mo	.400	.600	.400	.600
			12-23 mo	.700	1.200	.700	1.200
			2-65 yr	134.200	330.000	134.200	330.000
Nut Prods	Mono and diglycerides	5	0-5 mo	4.397	5.497	7.690	9.612
			6-11 mo	122.029	258.350	213.394	451.780
			12-23 mo	63.763	148.414	111.503	259.533
			2-65 yr	119.830	260.548	209.549	455.625
Reconst Veg	Mono and diglycerides	*	0-5 mo	---	---	---	---
			6-11 mo	---	---	---	---
			12-23 mo	---	---	---	---
			2-65 yr	6.451	10.752	6.866	11.443
Gravies	Mono and diglycerides	*	0-5 mo	.183	.349	.791	1.511
			6-11 mo	.515	.979	2.230	4.244
			12-23 mo	1.162	2.324	5.036	10.072
			2-65 yr	2.208	4.382	9.568	18.992
Imit Dairy	Mono and diglycerides	9	0-5 mo	.000	.000	.000	.000
			6-11 mo	105.764	133.160	140.436	176.814
			12-23 mo	52.882	95.570	70.218	126.900
			2-65 yr	36.954	108.312	49.068	143.820
Conf Frost	Mono and diglycerides	*	0-5 mo	1.400	1.400	2.800	2.800
			6-11 mo	5.600	15.400	11.200	30.800
			12-23 mo	18.200	39.200	36.400	78.400
			2-65 yr	23.800	43.400	47.600	86.800

TABLE 14

Food Category	Substance	# of Firms	Age	Average	High A	High B	Very High
Jam, Jelly	Mono and diglycerides	*	0-5 mo	7.000	10.000	21.000	30.000
			6-11 mo	183.000	410.000	549.000	1230.000
			12-23 mo	58.000	129.000	174.000	387.000
			2-65 yr	110.000	253.000	330.000	759.000
Sweet Sauce	Mono and diglycerides	*	0-5 mo	328.000	840.000	328.000	840.000
			6-11 mo	100.000	196.000	100.000	196.000
			12-23 mo	192.000	344.000	192.000	344.000
			2-65 yr	492.000	976.000	492.000	976.000
Gelatin Pud	Mono and diglycerides	5	0-5 mo	392.347	1205.422	538.401	1654.153
			6-11 mo	382.329	798.050	524.655	1095.131
			12-23 mo	335.582	692.867	460.505	950.794
			2-65 yr	515.894	1045.145	707.941	1434.210
Soups	Mono and diglycerides	*	0-5 mo	1.945	3.259	1.969	3.282
			6-11 mo	22.488	52.147	22,643	52.506
			12-23 mo	23.094	50.238	23.252	50.584
			2-65 yr	23.559	49.680	23.721	50.021
Snack Foods	Mono and diglycerides	*	0-5 mo	.600	1.00	1.500	2.500
			6-11 mo	2.000	3.600	5.000	9.000
			12-23 mo	3.400	7.000	8.500	17.500
			2-65 yr	4.200	9.200	10.500	23.000

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J. Amer. Pharm Assoc.
45: 282 - 284, 1956

Studies on the Toxicity of Acetoglycerides*

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Results of studies are presented on the toxicity of two "acetoglycerides" (acetostearin and aceto-olein), administered by various routes to albino rats, rabbits, and guinea pigs. No toxic effects were observed in rats receiving single oral doses of 4 Gm./Kg. nor in rats consuming diets containing 5, 10, or 20 per cent of the acetostearin for fourteen weeks, nor in rats on diets containing up to 4 per cent acetostearin or 1 per cent aceto-olein for fifty-seven weeks. No blood changes or major histopathological visceral damage were observed in rats used for the subacute and chronic toxicity experiments. Hypoplasia of the testes and suppression of spermatogenesis, which were observed with the acetoglycerides, are considered to be due to other factors. Acetostearin administered intravenously, in rabbits, in doses of 80 to 100 mg./Kg. daily for fifteen days was nontoxic, had questionable effect on clotting time, and was completely removed from circulating blood within thirty minutes. Daily inunction of guinea pigs with 4 ml. of a 30 per cent emulsion for forty-five days produced no local or systemic reactions. No sensitization or allergic responses were observed in guinea pigs.

THE ACETYLATION of saturated or unsaturated monoglycerides, prepared from animal fats and vegetable oils consisting primarily of stearic acid (acetostearin) and oleic acid (aceto-olein), has resulted in the formation of new-type fats of unusual properties (1-3). These modified fats, referred to collectively as "acetoglycerides," are of potential value in the preparation of pharmaceuticals and cosmetics (4) and as edible fats (5). Studies on the absorption and digestibility of various acetoglycerides have been presented in another publication (6).

In cooperation with the Southern Utilization Research Branch we have made a study on the acute, subacute, and chronic oral toxicities of these acetoglycerides.

EXPERIMENTAL PROCEDURES AND RESULTS

In the present studies two synthetic acetoglycerides have been investigated, namely, diacetomonostearin and diacetomono-olein. The acetostearin was prepared by acetylation of molecularly distilled monostearin and the aceto-olein was prepared from purified oleic acid by interesterification with glycerol prior to acetylation. Both were prepared and supplied by the Southern Utilization Research Branch.

Oral Administration–Single Dose by Stomach Tube–Rats.—The acute toxicity of a mixture of acetostearin and aceto-olein was determined in mature male and female rats by gastric intubation. For this purpose a 40% emulsion was prepared as follows: 20 Gm. of acetostearin and 20 Gm. of aceto-olein were emulsified with 2 Gm. of Span® 20[1] and 1 Gm. of Tween® 60[1]. Since the completion of these studies, we have found that a suitable emulsion can be prepared with glycerol-monostearate (Tegin®)[1]. A 20% emulsion of either acetostearin or aceto-olein was prepared with 2% Tegin®.

In twenty rats, ten of each sex, receiving single oral doses of 4 Gm./Kg., no obvious toxic symptoms or ill effects were observed within the first twenty-four hours following administration or during the next four weeks.

Intravenous Administration–Repeated Doses–Rabbits.—Studies were made in rabbits by daily intravenous administration of a 10% emulsion of acetostearin on clotting time and on rate of disappearance, as determined by turbimetric measurements, from circulating blood.

In rabbits, daily intravenous injections of 80 to 100 mg. of acetostearin for fifteen days were nontoxic. Histological examination of the visceral organs showed no pathological changes due to the acetoglycerides. On clotting time only a slight and transitory effect was observed. Disappearance of acetostearin from the blood, as determined turbimetrically, showed that the administered acetostearin, 100 mg./Kg., was completely removed from circulating blood (plasma) within fifteen to thirty minutes.

Skin Irritation and Allergic Responses–Guinea Pigs.—The effect of acetoglycerides upon the skin was determined on albino guinea pigs after daily cutaneous application of an emulsion of acetoglycerides, consisting of 20% acetostearin, 10% aceto-olein, 2% Span® 20, and 1% Tween® 60. The animals were prepared by removing superfluous hair from the flanks and ventral surface with clippers.

The daily inunction of guinea pigs, involving approximately one-third of the total body surface, with 4 ml. of the 30% emulsion for forty-five days resulted in no local irritation or systemic reactions. No sensitization or allergic response was observed in guinea pigs receiving the acetoglycerides by cutaneous application.

* Received August 29, 1955, from the Pharmacology Section, Western Utilization Research Branch, Agricultural Research Service, United States Department of Agriculture, Albany 10, California.

The authors wish to thank Drs. Alvin J. Cox, Jr., and Lelland J. Rather of the Department of Pathology, Stanford University Medical School, for histological examination of the sections and report on the pathology observed, and Mr. E. K. Doxtader of this Branch for preparation of the tissue sections.

We also wish to acknowledge the technical assistance of Miss Iva V. Streeter and Mr. M. Nonaka of the Poultry Meat Unit of this Branch for determining the peroxide values and vitamin E values, respectively.

[1] Brand of emulsifiers: Span and Tween supplied by Atlas Powder Company, and Tegin supplied by Goldschmidt Chemical Corporation. The use of proprietary names does not imply endorsement of these products.

Oral Administration in the Diet for 14 Weeks - Rats.- As a preliminary study to more extensive studies, five weanling female albino rats were placed on each of four diets for fourteen weeks, the composition of which is given in Table I. One group of rats was placed on the basal diet, and the remaining groups were placed on diets containing 5, 10 and 20% acetostearin. All rats had free access to their respective diets and water. Body weights and food consumption were recorded weekly.

This experiment was terminated at fourteen weeks, when all rats were autopsied. The differences in growth between the controls and experimental animals were not significant. Acetostearin consumption for rats on the 20% dietary intake varied from approximately 23 Gm./Kg./day during the first week to approximately 10 Gm./Kg./day at fourteen weeks. For rats on 5 and 10% acetostearin, the daily dietary intake/Kg. was proportionately lower. At necropsy, no gross postmortem changes were observed.

TABLE I.—COMPOSITION OF DIETS

Constituents	Basal	Experimental		
	%	%	%	%
Acetostearin	0	5	10	20
Corn meal (yellow degerminated)	73	68	63	51
Casein (ground)	10	10	11	12
Linseed oil cake meal	10	10	10	10
Alfalfa meal (ground)	2	2	2	2
Bone ash	1.5	1.5	1.5	1.5
Sodium chloride	0.5	0.5	0.5	0.5
Cod liver oil (U. S. P.)	3	3	3	3

Oral Administration in the Diet for 57 Weeks - Rats.—In these studies, two experiments were conducted in which groups of weanling male albino rats were used. In one experiment six groups of five rats each were fed on diets containing 0, 0.25, 0.5, 1, 2, and 4% acetostearin. In the second experiment four groups of five rats each were fed on diets containing 0, 0.25, 0.5, and 1% aceto-olein. The basal diet to which the acetoglycerides were added was the same as that used in the previous experiments, Table I. The vitamin E content of the basal diet was equivalent to approximately 20 μg./Gm. of diet and for the acetostearin approximately 68 μg./Gm. The peroxide values for the basal diet and for the diet containing 10% acetostearin after standing for three weeks at 25° were approximately 0.002 and 0.0026 meq./Gm. of diet respectively. These values are in close agreement with the peroxide values of the freshly prepared diets which for all practical purposes are negligible. All diets were fed *ad libitum* and free access to water was permitted at all times.

In Table II, data are summarized on the final body weights and organ weights of rats on the various dietary levels of acetostearin and aceto-olein for fifty-seven weeks (four hundred days). Growth of rats on the experimental diets was not significantly different from that of the controls. A few deaths occurred in different groups and these were not related to treatment and were therefore not significant.

Organ weights, consisting of liver, kidneys, testes, spleen, heart, and adrenals, were determined routinely on all rats. With the exception of the adrenals, the average organ weights, together with the standard error of the mean, are summarized in Table II. Except for the testes all organ weights compare favorably with those of the controls. Hypoplasia of the testes was observed with rats on the 0.25 and 0.5% acetostearin and on 0.25, 0.5, and 1% aceto-olein. The testes of rats on acetoglyceride diets were much smaller both in terms of absolute weight and as related to body weight than were those of the control rats. The significance of this observation will be discussed below in conjunction with histopathological studies and in more detail in a subsequent publication.

Histopathological studies on hematoxylin-eosin stained sections were made on all of the organs weighed and, in addition, on the thyroid, intestine, stomach, pancreas, and bladder. Except for the testes no outstanding gross or microscopic pathological differences were observed between the tissues of the experimental groups and those of the control groups. Testicular hypoplasia and suppression of spermatogenesis of varying degrees were observed in all rats. In the controls one out of nine rats showed severe suppression of spermatogenesis. With the acetostearins, mild to moderate suppression of spermatogenesis was observed in all rats on the 4% diet; in three on the 2% diet; none on the 1% diet; in two on the 0.5% diet, and in four on the 0.25% diet. With the aceto-oleins all rats showed variable degrees of suppression of spermatogenesis. The significance of the hypoplasia of the

TABLE II.—AVERAGE ORGAN WEIGHTS OF MALE RATS RECEIVING ACETOSTEARIN OR ACETO-OLEIN IN THEIR DIETS FOR 400 DAYS

Supplements Fed				Organ Wt. (Gm./100 Gm. Body Wt.) ± S. E.				
Name	Concn. (Wt. %)	No. of Rats	Body Wt. Gm.	Liver	Kidneys	Testes	Spleen	Heart
Acetostearin	0.00	4	383 ± 16.4	3.13 ± 0.13	0.59 ± 0.04	0.87 ± 0.08	0.17 ± 0.01	0.31 ± 0.02
	0.25	5	384 ± 14.1	3.57 ± 0.14	0.62 ± 0.02	0.58 ± 0.07*	0.17 ± 0.08	0.32 ± 0.02
	0.50	5	388 ± 10.0	2.96 ± 0.05	0.63 ± 0.03	0.57 ± 0.05*	0.17 ± 0.01	0.35 ± 0.02
	1.00	5	390 ± 10.5	3.06 ± 0.08	0.59 ± 0.02	0.72 ± 0.03	0.16 ± 0.01	0.30 ± 0.01
	2.00	5	388 ± 15.7	3.18 ± 0.11	0.63 ± 0.03	0.73 ± 0.06	0.16 ± 0.01	0.29 ± 0.02
	4.00	4	386 ± 5.7	3.22 ± 0.18	0.62 ± 0.03	0.62 ± 0.09	0.20 ± 0.04	0.33 ± 0.05
Aceto olein	0.00	5	358 ± 6.1	3.03 ± 0.15	0.62 ± 0.01	0.77 ± 0.09	0.17 ± 0.01	0.31 ± 0.01
	0.25	4	378 ± 5.3	2.95 ± 0.15	0.63 ± 0.02	0.63 ± 0.04*	0.18 ± 0.01	0.32 ± 0.01
	0.50	4	371 ± 17.4	3.20 ± 0.11	0.66 ± 0.06	0.60 ± 0.02*	0.17 ± 0.00	0.29 ± 0.01
	1.00	5	385 ± 8.4	3.17 ± 0.11	0.62 ± 0.07	0.55 ± 0.04*	0.18 ± 0.01	0.31 ± 0.02
Controls (Comb.)	0.00	9	369 ± 8.5	3.06 ± 0.11	0.61 ± 0.02	0.82 ± 0.06	0.17 ± 0.05	0.31 ± 0.01

* P = <0.01.

testes and the suppression of spermatogenesis with the acetoglycerides is not understood at this time, since it has also been observed in other studies (unpublished data) with materials totally unrelated to the acetoglycerides (feather-meal protein and allethrin). However, preliminary observations, in another series of studies with acetoglycerides, would suggest that the testicular hypoplasia and suppression of spermatogenesis was possibly due to an insufficient amount of vitamin E in the diet in the presence of stress (acetoglycerides) rather than to other causes (peroxide formation).

Hematological studies made at approximately four-month intervals showed no toxic effect on the blood at any of the dosages of the acetoglycerides.

SUMMARY

1. When acetoglycerides were administered to rats by gastric intubation in massive doses of 4 Gm./Kg., there was no evidence of any toxicity.

2. Intravenously, in rabbits, daily doses of 80 to 100 mg./Kg. of a 10% emulsion of acetostearin for fifteen days was nontoxic, had questionable effects on clotting time, and was completely removed from circulating blood within 30 minutes. Histological examination of the visceral organs showed no pathological changes.

3. In guinea pigs, daily inunctions for forty-five days with 4 ml. of a 30% emulsion of acetoglycerides produced no local irritation or systemic reactions. No sensitization or allergic response was observed in albino guinea pigs receiving the acetoglycerides by cutaneous application.

4. When acetostearin was fed to rats in concentrations of 5, 10, and 20% of the diet for fourteen weeks, there was no evidence of toxicity as determined by growth, food intake, and mortality. Rats maintained for fifty-seven weeks on diets containing acetostearin in concentrations of 4% or less of the diet and aceto-olein in concentrations of 1% or less of the diet exhibited no appreciable ill effects as ascertained by growth, food intake, mortality, periodic hematological examinations, and histopathological examination of visceral organs. However, hypoplasia of the testes and suppression of spermatogenesis, which were observed with the acetoglycerides, is thought to be due to other factors, since this phenomenon was also observed by us with other materials, such as feathermeal protein, to some extent with allethrin, and in vitamin E deficiencies (unpublished data).

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J. Nutr.
58: 113-124, 1956

STUDIES ON COMPARATIVE ABSORPTION AND DIGESTIBILITY OF ACETOGLYCERIDES

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(Received for publication August 22, 1955)

The substitution of acetyl groups for part of the fatty acids of ordinary fats and oils, such as lard or edible vegetable oils, has resulted in the development of new types of fats of unusual properties and of potential value as edible fats (Feuge, '55; Baur, '54). These modified fats, which have marked resistance to changes in consistency and to heat damage and are plastic over a wide temperature range and stable against oxidation and rancidity, have been referred to as "acetoglycerides" or "acetin" fats (Feuge, '55; Baur, '54). By proper selection of suitable fats and oils as starting materials and appropriate degrees of acetylation, the acetoglycerides can be tailored to fit a variety of needs (Feuge, '55).

The "acetoglycerides" differ from natural fats and oils, composed of mixed triglycerides of long-chain fatty acids, in that they are predominantly diacetotriglycerides of high molecular weight fatty acids, such as stearic acid or oleic acid. Other combinations are possible. However, as used here the term "acetoglycerides" refers to both acetostearins and acetooleins. To distinguish between the two, the acetylated monoglycerides derived from highly hydrogenated lard or vegetable oils are referred to as acetostearins, and those derived from unsaturated fats as acetooleins.

Uses for the acetoglycerides appear to be "unlimited." Aside from their use in cosmetics and pharmaceuticals, some of the more important suggested uses to the food industry

are as coating materials for edible foods, such as cheese, nut meats, rasins, in chocolate coatings for ice cream bars, etc.; in the manufacture of candy either as a slab dressing or as a component; in the preparation of plastic shortenings, dessert and filling fats; and in the preparation of margarine-type of spreads where monoglycerides are now permitted.

Although the "acetoglycerides" are not naturally-occurring substances, they are undoubtedly readily hydrolyzed in the alimentary tract to yield acetic acid, glycerol, fatty acid, and monoglycerides — all occurring physiologically in the normal course of the digestion of fats.

Since the acetoglycerides are new products, in the sense that they are being considered for the first time as *intentional additives* in foods, it remains to be proved that these new fats do not interfere with normal physiological functions in ways not previously revealed in studies with acetic acid, glycerol, fatty acids and monoglycerides. The present studies were therefore designed to compare the absorption and digestibility of acetostearin, acetoolein, mixtures of acetostearin and acetoolein and hydrogenated vegetable oil.[1]

EXPERIMENTAL

The source, description and composition of the various acetoglycerides (acetostearins and acetooleins) used in the present studies are shown in table 1. In studies with rats to be reported below, the various acetoglycerides were incorporated into the basal diet on a weight basis. The basal diet had the following percentage composition: casein 10, corn meal 73, linseed oil meal 10, alfalfa meal 2, bone ash 1.5, sodium chloride 0.5 and cod liver oil (U.S.P.) 3. To 80 gm of the basal diet, 20 gm of either of the acetoglycerides or of Crisco was added and fed ad libitum. The fats were warmed to 60°C. before they were added and thoroughly mixed with the basal diet.

[1] Crisco. The use of this product does not imply recommendation. Any other similar, commercially available product could have been employed.

TABLE 1

Physical and chemical characteristics of acetoglycerides (AG)[1]

SAMPLE NO.	DESCRIPTION	HYDROXYL VALUE (FREE)	SAPON. VALUE	IODINE VALUE	ACETYL	LINOLEIC ACID	FFA AS OLEIC ACID	UNSAPONIFIABLE MATTER	M.P.
					%	%	%	%	°C.
AG 3	Fully acetylated acetoolein from cottonseed oil	0.4	391	73.2	17.9	32.92	0.72	0.59	—2.0
AG 21	Fully acetylated acetoolein from prime steamed edible lard	0.4	401	42.0	19.8	6.01	0.92	0.23	5.0
AG 31	Partially acetylated acetostearin from hydrogenated lard	118.4	297	1.08	11.5	0.15	0.61	0.32	57.0
AG 26	Fully acetylated acetostearin from hydrogenated lard	0.8	380	0.96	18.5	0.13	1.14	0.30	32.5
AG 194	Fully acetylated acetostearin from hydrogenated lard	0.00	380	0.69	16.2	0.04	0.56	0.54	30.7

[1] Data supplied by Oilseed Section, Southern Utilization Research Branch.

Absorption

Studies on absorption were made over a 4-hour period in mature male albino rats weighing 200 to 300 gm each. Our procedure varied from that used by others in that the acetoglycerides were fed as an integral part of the diet rather than by stomach tube.

The diet containing 20% of one of the acetoglycerides or Crisco was fed to each rat previously fasted for 48 hours. Ten rats were used for each group. However, only those rats which consumed the greater portion of the offered diet within 30 minutes were used. The amount of diet consumed during this period was determined and the amount of fat calculated. Four hours later the rats were killed, the gastrointestinal tract, between the esophagus and caecum, was ligated and removed immediately. The contents of the ligated portion of the gut were washed out and extracted with diethyl ether as outlined by Deuel, Hallman and Leonard ('40). The ether extract was dehydrated with anhydrous sodium sulfate and the amount of ether soluble residue (fat) was determined. The absorption of the various acetoglycerides was determined for each rat separately. A similar experiment for endogenous fat was conducted on control rats fasted for 48 hours but fed no fat or acetoglycerides. A total of 70 rats was used in these studies. The percentage absorption of the respective acetoglycerides was calculated as follows:

$$\frac{\text{Ingested fat} - (\text{fat recovered} - \text{endogenous fat in fasting rats})}{\text{Ingested fat}} \times 100$$

The data are summarized in table 2.

Digestibility

Studies on the digestibility of the various acetoglycerides and the coefficient of digestibility were determined according to the procedure described by Augur, Rollman and Deuel ('47). Experiments were conducted with both male and female adult rats. Five rats of each sex were used. All rats

were housed in individual screen-bottom metabolism cages. Food and water were provided ad libitum. For determination of metabolic fat, one group of 10 rats was fed the control diet which contained no added fat, other than 3% of cod liver oil. All other rats in groups of 10 were placed on the experimental diets containing 20% of one of the acetoglycerides, mixtures of acetoglycerides, or Crisco. A total of 90 rats was used in these experiments. Following a preliminary adjustment period of 7 to 10 days on the respective diets, digestibility (of the various acetoglycerides) was determined

TABLE 2

Absorption of various acetoglycerides (AG) and Crisco from the gastrointestinal tract of fasting rats

Absorption time 4 hours

SUPPLEMENT FED[1]	FAT FED	NO. OF TESTS	FAT RECOVERED	FAT ABSORBED CORRECTED	
	mg		*mg*	*mg*	*% ± S.E.*[2]
Control	0	7	81	..	....
AG-3	696	10	248	529	76.1 ± 1.6
AG-21	741	9	249	573	77.3 ± 1.2
AG-194	694	9	318	457	65.9 ± 3.0
AG-26	717	10	350	448	62.5 ± 2.3
AG-31	693	7	319	455	65,7 ± 3.1
Crisco	700	9	308	473	67.6 ± 1.1

[1] See table 1 for description and characteristics of "Supplements Fed."
[2] SE = Standard error of the mean.

over a 7-day period. During the 7-day digestibility period accurate records of food consumed and feces excreted were kept for each rat. The feces from each rat were dried, weighed and ground to a coarse powder. The dried feces were analyzed for neutral fat and free fatty acids by Soxhlet extraction with diethyl ether. The fatty acids present as soaps were determined on the ether extracted residues after acidification with 50% sulfuric acid and re-extraction with diethyl ether. All diets were assayed for total ether extractives at the same time the experiments were run. The experiments and data are summarized in table 3. The coefficient of digesti-

bility was calculated for each rat, but only the average results for each sex and group, together with the standard error of the means, are reported. All values for digestibility have been corrected for metabolic fat from rats on the control diet in which no fat was added, other than cod liver oil which was present in the diet. A correction factor for metabolic fat for male and female rats of 79 and 75 mg/gm of dried feces, respectively, was obtained. The average, 77 mg/gm of dried feces, was used for correcting for metabolic fat. The coefficient of digestibility was calculated as follows:

$$\frac{\text{Ingested fat} - [\text{excreted fat} - \text{endogenous fat (wt. dried feces} \times 77)]}{\text{Ingested fat}} \times 100$$

RESULTS AND DISCUSSION

Absorption

A summary of the data on the absorption of the various acetoglycerides with the number of rats used in each group is given in table 2. Absorption of acetooleins (Ag-3 and Ag-21) was definitely better than the absorption of acetostearins or Crisco. On the other hand the absorption of the three acetostearins, the two fully acetylated (Ag-194 and Ag-26) and the one partially acetylated (Ag-31) were of about the same order as for a partially hydrogenated fat, for example, Crisco. These observations are in keeping with those in studies reported by others on the absorption of oils composed predominantly of unsaturated fatty acids as compared with fats consisting predominantly of saturated fatty acids (Steenbock, Irwin and Weber, '36; Deuel, Hallman and Leonard, '40; Crockett and Deuel, '47). No difference in absorption was observed between the acetostearin melting at 55°C. as compared with the acetostearins melting at 30.7°C. and 32.5°C., respectively. However, Crockett and Deuel ('47) did observe a difference in absorption between hydrogenated lard (M.P. 55°C.) and other fats with lower melting points.

Summary of digestibility of acetoglycerides (AG) and Crisco when fed to male and female rats as part of the diet
Values in the table are the averages of 5 rats of each sex per group

SUPPLEMENTS FED IN DIET [1]	PER CENT FAT IN DIET		SEX	BODY WT.	FOOD EATEN	FAT EATEN	WEIGHT DRIED FECES	LIPIDS EXCRETED AS				COEFFICIENT OF DIGESTIBILITY	
								Neutral fat + fatty acids	Fatty acids as soap	Total	Total corrected	Sexes	
	Added	Found										Separate	Combined
				gm	gm	gm	gm	gm	gm	gm	gm [2]	% ± S.E.[3]	% ± S.E.
Control			M	333	103.0	6.06	7.92	0.258	0.373	0.631			
	0	5.9	F	218	78.8	4.62	6.30	0.159	0.311	0.470			
AG-3			M	428	111.0	27.75	9.63	0.391	0.604	0.995	0.253	99.1 ± 0.3	
	20	25.0	F	274	81.2	20.30	6.73	0.203	0.388	0.591	0.073	99.4 ± 0.3	99.2 ± 0.2
AG-21			M	461	115.0	28.22	9.30	0.405	0.580	0.985	0.252	99.1 ± 0.8	
	20	24.5	F	289	88.0	21.59	7.41	0.255	0.431	0.686	0.111	99.5 ± 0.9	99.3 ± 0.1
AG-194			M	364	110.0	26.40	12.52	0.833	5.178	6.011	5.048	81.0 ± 0.8	
	20	24.0	F	244	80.4	19.30	9.82	0.599	3.798	4.397	3.641	79.0 ± 0.6	80.0 ± 0.6
AG-26			M	444	110.0	27.69	12.66	1.002	4.504	5.506	4.530	83.8 ± 1.3	
	20	25.2	F	277	77.4	19.49	8.90	0.573	2.846	3.419	2.730	86.1 ± 0.7	85.0 ± 0.8
AG-31			M	434	104.2	25.75	13.32	0.948	5.503	6.451	5.425	79.1 ± 1.4	
	20	24.7	F	273	79.4	19.62	9.23	0.600	3.415	4.015	3.304	82.5 ± 1.3	80.8 ± 1.0
AG-194, 15%			M	483	115.8	27.85	13.17	0.842	4.752	5.595	4.581	83.7 ± 1.2	
+ AG-3, 5%	20	24.1	F	292	78.6	18.90	8.25	0.495	2.561	3.056	2.419	86.5 ± 1.2	85.1 ± 0.9
AG-194, 10%			M	387	97.6	23.46	8.88	0.648	1.699	2.347	1.664	92.9 ± 0.5	
+ AG-3, 10%	20	24.0	F	253	74.2	17.86	6.25	0.409	1.066	1.475	0.942	94.4 ± 0.2	93.7 ± 0.6
Crisco			M	409	109.6	26.74	7.46	0.626	1.291	1.917	1.342	95.1 ± 0.1	
	20	24.4	F	266	80.0	19.50	5.84	0.400	0.659	1.059	0.609	96.8 ± 0.5	95.9 ± 0.4

[1] For description and characteristics of "Supplements Fed" see table 1.
[2] Corrected for metabolic fat (77 mg/gm dried feces).
[3] S.E. = Standard error of the mean.

Digestibility

Table 3 summarizes data on the comparative digestibility of the various acetoglycerides when fed to rats as an integral part of the diet. Data are also presented on the digestibility of mixtures of acetoglycerides, and for comparative purposes the digestibility of Crisco under our experimental conditions. Only average results are presented.

When diets containing 20% of acetoglycerides were fed to rats, the acetooleins had the highest coefficient of digestibility, while the acetostearins had much lower values. However, these values are substantially in agreement with those obtained in comparable studies by others with cottonseed oil and hydrogenated fats (Crockett and Deuel, '47; Hoagland and Snider, '40; Augur, Rollman and Deuel, '47; Cheng, Morehouse and Deuel, '49). When the diet containing 20% of one of the acetostearins (Ag-194) was modified to contain 5% of acetostearin and 5% of acetoolein (Ag-3) digestibility was slightly improved. However, when the diet was modified further (10% of acetostearin and 10% of acetoolein) digestibility was considerably improved. The digestibility of this mixture was 93.7% as compared to 95.9% for Crisco, which compares favorably with the digestibility of other partially hydrogenated fats and oils. It must be recalled that all of the acetostearins studied and reported here were prepared from oils which, for all practical purposes, were completely hydrogenated as judged by iodine values of one or less, while commercially available hydrogenated fats and oils are only partially hydrogenated to iodine values ranging from 60 to 75.

In the digestibility of various natural and synthetic fats, a number of factors have been considered: for example, melting point of the fat; composition of the fat, whether a simple or mixed triglyceride; presence of emulsifying agents, such as lecithin, monoglycerides, etc., and the presence of calcium and magnesium salts in the diet. The acetoglycerides reported here, with the exception of the acetooleins and mixtures of acetoolein and acetostearin, had melting points of 30.7, 32.5, and 57°C. for Ag-194, 26 and 31, respectively. The acetooleins and mixtures of acetooleins and acetostearin were all liquid at 25°C. With the exception of Ag-31, all were within the critical temperature of 50°C. for maximum digestibility.

Lyman ('17) and Mattil and Higgins ('45), as well as others, are of the opinion that the melting point of a fat is not as important a consideration as the composition of the fat (stearate content). These authors believe that stearates are indigestible and that they are better utilized when fed as a mixed glyceride rather than when fed as mixtures of simple triglycerides (tristearin mixed with triolein). In our studies with the three acetostearins, the melting point did not appear to be as important a consideration as the stearate content of the diet. All three acetostearins had approximately the same digestibility coefficients (80 to 85%). The digestibility of the acetostearins was indeed definitely better than the digestibility of monostearin (47.4%) (Cheng et al., '49). The acetostearins, perhaps, may be considered as mixed glycerides rather than as simple glycerides, since they are glycerol esters of acetic acid and stearic acid. Therefore, it might be expected that the acetostearins would be better utilized or digested than the monostearins.

A mixture in equal amounts of acetostearin (melting point of 30.7°C., digestibility coefficient of 80%, and containing approximately 64% stearic acid) and acetoolein (digestibility coefficient of 99.2%) was found to be approximately 93.7% digestible. This would suggest better utilization of acetostearins when mixed with a highly digestible fat, such as acetoolein. Comparing the digestibility of this mixture (93.7%) with that obtained for Crisco (95.9%), a partially hydrogenated fat, suggests that suitable mixtures of acetostearins and acetooleins would be utilized as well as or better than Crisco.

Augur et al. ('47) have reported data on the improvement in the digestibility of various hydrogenated fats with emulsi-

ring agents such as lecithin. Inasmuch as improvement in ie digestibility of the acetostearins was accomplished by ixing with acetoolein, the use of emulsifying agents was ot tried. However, there is no reason to believe that emulsiying agents would not also improve digestibility of the cetostearins, since Augur et al. ('47) found a definite improvement in digestibility of high-melting hydrogenated oils ith lecithin.

Cheng et al. ('49) have shown that the digestibility of lowielting fats was not influenced by the presence of calcium nd magnesium in the diet, but that the digestibility of highielting fats was decreased. In our studies, we have found no reat differences in the digestibility of the acetostearins on diet containing 0.7% of calcium and 0.08% of magnesium erived from natural constituents of the diet. X-ray examinaions of the long bones of the lower extremities of rats on iets containing 20% of acetoglycerides for approximately 00 days showed no difference in bone calcification as comared to rats on the basic diet alone, which would indicate hat the amount of calcium and magnesium in the diet was idequate for calcification. Our x-ray observations are in igreement with those in studies reported by Harris and Sherman ('54) on the effect on the length of the tibiae of ats when mono-, di-, or triglycerides, and a mixture of nono-, di- and triglycerides were fed in concentrations of approximately 25% of the diet.

Table 3 includes data on the amount of fatty acids excreted as soap. It will be noted that the amount of soap varied with the acetoglyceride fed. With the acetooleins the amount of fatty acids excreted as soap was the lowest, while with the acetostearins it was the highest. When acetostearin and acetoolein were fed together in equal amounts, the amount of fatty acids excreted as soap decreased. This would suggest that the acetostearins in a suitable mixture with other fats and oils, such as a margarine-like product, containing approximately 80% of acetoolein and 20% of highly hydrogenated

cottonseed oil, as proposed by Gros and Feuge ('54), would be completely digestible.

Our observations on the digestibility of acetoglycerides are consistent with the observations of others on the digestibility of oils predominantly unsaturated as compared with hydrogenated fats. It would appear, therefore, that as the percentage of stearic acid of a fat (or oil) is increased by hydrogenation, there is a concomitant decrease in digestibility and a concomitant increase in the amount of fatty acids excreted as soap.

SUMMARY AND CONCLUSIONS

Our results on the absorption and digestibility of acetoglycerides (acetostearin, acetoolein) justify the following conclusions:

1. The acetooleins appear to be better absorbed than the acetostearins over a 4-hour test period in rats. However, the acetostearins are absorbed at about the same rate as Crisco under the conditions of our experimental procedures.

2. The various acetoglycerides appear to be utilized by the rat in much the same way as natural fats. The digestibility coefficients obtained were, approximately 99% for the acetooleins; 80 and 80.8% for the acetostearins melting at 30.7°C. and 57°C., respectively, and 85% for the acetostearin melting at 32.5°C.; for the mixtures of 15% of acetostearin and 5% of acetoolein, and for 10% of acetostearin and 10% of acetoolein, the digestibilities were 85.1 and 93.7%, respectively.

3. Data obtained on the digestibility of the acetoglycerides indicate that the acetostearins are not as readily digestible as the acetooleins. This, in all probability, is due to the large amount of stearic acid formed during digestion, which combines with calcium to form calcium stearate which is not readily utilized.

4. The digestibility of acetostearin is markedly improved when mixed with acetoolein, as indicated by a decrease in the amount of fatty acid excreted as soap. This would suggest that the acetostearins in a suitable mixture with other edible

fats and oils, such as a margarine-like product containing approximately 80% of acetoolein and 20% of acetostearin would be completely digestible.

ACKNOWLEDGMENTS

The authors are grateful to the Southern Utilization Research Branch for making available, through Distillation Products Industries, adequate supplies of the various acetoglycerides studied and for the data contained in table 1.

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Food Research
23: 536-549, 1958

STUDIES ON THE LONG-TERM FEEDING OF ACETOGLYCERIDES TO RATS

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(Manuscript received April 26, 1958)

Acetylation of natural fats and oils by interesterification, acetylation of superglycerinated fats, or by the acetylation of purified monoglycerides of long chain fatty acids, such as stearic or oleic acids, has resulted in the synthesis of new type fats with usual properties and of potential value as edible fats. These modified fats have been referred to as "acetoglycerides" *(19)*, or "acetin" fats *(5)*. As used here the term "acetoglycerides" refers to both aceto-oleins and acetostearins. To distinguish between the two, the acetylated monoglycerides derived from highly hydrogenated lard or vegetable oils are referred to as acetostearins, and those derived from unsaturated fats as aceto-oleins. These acetoglycerides are undoubtedly the same type as those referred to as "acetylated monoglycerides" by Herting *et al. (21)*.

There is no apparent reason to suppose that acetoglycerides should be hydrolyzed differently *in vivo* than natural fats. There is also no reason to suppose that the metabolic end products should be more toxic than those arising from the metabolism of fats normally present in food. Partial hydrolysis of triglycerides to mono- and diglycerides has been reported to take place during baking and during digestion in man and experimental animals *(6, 23, 24, 27, 29)*. The nutritive value of these mono- and diglycerides has been shown to correspond to that of natural triglycerides of similar fatty acids *(11, 26)*, and the mode of digestion and nutritive value of acetylated monoglycerides have been shown to be the same *(21)*. The rate of absorption of the acetoglycerides was found by Ambrose and Robbins *(1)* to be as good or better than that of a partially hydrogenated shortening (Crisco), while the digestibility of acetostearins and aceto-oleins was comparable to that reported by others *(4, 13, 16, 22)* for completely hydrogenated and unhydrogenated fats. No difference was observed by Ames *et al. (2)* or Bourke and associates *(12)* in the nutritive value of purified monoglycerides or mixtures of mono- and diglycerides when compared with triglycerides.

Triacetin in amounts up to 55% of the diet of rats has been reported to be non-toxic, to support good growth, and to be converted to long chain fatty acids *(15)*. Acetic acid is rapidly utilized when fed to mammals *(18, 25)*, and has been described as occupying a key position in the metabolism of fats, carboyhydrates and certain amino acids *(7)*. By the use of labeled acetate it has been demonstrated that acetic acid disappears rapidly from the blood *(14)*, and labeled carbon dioxide appears in the expired air *(31)*. Acetic acid has been shown to take part in the biosynthesis of cholesterol in man *(20)* and animals *(8)*, in the synthesis of fatty acids *(30, 32)* and in the acetylation of amines *(3, 9, 10)*.

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In the light of this brief review it is difficult to visualize any toxicity resulting from the ingestion of acetoglycerides, particularly since the hydrolytic products, acetic acid, glycerol, free fatty acid, and monoglyceride all occur in the normal course of digestion and metabolism of fats, or are used as such in foods. Nevertheless, the acetoglycerides are new type fats and since they might enter the human diet in appreciable amounts, their investigation for possible chronic toxicity is a Federal Food and Drug Administration requirement.

EXPERIMENTAL

Weanling male and female albino rats of the Slonaker strain, weighing 43 to 51 g., were placed on an adequate basal diet, and on the same diet suitably modified to permit incorporation of acetoglycerides (AG). Table 1 provides information on the identity, and physical and chemical characteristics of the acetoglycerides studied. Since the various acetoglycerides were fed at levels of 5, 10, and 20% of the total diet, it was necessary to make minor adjustments in the composition of the diet to permit maintenance of protein levels and approximately the same caloric value per 100 g. of diet. The composition of the control and acetoglyceride-containing diets is given in Table 2. Two other supplements added to the basal control diet were also fed: (a) 3.3% triacetin and, (b) 6.1% sodium acetate containing 3 molecules of water of crystallization. On a theoretical basis each of these supplements would provide acetate ion equivalent to the amount derived from a diet containing 10% acetoglyceride.

TABLE 2

Percentage composition of diets

Components	Diet groups			
	1	2	3	4
Casein	10	12	11	10
Corn meal (degerm.)	72	50	61	67
Linseed oil meal	10	10	10	10
Alfalfa (ground)	2	2	2	2
Bone ash	1.5	1.5	1.5	1.5
Sodium chloride	0.5	0.5	0.5	0.5
Cod Liver oil (U.S.P.)	3.0	3.0	3.0	3.0
Brewers' yeast	1	1	1	1
Acetoglycerides	0	20	10	5
Protein percent	17.6	17.6	17.6	17.6
Calories/100 grams	360	469	414	388

Thirty rats of each sex were used on each of the acetoglyceride diets, 10 rats of each sex being placed on each of the 3 dietary levels of acetoglyceride. Five female rats were placed on each of the 2 diets containing triacetin and sodium acetate. For control purposes, 30 rats of each sex were placed on the basal diet (diet No. 1, Table 2). The rats were caged in groups of five in solid-bottom cages with wood shavings as litter. Free access to water and to the respective diets was permitted at all times. Food consumption for each group and the body weight of each rat in the group were recorded at regular weekly intervals during the first 300 days, only body weights being recorded thereafter at monthly or bimonthly intervals.

All rats on diets containing 20% of an acetoglyceride and an appropriate number of control rats were sacrificed at the end of 400 days, while rats on diets containing 5 and 10% acetoglyceride sample AG-194 were sacrificed at 600 days; all surviving rats on the 5 and 10% levels of the other acetoglycerides were sacrificed at the end of approximately 709 days. At time of sacrifice all organs were examined for gross evidence of injury. Livers, kidneys, spleens, hearts, and testes were removed, weighed and preserved in a 10% solution of neutralized formaldehyde for histopathological study. In addition, the following tissues were also preserved for histopathological study: bladder, stomach, intestine, pancreas, adrenal, thyroid, brain, hypophysis, parathyroid, ovaries, and uterus. Paraffin sections stained with hematoxylin-eosin were prepared, and in some instances frozen sections of liver were stained with Sudan IV.

TABLE 1

Description, physical and chemical characteristics of acetoglycerides (AG)[1]

Sample No.	Description	Hydroxyl value (free)	Sapon. value	Iodine value	Acetyl %	Linoleic acid %	FFA as oleic acid %	Unsaponifiable matter %	M.P. °C
AG-194	Fully acetylated acetostearin from hydrogenated lard	0.00	389	0.69	16.2	0.04	0.56	0.54	30.7
AG-26	Fully acetylated acetostearin from hydrogenated lard	0.8	380	0.96	18.5	0.13	1.14	0.30	32.5
AG-31	Partially acetylated aceto-stearin from hydrogenated lard	118.4	297	1.08	11.5	0.15	0.61	0.32	57.0
AG-21	Fully acetylated aceto-olein from prime steamed edible lard	0.4	401	42.0	19.8	6.01	0.92	0.23	5.0
AG-3	Fully acetylated aceto-olein from cottonseed oil	0.4	391	73.2	17.9	32.92	0.72	0.59	−2.0

[1] Data supplied by the Southern Utilization Research and Development Division, U. S. Department of Agriculture.

Studies on the effects of the acetoglycerides on reproduction were conducted on each of the 5 acetoglycerides. For this purpose 10 female and 5 male weanling rats were placed on a diet containing 10% of an acetoglyceride, and a similar number of rats of both sexes were placed on the basal diet (diet No. 1, Table 2). After approximately 3 months on the respective diets, all rats were mated and the number of siblings cast and weaned was recorded. Those acetoglycerides permitting successful reproduction in the first generation were studied on several successive generations. All rats receiving other acetoglycerides were remated several times before discontinuance.

RESULTS

Longevity. The survival data are summarized in Table 3. They indicate that none of the acetoglycerides even at the 20% level altered mortality during the first 400 days. Rats on 20% were not carried beyond 400 days. On the 5 and 10% levels most of the deaths occurred during the last 100 days of the 709-day test period. The greatest change in survival occurred in male and female rats receiving acetoglyceride sample AG-26, and in male rats receiving samples AG-21 and AG-3. Results obtained with diets containing triacetin or sodium acetate suggest that the acetate ion that might be derived from a 10% acetoglyceride diet did not contribute to an increased mortality.

Growth. The data on body weights are summarized in Table 4. With the possible exception of male rats receiving 20% of samples AG-194 and AG-31, and female rats receiving 20% of sample AG-31 there were no marked effects on growth rate and final body weights.

Organ weights. Tables 5 and 6 summarize the data on organ weights. As shown in Table 5, the only organs consistently affected by the 20% level of the various acetoglycerides were the testes and liver. The testes of rats on the 3 acetostearin diets (AG-194, AG-26, AG-31) were significantly smaller than those of the control rats or rats on the two aceto-olein diets (AG-21 and AG-3). The livers of male rats on 20% AG-194 and of both male and female rats on 20% AG-31 were significantly larger than those of control rats. Although the total ether extractives (fat) from these livers did not indicate fat storage, fat droplets were observed microscopically. The livers of female rats on 20% AG-21 and AG-3 were smaller than those of control rats, showed increased amounts of ether extractives, and exhibited fat droplets microscopically. Kidneys of female rats on 20% AG-31 were significantly larger than those of the controls.

Data on the 5 and 10% levels of the acetoglycerides for 600 days or more show no real differences between control and experimental groups except for a decrease in weights of testes of rats receiving AG-194 for 600 days or AG-31 for two years.

Liver fat and glycogen. In Table 7 data are summarized on liver fat and glycogen for rats on 20% acetoglyceride diets for 400 days. Although the liver fat values were consistent within experiments, considerable variation was encountered between the different acetoglycerides. Liver fat values for the males on the 3 acetostearins (AG-194, AG-26, AG-31) were slightly lower than those of the controls whereas the liver fat values for the 2 aceto-oleins (AG-21 and AG-3) were considerably higher. In the females no marked difference was observed between the controls and the experimental groups, except for the group of rats on AG-3. The actual liver weights in g./100 g. of body weight (Table 5)had no relationship to the fat found, and histologically no pathological changes were observed.

Liver glycogen values for both male and female rats, summarized in Table 7, suggest that the acetoglycerides were devoid of glycogenic activity. Where changes in liver glycogen were observed the values were always below those found for control rats on diet 1. These observations on liver glycogen storage after the ingestion of diets containing 20% acetoglycerides for 400 days are comparable to results reported on shorter experiments for natural fats and oils *(17)*, and for acetic acid *(28)*.

Efficiency of food utilization. The effect of the acetoglyceride diets on the efficiency of food utilization over a 100-day period is evident from the data summarized in Table 8. In general the acetostearins decreased the efficiency more than did the aceto-oleins, and the effect was related directly to the concentration in the diet.

TABLE 3

Survival data for rats on diets containing acetoglycerides

Supplements fed	Dietary conc. %	Number of survivors (days)							
		0	100	200	300	400	500	600	700
Males									
Controls	0	30	28	28	28	27[1]	19	18	14
AG-194	5	10	10	10	10	7	7	6	....
	10	10	10	10	9	8	5	5	....
	20	10	10	10	9	9	....	....	....
AG-26	5	10	10	10	10	9	9	9	4
	10	10	10	9	9	9	9	9	4
	20	10	8	8	8	8	....	....	....
AG-31	5	10	9	9	9	9	9	8	8
	10	10	8	8	7	7	7	6	6
	20	10	7	7	7	7	....	....	....
AG-21	5	10	10	10	10	10	10	9	5
	10	10	10	10	9	9	9	8	4
	20	10	9	9	9	9	....	....	....
AG-3	5	10	10	10	10	9	9	9	3
	10	10	8	8	7	5	5	5	2
	20	10	10	10	10	9	....	....	....
Females									
Controls	0	30	29	29	29	28[1]	18	17	11
AG-194	5	10	10	10	10	10	4	4	....
	10	10	10	10	10	10	8	8	....
	20	10	10	10	9	9	....	....	....
AG-26	5	10	9	9	9	8	7	5	4
	10	10	10	10	10	9	8	7	3
	20	10	9	9	9	9	....	....	....
AG-31	5	10	10	9	8	8	8	8	7
	10	10	10	10	10	10	10	9	8
	20	10	7	7	6	6	....	....	....
AG-21	5	10	10	10	10	10	10	10	8
	10	10	10	10	10	9	8	7	5
	20	10	9	9	8	8	....	....	....
AG-3	5	10	10	10	10	10	10	8	7
	10	10	10	10	10	10	10	8	6
	20	10	10	10	10	9	....	....	....
Triacetin	3.3	5	5	5	5	5	3	3	3
Sodium Acetate	6.1	5	5	5	5	5	5	5	5

[1] Seven male and eight female rats were sacrificed at 400 days.

TABLE 4

Growth data for rats on control and acetoglycerides supplemented diets

Supplements fed	% In diet	Average body weight at approximate time interval indicated (days)							
		0	50	100	200	300	400	600	700
		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Males		50	219	297	353	381	415	.....	.....
Control	0							424	413
AG-194	5	50	226	307	376	394	414	456	.....
	10	51	229	309	365	386	390	412	.....
	20	49	190	270	335	353	361	.....	.....
AG-26	5	49	229	304	335	381	405	429	428
	10	50	228	316	347	397	405	435	446
	20	49	204	281	326	367	393	.....	.....
AG-31	5	50	190	260	316	358	378	396	373
	10	50	185	240	292	352	370	398	385
	20	50	128	201	255	299	328	.....	.....
AG-21	5	49	211	290	333	376	398	417	410
	10	49	230	324	369	420	435	450	472
	20	50	203	291	326	368	393	.....	.....
AG-3	5	49	214	301	369	399	427	435	455
	10	49	195	292	360	392	426	452	447
	20	49	190	287	368	386	405	.....	.....
Females		43	160	205	252	277	306	.....	.....
Control	0							332	341
AG-194	5	43	157	201	245	265	271	339	.....
	10	43	167	207	255	277	297	352	.....
	20	44	154	197	229	274	288	.....	.....
AG-26	5	44	167	213	260	278	312	362	365
	10	44	160	204	251	257	305	321	323
	20	45	155	205	249	261	292	.....	.....
AG-31	5	43	161	205	255	279	299	337	340
	10	43	148	204	253	273	305	362	356
	20	43	130	189	253	247	251	.....	.....
AG-21	5	43	169	212	252	270	279	334	340
	10	44	163	215	258	283	311	348	358
	20	44	152	206	250	269	300	.....	.....
AG-3	5	43	154	213	282	310	337	365	378
	10	43	153	205	263	286	320	353	363
	20	44	161	219	282	307	345	.....	.....
Triacetin	3.3	49	169	208	244	286	293	305	313
Sodium Acetate	6.1	49	179	216	254	278	292	313	304

Reproduction. Rats on diets containing 10% aceto-olein (AG-3 and AG-21) supported reproduction and lactation through the test period of three generations. Each generation of rats was remated several times, and the rats remained fertile throughout. The total litters cast, the number of siblings per litter and the average body weights at weaning, and at 50 and 100 days for each generation were similar in the control and experimental groups. With the acetostearins, AG-194, AG-26, and AG-31, only AG-26 supported reproduction through 3 generations comparable to the controls. Both AG-194 and AG-31 failed to support reproduction beyond the first generation.

Gross pathology. On post-mortem examination no gross changes were seen that were not also seen in control rats, such as respiratory infection,

TABLE 5

Body and organ weights of rats on control and acetoglyceride supplemented diets for 400 days

Supplements fed	% in diet	No. of rats	Body weights grams	Organ weights (grams/100 body weight)				
				Liver	Kidneys	Testes	Spleen	Heart
			Mean ± S.E.[1]	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.
Males								
Control	0	8	418 ± 12.1	2.87 ± 0.08	0.55 ± 0.06	0.83 ± 0.05	0.18 ± 0.01	0.29 ± 0.01
AG-194	20	9	361 ± 7.7 *	3.54 ± 0.08 *	0.64 ± 0.01	0.50 ± 0.05 *	0.21 ± 0.08	0.38 ± 0.02
AG-26	20	8	393 ± 9.4	2.85 ± 0.02	0.57 ± 0.0	0.66 ± 0.01 *	0.16 ± 0.00	0.27 ± 0.01
AG-31	20	7	324 ± 19.7 *	3.41 ± 0.16 **	0.68 ± 0.08	0.41 ± 0.03 *	0.18 ± 0.03	0.29 ± 0.01
AG-21	20	9	403 ± 11.2	2.60 ± 0.08	0.52 ± 0.02	0.81 ± 0.01	0.14 ± 0.01	0.27 ± 0.02
AG-3	20	9	406 ± 9.7	2.72 ± 0.01	0.58 ± 0.01	0.78 ± 0.01	0.18 ± 0.02	0.30 ± 0.01
Females								
Control	0	9	291 ± 10.0	3.13 ± 0.05	0.53 ± 0.01		0.16 ± 0.0	0.30 ± 0.0
AG-194	20	9	288 ± 7.4	3.21 ± 0.03	0.61 ± 0.03		0.20 ± 0.0	0.35 ± 0.01
AG-26	20	9	290 ± 8.8	3.41 ± 0.04	0.66 ± 0.01		0.21 ± 0.01	0.32 ± 0.01
AG-31	20	6	251 ± 17.6	3.69 ± 0.14 *	0.90 ± 0.13*		0.23 ± 0.04	0.35 ± 0.03
AG-21	20	8	300 ± 9.8	2.75 ± 0.08 *	0.53 ± 0.01		0.16 ± 0.01	0.28 ± 0.01
AG-3	20	9	344 ± 6.9	2.59 ± 0.08 *	0.49 ± 0.01		0.16 ± 0.01	0.26 ± 0.0

[1] S.E.—Standard Error of the Mean
* = 0.001
** = 0.01

TABLE 6

Body and organ weights on control and acetoglyceride supplemented diets for approximately 2 years

Supplements Fed	% in diet	No. of rats	Days on diets	Bd. wts. grams Mean ± S.E.[1]	Organ weights (gram/100 grams body weight)				
					Liver	Kidneys	Testes	Spleen	Heart
					Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.
Males									
Control	0	11	709	408 ± 12.4	2.65 ± 0.13	0.65 ± 0.02	0.80 ± 0.02	0.17 ± 0.01	0.29 ± 0.01
AG-194	5	6	600	456 ± 17.9	2.63 ± 0.15	0.58 ± 0.01	0.49 ± 0.07	0.17 ± 0.01	0.28 ± 0.01
	10	5	600	411 ± 26.5	3.16 ± 0.11	0.63 ± 0.02	0.45 ± 0.11	0.19 ± 0.02	0.30 ± 0.02
AG-26	5	4	709	389 ± 38.2	2.70 ± 0.10	0.59 ± 0.03	0.86 ± 0.10	0.19 ± 0.03	0.29 ± 0.01
	10	4	709	451 ± 8.6	2.53 ± 0.11	0.59 ± 0.02	0.70 ± 0.05	0.16 ± 0.01	0.26 ± 0.01
AG-31	5	5	710	354 ± 23.7	2.62 ± 0.23	0.66 ± 0.05	0.26 ± 0.04	0.20 ± 0.03	0.32 ± 0.01
	10	4	711	387 ± 18.4	2.95 ± 0.21	0.63 ± 0.01	0.22 ± 0.02	0.20 ± 0.02	0.29 ± 0.03
AG-21	5	5	709	405 ± 20.2	2.67 ± 0.05	0.64 ± 0.01	0.79 ± 0.01	0.16 ± 0.01	0.27 ± 0.01
	10	4	709	454 ± 18.0	2.45 ± 0.12	0.54 ± 0.02	0.79 ± 0.02	0.15 ± 0.02	0.27 ± 0.02
AG-3	5	3	709	395 ± 11.1	2.86 ± 0.22	0.64	0.87	0.16 ± 0.32	0.38
	10	1	713	402	4.08	0.68	0.78	1.09	0.32
Females									
Control	0	10	707	301 ± 19.0	3.65 ± 0.26	0.68 ± 0.03		0.31 ± 0.05	0.34 ± 0.01
AG-194	5	4	600	338 ± 12.7	2.90 ± 0.18	0.55 ± 0.04		0.26 ± 0.05	0.27 ± 0.04
	10	8	600	353 ± 8.2	2.83 ± 0.10	0.54 ± 0.01		0.18 ± 0.01	0.28 ± 0.03
AG-26	5	4	707	372 ± 19.0	2.93 ± 0.17	0.55 ± 0.02		0.17 ± 0.02	0.28 ± 0.02
	10	3	707	339 ± 6.4	3.19 ± 0.22	0.63 ± 0.04		0.30 ± 0.01	0.34 ± 0.01
AG-31	5	6	714	318 ± 16.9	2.99 ± 0.23	0.61 ± 0.02		0.16 ± 0.01	0.30 ± 0.02
	10	5	714	315 ± 13.5	2.95 ± 0.14	0.61 ± 0.02		0.19 ± 0.01	0.32 ± 0.02
AG-21	5	7	707	335 ± 11.2	2.80 ± 0.11	0.53 ± 0.02		0.19 ± 0.02	0.30 ± 0.02
	10	5	707	335 ± 6.0	2.54 ± 0.12	0.52 ± 0.01		0.15 ± 0.01	0.27 ± 0.01
AG-3	5	5	715	361 ± 16.4	3.13 ± 0.13	0.57 ± 0.03		0.20 ± 0.01	0.31 ± 0.02
	10	6	715	343 ± 22.5	3.09 ± 0.18	0.57 ± 0.02		0.21 ± 0.02	0.31 ± 0.01
Triacetin	3.3	3	708	312 ± 17.1	4.10 ± 0.05	0.65 ± 0.03		0.19 ± 0.01	0.34 ± 0.01
Sodium acetate	6.1	4	708	311 ± 14.3	3.76 ± 0.01	0.65 ± 0.04		0.24 ± 0.03	0.37 ± 0.01

[1] S.E. = Standard Error of the Mean

TABLE 7

Fat and glycogen content of livers from rats on control and acetoglyceride supplemented diets

Supplement fed	% in diet	Days on diet	No. of rats		Body weight Grams		Liver weight Grams		% ± S.E. [1]			
									Fat		Glycogen	
			Male	Female	Male	Female	Male	Female	Males	Females	Males	Females
Control	0	400	8	9	418	291	12.03	9.11	6.34 ± 0.34	6.26 ± 0.49	2.71 ± 0.17	2.33 ± 0.09
AG-194	20	400	9	9	361	288	12.78	9.25	5.28 ± 0.34*	6.49 ± 0.33	2.17 ± 0.37	2.00 ± 0.18
AG-26	20	400	8	9	393	290	11.24	9.90	5.23 ± 0.17*	6.04 ± 0.59	1.86 ± 0.35*	1.93 ± 0.10**
AG-31	20	400	7	6	324	251	11.04	9.28	4.76 ± 0.25*	6.67 ± 1.16	2.05 ± 0.09	1.51 ± 0.26**
AG-21	20	400	9	8	403	300	10.49	8.26	8.19 ± 0.50**	6.73 ± 0.47	1.40 ± 0.02**	2.14 ± 0.19
AG-3	20	400	9	9	406	344	11.05	8.89	10.28 ± 0.68**	8.12 ± 0.53*	1.66 ± 0.24**	2.12 ± 0.01

* = Significant.
** = Highly Significant.
[1] = Standard Error of the mean.

TABLE 8

Body weight increases and efficiency of food utilization for rats on control and acetoglyceride supplemented diets for 100 days [1]

Supplements fed	% in diet	Males						Females					
		No. of rats	Body weights Start	End	Gain	Total food eaten	Efficiency of diet [1]	No. of rats	Body weights Start	End	Gain	Total food eaten	Efficiency of diet [1]
Control	0	10	50	290	240	1206	5.53	10	42	201	159	975	4.50
AG-194	5	10	50	306	256	1220	5.48	10	43	202	159	960	4.26
	10	10	51	308	257	1269	4.89	10	43	208	165	1015	3.93
	20	10	49	272	223	1097	4.33	10	44	197	153	917	3.55
AG-26	5	10	49	304	255	1207	5.44	9	44	213	169	932	4.67
	10	10	50	316	266	1246	5.16	10	44	204	160	951	4.06
	20	8	50	281	231	1170	4.21	9	45	205	160	907	3.76
AG-31	5	9		261	211	1135	4.79	10	43	204	161	978	4.24
	10	8	50	240	190	1188	3.86	10	43	202	159	995	3.86
	20	7	51	202	151	845	3.81	7	43	187	144	817	3.76
AG-21	5	10	49	292	243	1123	5.56	10	43	213	170	945	4.63
	10	10	49	324	275	1214	5.41	10	44	216	172	899	4.62
	20	9	50	296	246	1097	4.78	9	44	208	164	848	4.12
AG-3	5	10	49	300	251	1147	5.64	10	43	212	169	867	5.03
	10	8	49	292	243	1122	5.23	10	43	207	164	823	4.81
	20	10	49	283	234	1025	4.87	10	44	222	178	861	4.41
Triacetin	3.3							5	49	208	159	990	
Sodium Acetate	6.1							5	49	217	168	1123	
Control	0	5	45	268	223	1267	4.89						
Crisco	5	5	44	276	232	1306	4.58						
Crisco	10	5	45	286	241	1246	4.67						
Crisco	20	5	48	263	215	1057	4.29						

[1] Efficiency = gain in weight/calories consumed × 100

lung consolidations, adhesions, cysts, middle ear infection, and other infections. Bladder stones were found in one control rat autopsied at 700 days, and in 17 experimental rats, of which only one was a female. The greatest frequency of bladder stones occurred in rats fed 20% AG-31, AG-26, and AG-21 for 400 days, stones being found in 2, 5 and 3 rats respectively. Bladder stones occurred in one rat receiving 5% AG-194 for 600 days, and at the end of 700 days in one rat on 5% AG-31, one rat on 10% AG-26, and in 2 rats each on 5 and 10% AG-21. No bladder stones were found in rats on any dietary level of AG-3.

In at least 50% of the rats on all levels of AG-194, AG-31, AG-26 and AG-21, the uteri were discolored, "rusty." No discoloration of the uteri was observed in the rats in the control group or in rats on all levels of AG-3.

Testes of all rats on the diets containing 20% acetostearins for 400 days, on 5 and 10% AG-194 for 600 days, and 5 and 10% AG-31 for 700 days were considerably smaller than those of the controls. A total of 34 tumors was found and these occurred principally in rats that were 600 to 700 days old. The incidence of tumors was no greater in the experimental groups than it was in the control groups. Only 8 tumors were found between 400 and 600 days on the respective diets, the remaining 26 were found after 600 days.

Microscopic pathology. Sections of liver from control and experimental rats stained for fat showed the presence of more fatty material in the livers of animals fed various samples of acetoglycerides at dietary levels of 20% for 400 days than was seen in control livers. The variability of fat deposition within different animals of the same group was striking. Also, within the same liver the deposition was patchy, so that when some hepatic cells were greatly distorted by deposited lipid, other cells showed none. Similarly affected cells usually occurred in groups situated about central veins, but the distribution of the change within the liver lobules was not constant. The absence of other evidence of liver cell injury suggests that the fatty deposits represent a storage phenomenon. Hematoxylin-eosin stained liver sections showed these changes as vacuolation. Livers of animals receiving lower levels of acetoglycerides for 700 days showed about the same frequency and degree of vacuolation as noted on the 20% level after 400 days. Some of the animals receiving 10% of the various acetoglycerides in the diet for 700 days exhibited a little more irregularity in size of the hepatic cells than was seen in the controls. Occasional clusters of large cells were seen. They were all less than 1 mm. in diameter and their appearance gave no suggestion of malignancy.

A severe degree of testicular atrophy was present in rats receiving dietary levels of 20% AG-194 or AG-31 for 400 days, and the same damage existed in animals on 10% or 5% AG-31 for 700 days. Well-developed testicular atrophy was found in all animals receiving 10% AG-194 for 600 days, whereas the degree of atrophy was moderate in 4 of 6 animals receiving 5% AG-194 for 600 days, testicular structure being practically normal in the other two. The testes of animals receiving acetoglycerides AG-21, AG-26, and AG-3 were not distinctly different from those of the controls.

Kidneys from some animals in different experimental groups showed tiny focal deposits of basophilic material, presumably calcium, commonly associated with irregular tubular dilatation and patchy scarring. This calcification usually appeared within the tubules of the medullary pyramids and was apparently precipitated in the substance of casts. It was sometimes present

also as small lumps in the interstitial tissue, usually in the medulla, and sometimes it occurred as small calculi in the pelvis.

These kidney changes occurred principally in organs from animals which received 20% AG-194, 20% AG-26, and 20% AG-31. Most of these lesions did not distort the kidneys greatly but several of them were severe. The smaller doses of these substances for 600 and 700 days resulted in minor changes of the same type in a portion (less than half) of the animals. AG-21 and AG-3 produced essentially no calcification or scarring of the kidneys at any of the dose levels.

A suggestion of a sex difference could be observed in the kidney lesions noted above. Changes in the female kidneys were most severe after AG-26, while the most prominent lesions in the male kidneys were seen in the AG-194 and AG-31 animals. In each of the affected groups, however, there were some kidneys which showed very little or no alteration.

An interesting alteration of the fatty tissue occurred in many of the treated animals on AG-194, AG-26, and AG-31 for 400 days, but not in the animals on AG-21 or AG-3. Somewhat less prominent similar changes were present after 10% or 5% AG-194 for 600 days, and practically no such changes were found in the 700-day animals.

This lesion consisted of small foci of foreign body reaction in which macrophages and small giant cells were clustered about what appeared to be the residue of fat droplets. Each such structure presented a central space in which there were often fine clefts in a faintly stained matrix, suggesting that fine crystals had been dissolved out during preparation of the specimens. These suggest fatty acid crystals and are consistent with the idea that some focal change in the stored fat has caused it to act as a foreign body. Commonly several adjacent fat cells were similarly involved, producing a clump of fused nodules of foreign body reaction. The adjoining surrounding fatty tisue, however, appeared perfectly normal.

This change was seen most frequently and most prominently in the small amount of fatty tissue attached to the outer surface of the stomach, but it occurred also next to the small intestine, adjacent to the bladder and around the ovary. Occasionally a small focus was related to the spleen, pancreas, adrenal or testis. The reaction was not found around the renal pelvis or in the fatty tissue of the thorax or neck. It should be noted, however, that only small samples of fat were available inasmuch as they were present only incidentally to the preparation of sections of other organs.

The appearance of this lesion brings to mind the disease of human infants known as sclerema adiposum neonatorum, in which the fat of the adipose tissue has an abnormal, sometimes crystalline, structure and excites a foreign body reaction. One of the suggested causes of this disease is a deficiency of olein in the adipose tissue, causing the fat to solidify at a higher temperature than is usual *(33)*.

The other organs of the experimental animals showed no changes which could be clearly distinguished from those in controls. Pneumonia, and inflammation of the endometrium occurred fairly frequently without relation to the treatment. Similarly, several mammary adonomas in older animals and occasional other tumors cannot be ascribed to the treatment.

The animals which received triacetin and sodium acetate were not distinctly different from the controls.

SUMMARY

Three samples of acetostearin and two samples of aceto-olein were fed to albino rats at dietary levels of 5, 10 and 20% for periods ranging from 400 days at the 20% level to approximately 2 years on the lower levels.

No significant differences in growth were noted between the control animals and those fed acetoglycerides except in the case of male rats on dietary levels of 20% acetostearins AG-194 and AG-31, and female rats receiving 20% AG-31.

Testes of rats receiving the three acetostearin diets were significantly smaller than those of the control rats or rats fed the two aceto-oleins. The livers of male rats fed 20% acetostearin AG-194 and of both male and female rats fed 20% acetostearin AG-31 were significantly larger than those of the control rats. Livers of female rats fed 20% of either aceto-olein were smaller than those of the control rats. Kidneys of female rats fed 20% acetostearin AG-31 were significantly larger than those of the controls.

The storage of liver glycogen by rats on the various acetoglyceride-containing diets was not enhanced. Excess fat observed in rats fed the various acetoglycerides appeared to be a storage phenomenon since it was unaccompanied by evidence of cellular damage.

Acetostearins produced a foreign body reaction in the fatty tissue of a number of the organs, presumably due to crystalline deposits of a high melting point fat.

Acetostearins AG-194, AG-26, and AG-31 produced some scarring of kidney tissue, presumably due to deposited calcium.

Acetostearins AG-194 and AG-31 inhibited reproduction. This finding correlates well with the decrease in weight of the testes and the histopathological evidence of testicular atrophy produced by these two acetostearins. It also correlates with the "rusty" discoloration of the uteri observed in at least half of the rats receiving these acetostearins and suggests an increase in vitamin E requirement. The fact that discolored uteri were also seen in rats receiving acetostearin AG-26 and aceto-olein AG-21, and that the former gave rise to small testes without microscopic evidence of atrophy suggest the increase in E requirement was not great enough to interfere with reproduction in these instances.

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Molecularly Distilled Monoglycerides. III. Nutritional Studies on Monoglycerides Derived From Cottonseed Oil[1,2]

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TRIGLYCERIDES, from a quantitative viewpoint, are the most important lipids of foods and of tissues and have been of prime interest to the lipid biochemist for a long time. However certain intermediate products of triglyceride metabolism, mono-, and di-glycerides, have received attention in recent years. Monoglycerides, in particular, have been studied with respect to their beneficial role in fat absorption from the intestinal tract (1) and to their presence normally in pancreas and other tissues (2). Furthermore Tidwell (3) has shown that monoglycerides of olive oil fatty acids were more efficiently absorbed than olive oil. Also the nutritive properties of monostearin and monolinolein were investigated by Braun and Shrewsbury (4), who indicated that these two monoglycerides were "practically equal" to lard in promoting the growth of young rats.

These experiments were designed to investigate the problem of whether relatively large amounts of monoglycerides of a natural mixture of fatty acids would be utilized *in vivo*, as efficiently as natural fats composed of triglycerides of the same mixture of fatty acids. Monoglycerides of the fatty acids of cottonseed oil have been compared with cottonseed oil itself at 15% and at 25% levels in the diet of three generations of rats, for adequacy in supporting growth, reproduction, and lactation. Results indicate that for all these functions cottonseed oil monoglycerides are equal to the fat from which they were prepared.

Experimental

Diets. The detailed composition of the diets is given in Table I. The additional fat was added at the expense of a portion of both cerelose and starch. The cottonseed oil was deodorized, refined salad oil (Wesson oil), and the cottonseed oil monoglycerides were a commercial product (Myverol Type 18-85 Monoglyceride) made by molecular distillation (5). Each diet was prepared to contain 400 mg. of vitamin E/kg. The cottonseed oil contained 0.75 mg./gm. of vitamin E and the cottonseed oil monoglyceride, 0.22 mg./gm.; the extra amount needed was furnished as distilled d,α-tocopherol concentrate. Fresh diets were prepared weekly, and the prepared diets were stored in the refrigerator until used. No increase in peroxide value (about 2) of the diet was observed on storage for one week. All diets were fed *ad libitum*.

TABLE I

Composition of Diets

Ingredient	Content (%)
Casein, crude	16.0
Casein, vitaminized	2.0
Salt mixture, U.S.P. No. 2	4.0
Yeast, dried brewers	7.8
Liver extract, Wilson's 1-10	0.2
Lipid, cottonseed oil or monoglycerides	15.0 or 25.0
Corn starch	36.7 or 30.0
Dextrose (Cerelose)	18.3 or 15.0

Vitamins: per 10 grams of diet—

A—400 I.U.	B_1—100 μg.	Ca-pantothenate—250 μg.
D— 40 I.U.	B_2—100 μg.	Niacin—200 μg.
E— 4 mg.	B_6—100 μg.	Choline—10 mg.
K—500 μg.		Inositol—5 mg.

Treatment of Animals. Eighty albino rats, forty males and forty females, were selected at time of weaning from our stock colony. The animals (10 per group) were placed immediately on the four diets previously described: 15% and 25% cottonseed oil, and 15% and 25% cottonseed oil monoglycerides. The growth of these animals was observed for an 8-week period, at which time reproduction and digestibility experiments were started. Six females and two males were selected from each dietary group for breeding purposes. Throughout the breeding and lactation period the females were maintained on the same diets previously fed. The males were interchanged between breeding cages and subsisted for short periods on diets other than those to which they were originally assigned. The young of the original ani-

TABLE II

Growth Response of Male Rats (8-Week Period)

Level of Fat	Generation	Mean Gain ± S.E. (gms.) Cottonseed Oil	Monoglycerides
15%	1	219.3 ± 9.5	213.9 ± 6.5
	2	251.1 ± 9.4	220 ± 5.9
	3	225.7 ± 2.7	235.9 ± 6.7
25%	1	217.3 ± 9.5	228.0 ± 8.5
	2	226.9 ± 4.2	219.0 ± 7.7
	3	226.5 ± 10.5	219.8 ± 5.3
Over all Mean		227.8 ± 4.0	222.9 ± 2.8

Diff. of Means = 4.9; S.E. of Diff. = 5.0; t = 0.98; p = 0.3-0.4.

Each group contained approximately 10 animals. The over all mean gain for cottonseed oil is based on 61 animals and for monoglycerides on 59 animals.

[1] Presented at San Francisco fall meeting, American Oil Chemists' Society, Sept. 26-28, 1950.
[2] Communication No. 174.

TABLE III

Growth Response of Female Rats (8 Week Period)

Level of Fat	Generation	Mean Gain ± S.E. (gms.)	
		Cottonseed Oil	Monoglycerides
15%	1	151.1±6.0	139.9±4.7
	2	152.3±3.8	141.9±2.7
	3	143.9±4.5	152.0±6.0
25%	1	143.6±4.4	136.8±3.6
	2	149.7±5.7	141.5±2.3
	3	147.3±6.7	147.3±2.4
Over-all Mean		149.2±2.5	143.6±1.5

Diff. of Means = 5.6; S.E. of Diff. = 2.9; t = 1.96; p = 0.05-0.06.

Each group contained approximately 10 animals. The over-all mean gain for cottonseed oil is based on 58 animals and for monoglycerides on 67 animals.

mals were termed second generation animals. They were treated in a similar manner throughout growth, breeding, and lactation. The young of the second generation (or third-generation animals) were treated in similar manner throughout the 8 weeks' growth study period. No attempt was made to breed these animals, and they are being retained for survival tests.

Growth. The results of the 8 weeks' growth studies of the three generations of rats fed the four diets under examination are summarized in Tables II and III. It was found by statistical analysis that there was practically no difference in rate of growth between diets containing 15% and 25% fat or between generations of animals. The data are therefore summarized so that a direct comparison can be made between the diets containing cottonseed oil on one hand and those containing cottonseed oil monoglycerides. The results of the conventional "t" test indicate that there was no significant difference in the over-all mean weight gains of the male rats between the diets containing the two types of fat (p = 0.3-0.4). The difference in mean weight gains for the female rats was slightly greater but was not statistically significant (p = 0.05-0.06).

Reproduction. The reproductive performance of the females of the first- and second-generation animals is summarized in Table IV. Comparable fertility was observed irrespective of type of fat, level of fat, or generation. The mean size of the litters and the mean weight of the young at birth are also comparable, irrespective of treatment. No differences in reproductive performance were observed in females fed the different diets under consideration.

Lactation. The body weight of the young at weaning was used as the criterion of lactation. Lactation performance is summarized in Table V. There was no significant difference in the weaning weights between sex or between level of fat. The data are therefore summarized so that a direct comparison can be made between the cottonseed oil and cottonseed oil monoglyceride diets. The results of the conventional "t" test indicate that diets containing the two fats are comparable in their effect on the weaning weight of the young (p = 0.1-0.2).

TABLE IV

Reproductive Performance

Diet	Generation	Females Pregnant /Mated	Litters		
			Total Live Young	Mean Litter Size	Mean Wt. at Birth (gms.)
15% Fat Cottonseed Oil	1	6/6	52	8.7	....
	2	6/6	41	6.9	5.6
Monoglycerides	1	5/6	42	8.4	....
	2	6/6	54	9.0	6.1
25% Fat Cottonseed Oil	1	4/4	27	6.8	....
	2	6/6	50	8.3	5.8
Monoglycerides	1	5/6	39	7.8	....
	2	6/6	55	9.2	6.7

TABLE V

Lactation (Body Weight of Young at Weaning, 21 Days)

Sex	Level of Fat	Mean Weight at Weaning ± S.E. (gms.)	
		Cottonseed Oil	Monoglycerides
Male	15%	50.4±2.2	43.1±0.9
Male	25%	43.2±2.2	49.1±1.8
Female	15%	49.9±2.3	41.7±1.3
Female	25%	43.8±3.3	47.0±1.6
Over-all Mean		47.4±1.3	45.4±0.8

Diff. of Means = 1.4; S.E. of Diff. = 2.0; t = 1.40; p = 0.1-0.2.

Digestibility. Eight male rats on each of the two diets containing 25% fat, one containing cottonseed oil, the other cottonseed oil monoglycerides, were segregated following the first generation, 8 weeks' growth experiment. The feces were collected, and food consumption was determined daily for a period of seven days. In order to determine the excretion of metabolic fat the animals were then placed on the corresponding fat-free diet. After an equilibration period of seven days feces were again collected and food consumption determined on a daily basis for seven days. The fat content of the feces and the digestive coefficient were determined according to the procedures described by Hoagland and Snider (8). The results of food consumption and digestibility studies are given in Table VI. There was no difference in the mean digestive coefficient between the rats fed the diet containing 25% cottonseed oil and the diet containing 25% cottonseed oil monoglycerides. In view of the high digestive coefficients observed with the diets containing 25% fat, no experiments were performed with the 15% fat diets.

TABLE VI

Food Consumption and Digestibility
(Male Rats; Diets Containing 25% Fat)

Type of Fat	Mean Daily Food Consumption (9th experimental week)	Coefficient of Digestibility
	(gms.±S.E.)	*(%±S.E.)*
Cottonseed Oil	13.8±0.4	96.8±0.5
Monoglycerides	13.6±0.5	97.8±0.4

Food Consumption: Diff. = 0.14; S.E. of Diff. = 0.6; t = 0.22; p = 0.8-0.9.

Coef. of Digestibility: Diff. = 0.94; S.E. of Diff. = 0.6; t = 1.52; p = 0.1-0.2.

There was likewise no difference in the mean daily food consumption between rats on the two diets.

Discussion

Prior to this study it was interesting to speculate that monoglycerides of the fatty acids of cottonseed oil would be superior, nutritionally, to cottonseed oil

itself. This speculation was based on the concept of Frazer and co-workers (1) that monoglycerides are essential elements in the mechanism of intestinal absorption of lipids and on the results obtained by Tidwell (3), showing that monoglycerides of olive oil fatty acids were more efficiently absorbed than olive oil. These facts and the successful use of monoglycerides in intravenous feeding experiments (6, 7) are compatible with the concept that monoglycerides of natural fats might be superior in nutritive value to the original fat. However from the results obtained in the present study it is evident that monoglycerides of fatty acids of cottonseed oil are not significantly better than cottonseed oil itself and the two types of lipids must be considered nutritionally equal.

Summary and Conclusions

Monoglycerides, prepared from cottonseed oil, were fed to three generations of rats at a 15% and a 25% level as the sole source of fat in the diet. Refined cottonseed oil was fed to comparable groups of rats at the same levels.

No significant differences were found between the monoglycerides and the cottonseed oil in their nutritive value as measured by growth response, reproduction ability, and lactation performance. Absorption of fatty acids, either as monoglycerides or as the original oil, from the intestinal tract was the same as shown by essentially equal coefficients of absorption for the two types of lipid at a 25% level in the diet.

Acknowledgment

Appreciation is expressed to Hugh Risley, who performed the analyses associated with the digestibility experiments.

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[Received October 5, 1950]

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J. Am. Pharm. Assoc.
39: 583-585, 1959

Toxicological Studies on Synthetic Glycerin*

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Acute and prolonged studies have failed to show any differences in the toxicity of natural and synthetic glycerin. The acute toxicities in mice following intravenous and oral administration are reported. Pathological changes in rats are compared after treatment for six months with natural and synthetic glycerin.

GLYCERIN, a trihydric alcohol, was discovered by Scheele in 1783 but was not used in pharmacy until many years later when it was employed as an emollient for retaining moisture on surfaces having a tendency to dry and crack. It first appeared in the third edition of the United States Pharmacopoeia, which became official in 1850. Today the use of glycerin is widespread and millions of pounds are consumed annually in the pharmaceutical industry alone. Lesser (1) has reviewed the many formulas where it is employed. Other uses include the manufacture of cigarettes, cosmetics, explosives, liqueurs, printing inks, liquid soaps, and lubricants.

* Received May 5, 1950, from the Lilly Research Laboratories, Indianapolis 6, Ind.
Presented to the Scientific Section, A. Ph. A., Atlantic City meeting, May, 1950.
† The authors wish to acknowledge the assistance of Marian Ellaby, Nila Maze, John Owen, John S. Welles, and Harold Worth in these studies.

Glycerin has been obtained for the most part as a by-product from the manufacture of soaps and fatty acids. During the war when large quantities were essential in the manufacture of explosives, other glycols were substituted in pharmaceuticals where possible. None seemed to be as universally acceptable as glycerin. It now appears that the use of detergents is increasing and if the manufacture of soaps declines, the supply of natural glycerin may become inadequate. The development of a process on a commercial scale for the production of synthetic glycerin will alleviate the necessity of finding substitutes.

Before synthetic glycerin can be used in products designed for human consumption, it must be shown to contain no harmful impurities. The toxicity of natural glycerin has been studied by numerous workers including Deichmann (2), von Oettingen (3), and Holck (4). Hart (5) could find no difference in the toxicity of natural and synthetic glycerin following oral administration of single doses to rats. We have attempted to compare the acute and chronic toxicities of the two products.

EXPERIMENTAL

The synthetic glycerin used in our studies was supplied by the Shell Chemical Corporation. It was a clear, colorless, syrupy liquid having a sweet taste, a slight characteristic odor, and was neutral to litmus. Other physical properties follow:

Specific gravity, 20/20°	1.2631
Purity, % by weight	99.80
Total chlorine, % by weight	0.009
Water, % by weight	0.30
Ash, % by weight	0.0004

The natural glycerin used was a pooled sample from various suppliers, all lots being U. S. P. quality or better.

Acute Toxicity in Mice.—For intravenous studies, 30% solutions by weight were prepared. This gave an injectable material which could be measured conveniently within practical limits. For oral studies, undiluted samples were administered by stomach tube. All animals were observed for one week and the LD_{50} for each study was computed by the method of Bliss (6). The results are found in Table I. All deaths occurred soon after intravenous injection but were somewhat delayed following oral administration. In the latter case, some mice died within ninety minutes and all died within forty-eight hours after ingestion. Obviously, no statistical difference can be shown between the toxicities of the two products.

Chronic Toxicity.—Fifteen female rats, six to eight weeks old, were housed in individual cages in temperature-controlled quarters. All were allowed to eat ground rat ration *ad libitum*. Five animals, which served as controls, were given tap water to drink; a second group of five received tap water containing natural glycerin 5% by volume, while the third group received the same concentration of synthetic glycerin as drinking water. All rats were weighed at weekly intervals. Hemoglobin was determined, and erythrocytes, leucocytes, and differentials were counted monthly for a period of six months. The animals were then sacrificed and submitted to necropsy. Both gross and microscopic studies were made of the heart, lungs, liver, spleen, stomach, intestines, kidneys, thymus, thyroid, and adrenals.

Effect on Growth.—The growth curves for each group are found in Fig. 1. The rate of growth in the control group is typical of female rats of the same strain and has been checked several times. When the body weight reaches 200–225 Gm., the gain levels off. Both groups which received 5% glycerin in the drinking water gained weight to about the same degree as the controls.

Effect on Hemoglobin.—The mean hemoglobin values determined as acid-hematin by the Fisher electrohemometer are found in Table II. The figures are quite comparable to those found for the controls.

TABLE II.—AVERAGE HEMOGLOBIN VALUES IN PER CENT (15.6 GM./100 CC. EQUALS 100%)

Date	Control Group	Natural Glycerin	Synthetic Glycerin
3-21-49 (Normal)	87.8	90.6	79.2
4-18-49	101.2	108.2	104.8
5-24-49	114.8	110.2	109.0
7- 5-49	113.8	110.8	109.2
8- 2-49	113.6	104.6	96.8
8-25-49	109.8	104.6	106.3
9-27-49	112.6	109.8	105.3

Effect on Red Blood Cells.—The mean erythrocyte count for each group is found in Table III. Here, also, no variation from the control group is found, the number in general rising from the initial value recorded prior to treatment.

Effect on White Blood Cells.—The mean number of leucocytes is found in Table IV. An increase is noted at times, which is true in all groups. It has previously been found that rats, subjected to fre-

TABLE I.—ACUTE TOXICITY OF GLYCERIN IN MICE

Mode of Administration	Lot Used	Dose	No. Died/ No. Used	LD_{50} ± S. E.
		Gm./Kg.		
Intravenous	Natural	3.75	1/10	4.37 ± 0.14 Gm./Kg.
		4.20	5/10	
		4.80	6/10	
		5.40	10/10	
	Synthetic	3.75	2/10	4.25 ± 0.16 Gm./Kg
		4.20	5/10	
		4.80	8/10	
		5.40	9/10	
		Cc./Kg.		
Oral	Natural	16.0	1/10	20.65 ± 0.47 Cc./Kg.
		18.0	4/10	
		20.0	5/15	
		22.5	7/10	
		25.0	8/10	
		30.0	5/5	
	Synthetic	16.0	1/10	20.81 ± 0.58 Cc./Kg.
		18.0	2/10	
		20.0	6/15	
		22.5	7/10	
		25.0	8/10	
		30.0	5/5	

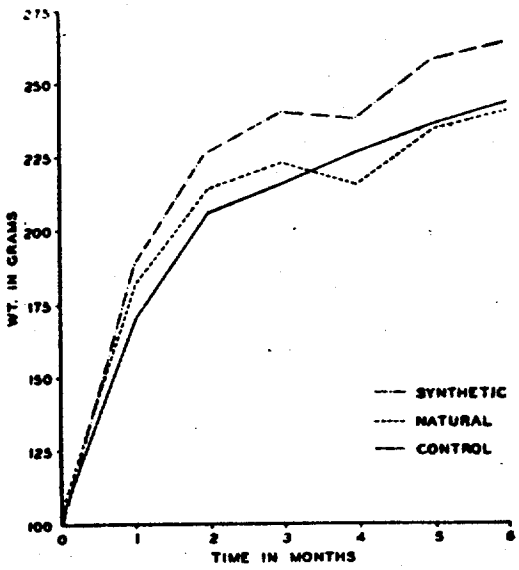

Fig. 1.—Effect on growth of rats receiving glycerin (5%) in the drinking water.

quent bleedings from the tail, often develop a mild leucocytosis.

Although differential counts were made monthly on all groups, no differences were found among the three groups that would justify the use of the necessary space for the voluminous records.

Pathological Findings: Control Group.—All rats were normal, grossly and microscopically.

TABLE III.—AVERAGE RED BLOOD COUNT (IN MILLIONS)

Date	Control Group	Natural Glycerin	Synthetic Glycerin
3-21-49 (Normal)	7.92	7.95	7.37
4-18-49	8.28	7.38	7.25
5-24-49	9.87	8.79	9.32
7- 5-49	7.95	8.18	7.75
8- 2-49	9.95	9.64	8.77
8-25-49	9.24	8.51	8.78
9-27-49	9.63	9.48	8.99

Natural Glycerin.—Grossly, all were normal except for a small thymus in two. One rat had a slight focal interstitial pneumonia not attributable to the drug. All microscopic sections were normal except those of the kidneys. In three out of the five, several tubules near the junction of the cortex and medulla contained calcified masses.

Synthetic Glycerin.—One rat died after four months. It was emaciated, the liver was small and moderately congested; the spleen was small and the thymus was atrophic; the stomach and intestines were empty. Microscopic examination of the spleen revealed small lymph nodules and moderate deposits of hemosiderin in reticular cells. It seems improbable that these changes were due to synthetic glycerin, for similar ones were not found in the other animals. Except for the kidneys, the tissues of all other rats were grossly and microscopically normal. As with natural glycerin, three out of the five rats had calcified masses in the tubules near the junction of the cortex and medulla.

TABLE IV. AVERAGE WHITE BLOOD COUNT (IN THOUSANDS)

Date	Control Group	Natural Glycerin	Synthetic Glycerin
3-21-49 (Normal)	17.4	15.9	17.2
4-26-49	28.1	24.8	23.6
5-24-49	18.4	20.7	23.2
6-21-49	20.6	24.5	20.2
7-19-49	20.0	16.0	16.1
8-23-49	14.4	17.2	17.7
9-27-49	17.2	16.8	16.8

CONCLUSIONS

1. No difference has been shown between the toxicity of natural and synthetic glycerin following intravenous and oral administration to albino mice.

2. Rats that received 5 per cent glycerin (either natural or synthetic) as drinking water for six months gained weight comparable to that of the controls. No change was found in the hematopoietic system and the mild damage to the kidneys occurred with glycerin from both sources. No other visceral damage was apparent.

3. Synthetic glycerin appears to be as harmless as natural glycerin to laboratory animals. However, additional work may be necessary before it is used in pharmaceutical products for human consumption. Since large quantities serve in the manufacture of cosmetics, prolonged studies by local application must be also completed before its use in this industry may be justified.

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Acta path. et microbiol. scandinav. 61, 161-180, 1964

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HEATED FATS AND ALLIED COMPOUNDS AS CARCINOGENS

Studied by the Newt Test

By

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Received 4.x.63

Extensive studies through decades have failed to clarify whether heated and oxidated fats have carcinogenic properties. This appeared from a review of accumulated experimental results (*Arffmann* 1960) pointing to the incapability of the conventional methods of bioassay to yield conclusive evidence. It seems therefore highly desirable to develop new methods for the demonstration of carcinogenicity which at the same time may be expected to reveal unknown effects of carcinogens. A series of experiments on the newt test (*Arffmann & Christensen* 1961, *Arffmann* 1962, 1963 and 1964) has shown a specificity sufficient for a screening method, if the chemical compounds are given in oil, which makes the method convenient for the study of heated fats and allied compounds.

Heating and Oxidation of Fats.

The chemical changes induced by heating of lipids depend on the presence or absence of oxygen, the active components being primarily the unsaturated fatty acids (*Axt* 1959). Most important is the oxidative transformation, corresponding to autoxidation which has found excellent description in works by *Paquot* (1958), *Axt* (1959) and *Glavind* (1963). The first step is the formation of hydroperoxides (lipoperoxides) formed by a free-radical mechanism and proceeding as a chain reaction. This is terminated by polymerization. Decomposition of the peroxides leads amongst others to a formation of hydroxyl-, aldehyde- and ketone groups as well as acids, and the products include a series of low-mole-

The author's thanks are due to Director, cand. polyt. *A. Herlow* and cand. polyt. *O. Tolboe*, Grindstedvaerket, Viby J., for fruitful discussions on the chemistry of heated fats, and for technical assistance in numerous ways. The author is indebted to *Johs. Glavind*, D.Sc., Polytechnic Institute, Copenhagen, for his experienced guidance into the chemistry and biochemistry of lipoperoxides and for inspiring suggestions.

cular-weight unsaturated carbonyl compounds. Also epoxides are formed late in the process.

If oxygen is absent during heating oxygen-free polymers, often of a cyclic structure, are formed. Decomposition products are paraffins, aldehydes, acids and ketones.

The most simple analytical methods to follow the oxidation are determinations of the peroxide value and of the carbonyl (aldehyde) value. The peroxide value expresses the actual quantity of hydroperoxide groups and is therefore not a measure of the total oxidation (*Deutsch et al.* 1941). The carbonyl value measures the secondary oxidation products, which are determined as aldehydes (*Holm et al.* 1957).

The present knowledge of the chemical changes in heating and oxidation of fats is, however, only elementary. In summary, complicated processes of oxidation, polymerization and decomposition lead to a vast mixture of reaction products, including polymers, peroxides, aldehydes, ketones, epoxides, lactones and free fatty acids (*Wurziger & Osterthun* 1960).

Heating of cholesterol probably leads to oxidation, and the formation of ketonic steroids is suggested by *Haven & Bloor* (1956). The products may possess hydroperoxide- or epoxide groups (*Fieser* 1954).

MATERIAL AND METHODS

Substances. Soyabean oil was chosen as test object for the study of heated fats. This oil is used for the preparation of edible emulsifiers, especially for the manufacture of margarine and baking compounds, and besides its content of the more unsaturated fatty acids is rather high.

Simple heating was performed on an infrared radiation bath (BB Infrabath, Bie & Berntsen, Copenhagen) furnished with a relay and a contact thermometer. The fresh soyabean oil (supplied by Dansk Sojakagefabrik) was contained in a Pyrex beaker or retort and the contact thermometer put into the oil. A temperature of 350° C was reached after 45 to 60 minutes and maintained for exactly ½ or 1 hour. During this time the temperature was not below 350° C and not above 355° C. After the heating period the oil was immediately removed from the warm plate. Air had free admittance, but was not bubbled through. The end-product was a dark, brownish and distinctly viscous material.

Some of the soyabean oil was heated in nitrogen atmosphere. The fresh oil was bubbled through with nitrogen for 6 hrs. 20 min. before being poured into a retort, which was sealed from the surrounding air. The warm-up time was 105 minutes, and a strong current of nitrogen through the retort was maintained until 300°-320° C was reached after about 70 minutes. The sealed system was heated at 350°-355° C for exactly 1 hour. The nitrogen current was reestablished during the first hour of cooling, and then the retort was closed. The end-product was of a lighter colour and less viscous than that after heating in the presence of air.

Chemical data of the polymerized soyabean oils are given in Table 1.

The viscosity of the heated oils made it necessary to dilute the products with 3 parts of fresh soyabean oil (v/v) before injection.

Commercially polymerized soyabean oil was kindly supplied by Grindstedvaerket, Viby J., Denmark. It is used in an edible emulsifier and produced by heating at 300° C in vacuum (about 20 mm Hg) for about 48 hours until the desired viscosity has been reached. Chromatographic analysis (performed by Professor Jacini, Milan) after saponification of the oil showed the following approximate percentages: monomeric acids: 43-50, dimeric acids: 32-38, polymeric acids: 15-16.5. Molecular weight

of the monomeric glyceryl fraction was about 920, while total oil molecular weight was 2470–3120. Because of the viscosity the product was administered as a 25 per cent in one experiment 50 per cent solution in fresh soyabean oil (w/w).

TABLE 1

Chemical Analyses of Heat-Polymerized Soyabean Oil.

Substance	Peroxide value m.e./kg	Carbonyl value mg%	Iodine value	% Urea filtrate Acids	% Non-distillable methyl esters
Soyabean oil	1.4	6.2	131	6	5
Soyabean oil heated in N-atm. at 350° C for 1 hr.	4.7	217	75	58	53
Soyabean oil heated in air at 350° C for 1 hr.	5.1	206	73	66	56
Soyabean oil heated in air at 350° C for ½ hr.	...	...	...	...	51

The peroxidized soyabean oils were prepared by the Laboratory at Grindstedvaerket. Moderate heating, vigorous stirring of the oil and a constant current of air through the retort were maintained until the peroxide content had risen to the highest obtainable values. Before that, specimens had been taken out at lower levels with proper intervals. In experiments Nos. 11 and 12 (Table 3) the P.V.[1] 4.5 oil was oxidated at 37° C for 131 hours, while the P.V. 858 specimen was made at 100° C for 21 hours. The same temperature was applied to the P.V. 293 oil through 28 hours, but after the oxidation this specimen was mixed with equal part of fresh soyabean oil (the peroxide value is determined on the mixture). In spite of several trials it proved impossible once more to reach the high peroxide value of 8–900 m.e./kg. The applied temperatures were 70° C and down to 40° C in order to avoid heat-induced reversion of the process which ran for up to 95 days. The highest peroxide value obtained in these latter experiments was 409 m.e./kg.

For parallel control, specimens of the peroxidized oils were quickly heated at 200° C for 10 minutes at 10 mm Hg in order to destroy the peroxides. These samples are designated B in the tables.

The peroxidized soyabean oils were—with a few exceptions—injected without any dilution.

Ethyl linoleate hydroperoxides were kindly prepared by Johs. Glavind, D.Sc., Polytechnic Institute, Copenhagen, who also supplied the ethyl linoleate. The technique of preparation is described by *Glavind, Søndergaard & Dam* (1961). The peroxide values (*Glavind & Hartmann* 1955) of the three specimens were 2000, 1500 and 750 m.e./kg. Part of the P.V. 1500 sample was reduced following the method of *Privett & Nickell* (1956), and the peroxide value thereby lowered to 70 m.e./kg. The reduction lasted 1 hour. In most experiments the ethyl linoleate preparations were solved in peanut oil to reduce toxicity, concentrations being v/v.

Epoxidized soyabean oil was obtained from General Mills, U.S.A., and is designated Epoxygen (type 90 premium grade). The following analytical values are specified: Epoxy content, per cent of theoretical: 90 per cent min. Oxirane content: 6.8 per cent min. Iodine value (Wijs): 2.0 max. Acid value: 0.2 max. Saponification value: 180–184. Molecular weight: 1000 appr. Because of viscosity the epoxygen had to be diluted with fresh soyabean oil before injection to make a 25 per cent solution (v/v).

The various isomers of diepoxybutane (*Feit* 1960) were kindly supplied by Dr. P. W. Feit, the Leo Pharmaceutical Products, Copenhagen. The pure d-, l-, meso- and dl- compounds were all easily solved in peanut oil without the use of heat. The concentrations are weight/volume except in experiment No. 65 (v/v).

16 α, 17 α-epoxyoestratrien-3-ol, synthetisized and kindly provided by Professor H. Breuer, Bonn, was tested in the newts. This monoepoxide, which is a naturally

[1] P.V. = peroxide value, determined a.m. Wheeler (DGF Einheitsmethoden C-VI 6a (57)).

occurring product in estrogen metabolism, is closely related to oestradiol, which was chosen for parallel control and kindly supplied by the Leo Pharmaceutical Products. Both substances were found difficult to solve in peanut oil requiring vigorous stirring and heating on waterbath (<100° C) for 5 to 11 minutes to obtain 0.5 per cent solutions. In most instances slight secondary precipitation after cooling was eliminated before injection by heating over open fire for a few seconds.

Cholesterol was obtained from The British Drug Houses Ltd. (Laboratory Reagent. Melting point: 145° to 149° C. Specific rotation (α) $^{20}_{D}$ (in chloroform): −38°.5 to −40°.5. Moisture: Not more than 0.5 per cent. Sulphated ash: Not more than 0.1 per cent). Cholesterol was heated on the BB Infrabath the compound being placed in a Pyrex glass, which together with the contact thermometer was put into a beaker with silicon oil. One sample was heated at 350° C, another at 200° C, both for exactly 1 hour. The warm-up time was 30 and 14 minutes, respectively. After the 350° C heating cholesterol appeared darkbrown to black and after cooling was paste-like. The lower temperature changed the white colour to light yellow-brown. Both specimens were, like untreated cholesterol, easily solved in oils by the use of stirring and heating on waterbath (<100° C) for 1–1½ minutes.

Technical procedures have been described before (*Arffmann* & *Christensen* 1961, *Arffmann* 1962, 1963). In a few experiments Triton marmoratus was used instead of Triton cristatus, the latter being at that time not available.

RESULTS

The results are shown in Tables 2–10. Negative and positive controls are included. The various heat-polymerized soyabean oils gave a negative outcome in all experiments (Table 2).

Strongly peroxidized soyabean oil was submitted to two tests (Table 3). The results are slightly varying, probably because of a less reliable technique in these early experiments, but indicate clearly a weak or moderate, positive reaction to the two oils with a peroxide value of 858 and 293 m.e./kg respectively. In contrast, the P.V. 4.5 oil gave a result not different from that seen after fresh oil, while heavy dilution (2 per

TABLE

Experiments with Heat-Polymerized Soyabean Oil.

Experiment No.	35-36			10						11			
Substance	Soyabean oil	Soyabean oil heated in air at 350°C for ½ hr. 25% in soyabean oil	Dibenz-[a,h]anthracene 0.1% in soyabean oil	Soyabean oil		Soyabean oil heated in N-atm. at 350°C for 1 hr. 25% in soyabean oil		Soyabean oil heated in air at 350°C for 1 hr. 25% in soyabean oil		Soyabean oil		Soyabean oil heated in N-atm. at 350°C for 1 hr. 25% in soyabean oil	
Site of application	Tail		Back	Tail	Back	Tail	Back	Tail	Back	Tail	Back	Tail	Back
Number of animals (Triton cristatus)	4	8	8	4	4	4	4	4	4	4	4	4	4
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on													
3.- 4. day													
6.- 7.													
8.-10.													
11.-12.													
15.													
18.													
Animals died during experiment	0	1 (included)	3 (2 included)	1	0	3 (1 included)	1	2	0	0	0	2 (included)	0

Experiment No.	12						30			31			32		
Substance	Soyabean oil heated in air at 350°C for 1 hr. 25% in soyabean oil		Soyabean oil	Soyabean oil heated in N-atm. at 350°C for 1 hr. 25% in soyabean oil	Soyabean oil heated in air at 350°C for 1 hr. 25% in soyabean oil	Benzo-a pyrene 0.5% in soyabean oil	Soyabean oil	Commercially polymerized soyabean oil 25% in soyabean oil	Benzo-a pyrene 0.1% in soyabean oil	Soyabean oil	Commercially polymerized soyabean oil 25% in soyabean oil	Benzo-a pyrene 0.1% in soyabean oil	Soyabean oil	Commercially polymerized soyabean oil 50% in soyabean oil	Chrysene 0.5% in peanut oil
Site of application	Tail	Back	Tail				Tail			Tail			Tail		
Number of animals (Triton cristatus)	4	4	8	8	8	8	8	8	8	8	8	8	8	8	8
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on															
3.- 4. day															
6.- 7.															
8.-10.															
11.-12.															
15.															
18.															
Animals died during experiment	1	1	3	2 (included)	3 (1 included)	2 (1 included)	0	2	1 (included)	1	1	1 (included)	0	0	0

* Unsuited because of epidermolysis.

TABLE 2

TABLE 3

Experiments with Strongly peroxidized Soyabean Oil.

Experiment No.	11								12								
Substance	Peanut oil	Perox. soya-bean oil (P.V.* 4.5)	Perox. soya-bean oil (P.V. 293)	Perox. soya-bean oil (P.V. 858)	Perox. soya-bean oil (P.V. 4.5) 2% in peanut oil	Perox. soya-bean oil (P.V. 293) 2% in peanut oil	Perox. soya-bean oil (P.V. 858) 2% in peanut oil	3-Methyl-cholanthrene 0.1% in peanut oil	Soya-bean oil	Perox. soya-bean oil (P.V. 4.5)	Perox. soya-bean oil (P.V. 293)	Perox. soya-bean oil (P.V. 858)	Perox. soya-bean oil (P.V. 4.5) 2% in soya-bean oil	Perox. soya-bean oil (P.V. 293) 2% in soya-bean oil	Perox. soya-bean oil (P.V. 858) 2% in soya-bean oil	3-Methyl-cholanthrene 0.1% in soya-bean oil	
Site of application	Tail								Tail								
Number of animals (Triton cristatus)	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on																	
3. day	—									(+)	—	+	—		+		
6. "	—		(+)					(+)			—	+	—	—	+†	—	
9. "	—		+		—		(+)			+†	—	+	—	—	—	+	
12. "	—		—	+	+†				—	—	—	+	—	—	—	(+)	
15. "	—	—	+		—		—	+	—	—	—	—	—	—	(+)	+	
18. "	—	—	+	+†	—		—	+		—	+	—	—	—	+†	—	
21. "	—	—			—	—	—	—	—	—	—	+	—	—	—	—	
Animals died during experiment	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

* P.V. = peroxide value (m.e./kg).
† No signs of the injected oil.
[illegible] [illegible]ations technically bad.

TABLE 4

Experiments with Peroxidized Soyabean Oil before (A) and after (B) Heat-Reduction.

Experiment No.			45	46	47	49	50	51	52	54	69	70
Substance	P.V.*	C.V.§	Positive reactions Total number of animals									
Oil 0	1.9	12.5	1/5	0/5	0/6	...	...	...	...	...	...	...
Oil 0	2.2	3.7	...	...	...	0/8	0/8	0/7	0/8	...	...	...
Oil 0	1.4	10.2	...	...	...	...	...	...	...	1/6	...	...
Oil 0	3.0	2.5	...	...	...	...	...	...	...	...	1/8	0/8
Oil 1A	33.0	9.5	0/7	0/6	...	...	...	...	...	...	...	...
Oil 2A	55.0	...	...	...	...	0/8	...	...	...	0/6	...	...
Oil 3A	70.0	9.1	0/6	0/6	...	...	...	...	...	...	...	...
Oil 4A	104.0	...	...	...	...	0/8	...	...	...	0/6	...	...
Oil 5A	161.0	13.1	2/7	1/6	...	...	...	...	...	...	...	...
Oil 6A	191.0	...	...	...	...	0/8	...	1/8	...	0/6	...	...
Oil 7A	296.0	22.0	0/2	0/5	2/4	...	...	...	...	...	...	...
Oil 8A	362.0	...	...	...	...	0/8	0/8	0/8	0/8	0/6	...	...
Oil 9A	409.0	57.2	...	...	...	...	...	...	...	...	2/8	3/16
Oil 1B	1.5	47.0	0/3	0/5	...	...	...	...	...	...	...	...
Oil 2B	0.6	67.0	...	...	...	0/8	...	...	...	0/5	...	...
Oil 3B	1.8	90.0	0/6	2/5	...	...	...	...	...	...	...	...
Oil 4B	0.7	137.0	...	...	...	0/8	...	...	...	0/5	...	...
Oil 5B	2.6	162.0	0/6	0/6	...	...	...	...	...	...	...	...
Oil 6B	1.3	310.0	...	...	...	0/8	...	0/7	...	0/6	...	...
Oil 7B	4.1	165.0	0/3	0/6	0/4	...	...	...	...	...	...	...
Oil 8B	3.8	640.0	...	...	...	1/8	0/7	0/8	0/8	0/6	...	...
Oil 9B	3.1	336.0	...	...	...	...	...	...	...	...	2/7	3/16
Dibenz(a,h)-anthracene 0.1%			2/2	...	...	...	...	...	...	...	...	...
Chrysene 0.5%			...	2/5	...	...	...	...	3/8	...	...	...
Benzo(a)-pyrene 0.1%			...	...	3/6	...	1/8	4/8	...	...	5/7	...
— 0.5%			...	...	...	...	...	...	...	...	...	8/8
Benz(a)-anthracene 0.1%			...	...	...	0/6	...	...	...	...	...	...
— 0.5%			...	...	...	...	...	...	...	2/5	...	...

* P.V. = peroxide value (m.e. kg).
§ C.V. = carbonyl value (mg%).

cent) of the P.V. 858 and 293 oils weakened the positive response distinctly.

The following extensive experiments on the reactivity of the newt epidermis to peroxidized soyabean oil are summarized in Table 4. The oil numbering is arbitrary and in series A refers to the increasing peroxide value of the samples, while series B represents the same specimens after heat destruction of the peroxides. O oils are untreated, fresh soyabean oils. Positive reactions cover + and (+) responses, while doubtful reactions (±) are not included. Total number of animals represents effective total. Eleven positive reactions to soyabean oils with a peroxide value of 161 or more were seen among 108 newts. With one exception all of these responses fall in experiments 45–47, being made in the autumn, and in experiments 69–70, using hibernating animals from the preceding year's batch, which in both cases means that

TABLE 5

Experiments on a Possible Cocarcinogenic Effect of Oxidated Soyabean Oil.

Experiment No.	47												70																	
Substance	Soyabean oil 0* (P.V. 1.0 C.V. 22.5)§	Soyabean oil 7A (P.V. 4.1 C.V. 165)	Soyabean oil 7A (P.V. 296 C.V. 221)	Benzo[a]pyrene 0.05 % in soyabean oil 0	Benzo[a]pyrene 0.05 % in soyabean oil 7A	Benzo[a]pyrene 0.05 % in soyabean oil 7A	Benzo[a]pyrene 0.1 % in soyabean oil 0	Soyabean oil 0 (P.V. 3.0 C.V. 2.5)	Soyabean oil 9B (P.V. 3.1 C.V. 336)	Soyabean oil 9A (P.V. 400 C.V. 57.2)	Benzo[a]pyrene 0.025 % in soyabean oil 0	Benzo[a]pyrene 0.025 % in soyabean oil 9B	Benzo[a]pyrene 0.025 % in soyabean oil 9A	Benzo[a]pyrene 0.025 % in soyabean oil 0	Benzo[a]pyrene 0.1 % in soyabean oil 0	Soyabean oil 0 (P.V. 3.0 C.V. 2.5)	Soyabean oil 9B (P.V. 3.1 C.V. 336)	Soyabean oil 9A (P.V. 400 C.V. 57.23)	Benzo[a]pyrene 0.025 % in soyabean oil 9A	Benzo[a]pyrene 0.025 % in soyabean oil 0	Benzo[a]pyrene 0.025 % in soyabean oil 9B	Benzo[a]pyrene 0.025 % in soyabean oil 9A	Benzo[a]pyrene 0.5 % in soyabean oil 0
Site of application	Tail									Tail					Tail Pr. T.††		Tail Pr. T.		Tail Pr. T.		Tail Pr. T.		Tail Pr. T.		Tail Pr. T.		Tail Pr. T.		Tail Pr. T.
Number of animals (Triton cristatus)§	6	6	6	6	6	6	6	8	9	8	8	8	4	4	3	3	8	8	8	8	3	3	3	3	3	3	3	3	3	3
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on 3.-4. day								(+)				(+)																		
6. -																														
8. -	+							–	–	–	+																			
12. -	–	–		–	–	(+)	–	(+)	–	+	(+)		–	–			–	–	–	–	–	–	+		+		+		+	
14.-15. -	–	–		(+)	–	(+)	+	–	+	–	–		–	–	(+)		–	–	–	–	+	+			+			+	+	+
18. -	–	–			+	–	+	–	–	–	–		–	–		(+)	–	+	+	–	–	–		+	+	–	+		+	
Animals died during experiment	1 (included)	2 (1 included)	2	0	0	0	0	2 (included)	2 (1 included)	0	1 (included)	1	0	0	0	0	0	0	1 (included)	1 (included)	1		0	0	1	0	0	1	0	0

* Numbering of the oils is the same as in Table 4.
P.V. = peroxide value (m.e./kg). C.V. = Carbonyl value (mg%).
§ Triton marmoratus in exper. no. 47.

†† Unsuited because of heavy skin-infection.
†† Part of tail proximal to secondary tail.

the animals have been in a rather sensitive state (cfr. *Arffmann* 1964). Experiments 49–54, holding only one positive response, were made in the spring on newly received and therefore more resistant animals. This is reflected by the positive controls in experiments 49–50. In the B series the result of experiments 69–70 equals that in the A series. Besides, the positive reactions are few, but it is difficult to explain why 2 out of 5 animals in experiment No. 46 react to an oil with carbonyl value 90 considering the many negative responses at higher carbonyl values.

A possible cocarcinogenic effect of oxidated soyabean oil was examined in three experiments shown in Table 5. The first experiment (No. 47) was indicative of such an effect, but could not be confirmed. Unfortunately an unexpected high reactivity to benzpyrene was seen at a concentration of 0.025 per cent, and signs of promotion could not be demonstrated, when oxidated soyabean oil was used as vehicle instead of the fresh oil.

The first experiment with ethyl linoleate hydroperoxide is not tabulated. Since the preparation of the compound several months earlier the peroxide value had fallen from 3000 to 2000 m.e./kg. The oil was injected undiluted into 11 Triton marmoratus and 1 Triton cristatus. From the 4th day severe toxic lesions with extensive necrosis and deep ulceration were seen in the tails, so that the experiment had to be terminated within 9 days. The lesions were grossly like those seen after tributyrin (*Arffmann* 1962), but microscopically the epithelial reaction was of quite different degree with extensive and strong hyperplasia. Less striking was the slight or moderate infiltrative downgrowth at the edges or in the bottom of the ulceration. The severe inflammatory lesions made it impossible to estimate the epithelial reaction exactly in terms of degree, but the changes strongly indicated further experimentation.

Tables 6 and 7 show the results with ethyl linoleate hydroperoxide of peroxide value 750 and 1500 m.e./kg and in various concentrations, compared to the effect of reduced ethyl linoleate hydroperoxide and pure ethyl linoleate. The degree of toxic symptoms was practically equal

TABLE 6

Experiments with Ethyl Linoleate Hydroperoxide (High Concentrations).

Experiment No.	54				57					55	58									61			
Substance	Soyabean oil	Ethyl linoleate hydroperoxide 750 m.e./kg	Ethyl linoleate hydroperoxide 1500 m.e./kg	Benz-a-anthracene 0.5% in soyabean oil	Soyabean oil	Ethyl linoleate	Dibenz-a,h-anthracene 0.025% in soyabean oil	Peanut oil	Ethyl linoleate hydroperoxide 750 m.e./kg 50% in peanut oil	Ethyl linoleate hydroperoxide 1500 m.e./kg 50% in peanut oil	[illegible] in peanut oil	As_2O_3 in peanut oil (0.5% suspension)	Peanut oil	Ethyl linoleate 50% in peanut oil	Reduc. ethyl linoleate hydroperoxide 1500→70 m.e./kg 50% in peanut oil	Ethyl linoleate hydroperoxide 750 m.e./kg 50% in peanut oil	Ethyl linoleate hydroperoxide 1500 m.e./kg 50% in peanut oil	Dibenz-a,h-anthracene 0.025% in peanut oil	Peanut oil	Ethyl linoleate 25% in peanut oil	Reduc. ethyl linoleate hydroperoxide 1500→70 m.e./kg 25% in peanut oil	Ethyl linoleate hydroperoxide 1500 m.e./kg 25% in peanut oil	Benzo-a-pyrene 0.5% in peanut oil
Site of application	Tail				Tail					Tail	Tail								Tail				Back
Number of animals (Triton cristatus)	6	6	6	6	8	8	8	8	8	8	8	8	8	8	8	8	8	8	4	8	8	8	8
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on																							
3.-7. day		-*	-*																				
8.-9. -	+	-*	-*	-	- -	(+) -	+ +	-	-	-	(+)	(+)	-	-	(+)	- -	-	- +	-	- -	- ±	+ (+)	+ +
12.-13. -	- -	+ +	+ -* + +	+ -	- -	- -	+ +	- -	-* +	- (-)	-	- ±	- -	- -	- -	- -	- +	+ -	-	- -	+ -	- -	- +
14.-15. -	- -	(+) -*		+ -	- -	- -	+ -	- -	- (-)	+ (-)	-	- -	- -	- -	+ -	- -	+ +	+ -	-	- -	- -	- +	+ +
16.-19. -	-			-+	- -	- -	+ -	- -	-	(+) +	-	- -	-	-	(-) -	+ -	-	+ +	-	- -	- -	-	+ (+)
Animals died during experiment	0	1 (included)	0	0	0	1 (included)	0	0	0	0	[illegible]	1 (included)	1	1	0	0	1	0	0	0	0	1	0

* Severe necrosis. † Sections technically bad.

for the two peroxides (Fig. 1) and the reduced form, but decreased with concentration, being absent at 10 per cent. Undiluted ethyl linoleate had negligible toxicity. A few cases (in experiments 54 and 55) could not be classified because of severe necrosis. Ethyl linoleate hydroperoxide P.V. 1500 induced a strong, positive reaction in the newt when undiluted and as a 50 per cent solution in peanut oil (Fig. 2). The response decreased with the concentration and was totally negative at 1 per cent. The reaction to the reduced compound was a little less, but also positive. Ethyl linoleate hydroperoxide P.V. 750 induced a clearly weaker positive response than the P.V. 1500 compound, and pure ethyl linoleate can be estimated as negative.

The toxic lesions being similar to those observed after the injection of tributyrin made it interesting to compare the latter compound with ethyl linoleate hydroperoxide in a few experiments. Toxicity showed to be of the same order. In one experiment (No. 58) a 50 per cent solution (v/v) of tributyrin in peanut oil had the same carcinogen-like effect as ethyl linoleate hydroperoxide P.V. 750, and even at 10 and 1 per cent single positive reactions were seen. This result seems to indicate that tributyrin is not a totally indifferent compound (cfr. *Arffmann* 1962). Peroxide value of the tributyrin was 0 and carbonyl value 1.4.

A completely negative result followed the injection of epoxidized soyabean oil in three experiments (Table 8). The same applies to 16 α, 17 α-epoxyoestratrien-3-ol which was submitted to three separate tests comprising 24 animals with intact tails. Also estradiol appeared newt-negative, while 0.5 per cent benzo(a)pyrene in the positive control experiments provoked epithelial proliferation in 20 out of 24 animals.

In contrast, the testing of a low-molecular diepoxide, diepoxybutane, showed positive reaction to all isomers (Table 9). A preliminary experiment (No. 65) with di-diepoxybutane revealed a moderate irritative

TABLE

Experiments with Ethyl Linoleate Hydroperoxide (Low Concentrations).

Experiment No.	50					56			66									
Substance	Peanut oil	Ethyl linoleate 10% in peanut oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 10% in peanut oil	Reduc. ethyl linoleate hydroperoxide 1500→70 m.e. kg 10% in peanut oil	Dibenz-(a,h)anthracene 0.00035 % in peanut oil	Peanut oil	Tributyrin 1% in peanut oil	Tributyrin 10% in peanut oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 1% in peanut oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 10% in peanut oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 1% in peanut oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 10% in peanut oil	Benzo-a-pyrene 0.5% in peanut oil	Peanut oil	Ethyl linoleate 1% in peanut oil	Reduc. ethyl linoleate hydroperoxide 1500→70 m.e. kg 1% in peanut oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 1% in peanut oil	Benzo-a-pyrene 1% in peanut oil
Site of application	Tail								Tail							Tail		
Number of animals (Triton cristatus)	8	8	8	8	8	8 (Tr. crist. & vulg.)	8	[illegible]	[illegible]	8	8	8	8 (Tr. crist. & vulg.)	4	8	8	8	8
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on																		
5.- 7. day											–							
8.- 9. -	±	– –	– +	– –	– –	±	– –	– –	– –	– –	– –	– –	±	–	– –	– ±	– –	+ +
12.-13. -	±	– –	– +	– +	+ +	±	– –	– –	– –	– –	±	– –	±	–	– –	– –	– –	– +
14.-15. -	–	– –	– –	– –	+ +	– –	+ –	– –	– –	– –	– –	– –	±	–	– –	– ±	– –	+ +
18.-19. -	±	– –	– –	±	+ –	±	– –	+	– –	–	–	– –	± +	–	– –	– –	– –	+ +
Animals died during experiment	0	0	0	0	0	0	0		[illegible]	1	1 (included)	0	0	0	0	0	1 (included)	0

effect at a concentration of 10 per cent, but at 1 and 0.5 per cent such lesions were not seen. In two out of three experiments the positive reaction to the meso-compound was slight, but else there was no significant difference between the various isomers (Fig. 3).

In seven different experiments 0.1 or 0.5 per cent solutions of cholesterol induced no epithelial reaction in newts. After heating of the cholesterol at 200° C for 1 hour, one single positive response occurred among 16 animals, injected with 0.5 per cent solutions. Preheating at 350° C, however, formed a product which in the same concentration provoked epithelial reaction in about half the animals (Fig. 4). Experiment No. 66 confirmed this finding, being made 7 months later. A 0.1 per cent solution of the heated cholesterol had no effect. (Table 10).

DISCUSSION

The present results with peroxides suggest a weak carcinogenicity of such compounds. Most impressing was the reaction to ethyl linoleate hydroperoxide at high peroxide value and in high concentrations, while peroxidized soyabean oil had a weak and inconstant effect. This difference parallels the variation in peroxide value, indicating a causality which is further supported by experiments Nos. 11 and 12, and by the quantitatively different response to the two ethyl linoleate hydroperoxides (P.V. 1500 and 750). The results may, however, have been influenced by the fact that the linoleate peroxide is a low-molecular, more diffusible and therefore immediately active compound, while the peroxy groups in the soyabean oil are connected to larger and less mobile molecules. The severe toxic reaction to ethyl linoleate hydroperoxide is another expression of this circumstance and may have added to the

epithelial response. The carcinogenicity of peroxides is considered weak, because positive reaction demands a very high peroxide value of the oil or the use of sensitive animals.

The positive results obtained with reduced peroxides indicate that the activity is connected with other oxidation products as well, even if peroxy groups seem to be the most active. The carbonyl value is a summarized expression of the secondary oxidation products in the thermo-reduced soyabean oils (B series), while reduction of ethyl linoleate hydroperoxide probably leads to the formation of alcohol groups (*Glavind* 1963). The exact nature and quantities of these various reaction products is not known.

That the observed effect is connected with oxidation of the oils is supported by the other results of the present experiments. Heating of cholesterol is supposed to yield oxidative transformation products (*Fieser* 1954, *Haven* & *Bloor* 1956), and cholesterol heated at 350° C provoked a positive response in newts. Diepoxybutane likewise showed a distinct activity, while the negative result with epoxidized soyabean oil may be explained by the high molecular weight and low mobility of the active groups. The results with 16 α, 17 α-epoxyoestratrien-3-ol may express inactivity of monoepoxides.

Commercially polymerized soyabean oil is heated in vacuum, and the soyabean oil heated at 350° C in the presence of air showed only a mild degree of oxidation, the carbonyl value not exceeding that seen after heating in nitrogen atmosphere. Also *Wurziger* & *Ostertag* (1960) found a moderate rise in carbonyl value on heating of oils in a current of nitrogen. Table 4 shows that with a few exceptions oils in series B with a carbonyl value below 300 gave no positive reactions. Furthermore, the high-molecular-weight of polymerized oils will restrain the effectivity of any oxidized groups. So the negative effect of heat-polymerized soyabean oil is in accordance with the hypothesis that oxidation is the major factor responsible for the carcinogen-like activity of some heated lipids in newts.

Figs. 1–4.

Fig. 1. Large ulcerative defect with necrotic tissue remnants on 12th day after injection of ethyl linoleate hydroperoxide (P.V. 1500), 50 per cent in peanut oil. Strong epithelial hyperplasia is seen at the edge of the ulceration and on the other side of the tail. (10 ×).

Fig. 2. Epithelial hyperplasia and infiltrative downgrowth on 19th day after injection of ethyl linoleate hydroperoxide (P.V. 1500), 50 per cent in peanut oil. (40 ×).

Fig. 3. Strong epithelial hyperplasia and infiltrative downgrowth on 15th day after injection of 1-diepoxybutane, 1 per cent in peanut oil. (40 ×).

Fig. 4. Epithelial hyperplasia and downgrowth on 18th day after injection of heat cholesterol (350° C for 1 hour), 0.5 per cent in peanut oil. (40 ×).

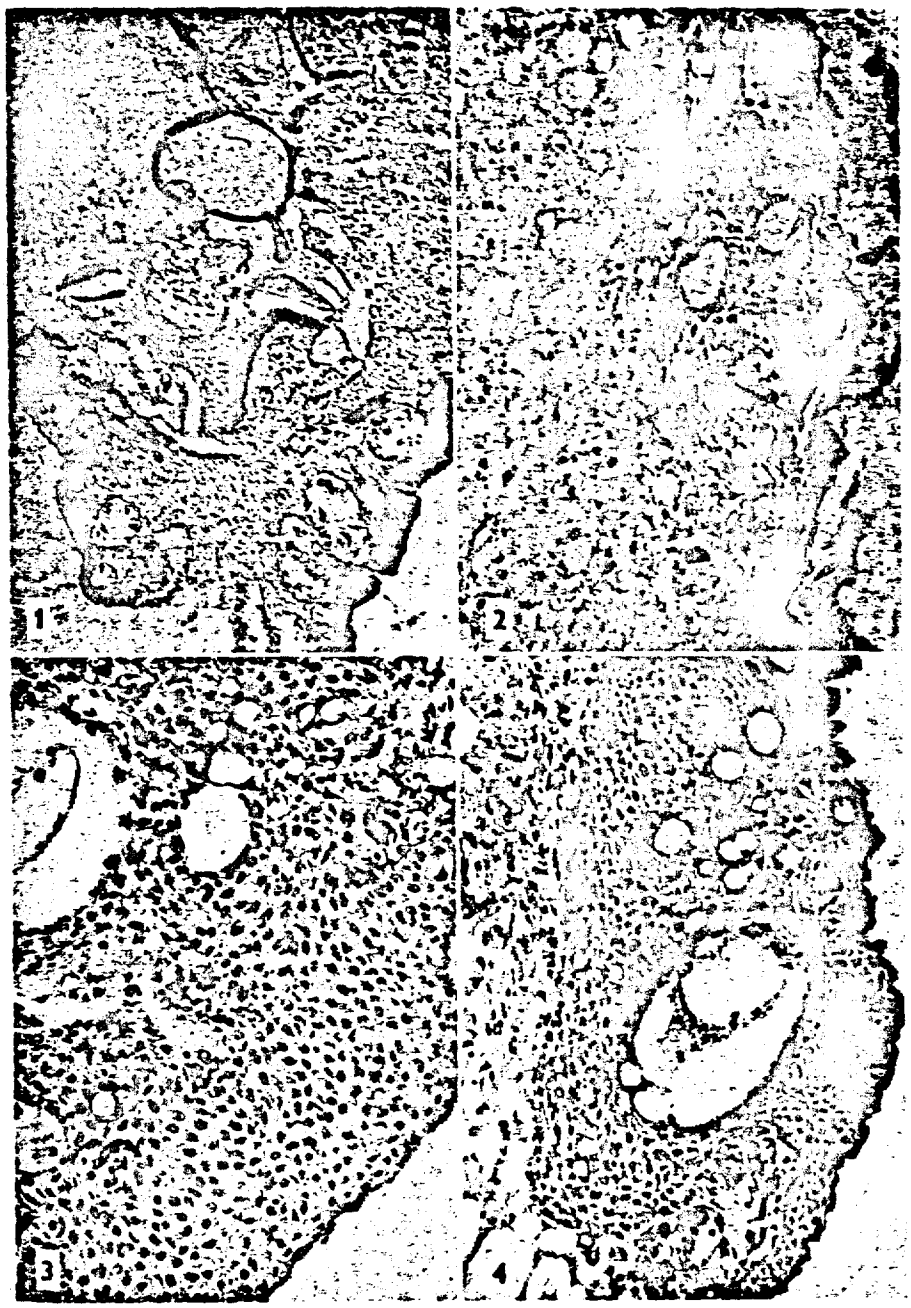

Fig. 1–4.

TABLE 8

Experiments with Epoxidized Soyabean Oil.

Experiment No.	39						40				41						
Substance	Soyabean oil		Epoxy soyabean oil 25 % in soyabean oil		Benz[a]-anthracene 0.1 % in soyabean oil		Soyabean oil		Epoxy soyabean oil 25 % in soyabean oil		Soyabean oil		Epoxy soyabean oil 25 % in soyabean oil				
Site of application	Tail	Back	Tail	Back	Tail	Back	Tail	Back	Tail	Back	Tail	Back	Tail	Back			
Number of animals (Triton cristatus)	4	4	4	4	4	4	4	4	4	4	4	4	4	4			
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on																	
5. day		−				±											
9. -	−		−	−	−			−	−	−	−	−		−			
11.-12. -	±	−	−	−			−	−	−	− −	−	−		−			
15. -	−	−		−	+	−	−	−	−	−	−	−	−	−			
18. -	±		−	−	+	−	−	−	−	−	−	−		−			
Animals died during experiment	0	0	0	0	0	1 (included)	1	0	0	2 (included)	0	0	3	0			

TABLE 9

Experiments with Diepoxybutane.

Experiment No.	65		66					67					68					
Substance	dl-Diepoxybutane 10% in peanut oil	dl-Diepoxybutane 1% in peanut oil	Peanut oil	d-Diepoxybutane 0.5% in peanut oil	l-Diepoxybutane 0.5% in peanut oil	meso-Diepoxybutane 0.5% in peanut oil	Benzo[a]pyrene 0.5% in peanut oil	Peanut oil	d-Diepoxybutane 1% in peanut oil	l-Diepoxybutane 1% in peanut oil	meso-Diepoxybutane 1% in peanut oil	Benzo[a]pyrene 1% in peanut oil	Peanut oil	d-Diepoxybutane 1% in peanut oil	l-Diepoxybutane 1% in peanut oil	dl-Diepoxybutane 1% in peanut oil	meso-Diepoxybutane 1% in peanut oil	Benzo[a]pyrene 1% in peanut oil
Site of application	Part of tail proximal to secondary tail		Part of tail prox. to secondary tail					Part of tail prox. to secondary tail					Part of tail proximal to secondary tail					
Number of animals (Triton cristatus)	6	6	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on																		
6. day																		
9. -	(+)	—	— —	—	—	—	+ +	— —	—	— —	—	+ +	— +	+ —	— —	— +		+
12. -	+	(+)	— —	(+) —	(+)		+ +		(+)(+)	+ +	— +	+ —	— —	—	—	(+) —	— —	+ +
15. -	(+) —	— (+)	— —	+	+ +	— (+)	+ —	— —	(+) —	± —	(+)(+)	+ +	—	(+) —	±	+ +	— —	+ +
18.-19. -	—	+	— —	(+) —	+	—	+ +	— +	+	— (+)	+ —	+ +	— —	(+)(+)	— +	— +	— —	— +
Animals died during experiment	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3 (1 included)	1 (included)

Experiments with Chol[illegible] and Heated Cholesterol.

Experiment No.	38		41			57					58			59					60					61			
Substance	Peanut oil	Cholesterol 0.5% in peanut oil	Soyabean oil	Cholesterol 0.5% in soyabean oil	Dibenz-a,h anthracene 0.1% in soyabean oil	Soyabean oil	Cholesterol 0.1% in soyabean oil	Cholesterol heated at 350° C for 1 hr 0.1% in soyabean oil	Dibenz-a,h anthracene 0.025% in soyabean oil	Peanut oil	Cholesterol 0.5% in peanut oil	Cholesterol heated at 350° C for 1 hr 0.5% in peanut oil	Dibenz-a,h anthracene 0.05% in peanut oil	Peanut oil	Cholesterol 0.5% in peanut oil	Cholesterol heated at 350° C for 1 hr 0.5% in peanut oil	Dibenz-a,h anthracene 0.025% in peanut oil	Peanut oil	Cholesterol heated at 350° C for 1 hr 0.5% in peanut oil	Benzo-a-pyrene 0.5% in peanut oil	Peanut oil	Cholesterol 0.5% in peanut oil	Cholesterol heated at 300° C for 1 hr 0.5% in peanut oil	Benzo-a-pyrene 1% in peanut oil	Peanut oil	Cholesterol 0.5% in peanut oil	Cholesterol heated at 300° C for 1 hr 0.5% in peanut oil	Benzo-a-pyrene 0.5% in peanut oil
Site of application	Tail		Part of tail proximal to secondary tail			Tail					Tail			Tail					Part of tail proximal to secondary tail			Tail					Tail		Back
Number of animals (Triton cristatus)	6	6	6	6	6	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on																												
9. day							–		–										–			–				–	–	–
12. "	–	–	–			–		–															–			–	–	–
15. "	–	–		–		–	–	–	–			+	–	–	–	–	–			–	–	–	–			–	–	–
18. "	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Animals died during experiment	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0

* Minimal signs of the injected oil.

SUMMARY

Some heated and oxidated fats and a few allied compounds were tested in newts. Positive results of varying degree were obtained with peroxidized soyabean oil and ethyl linoleate hydroperoxide, but the carcinogen-like activity of the peroxides is considered rather weak, when the response is compared with the magnitude of peroxide values and the sensitivity of the animals. Reduction of the peroxides through heating or by chemical means weakened the activity only slightly. Epoxidized soyabean oil and 16 α, 17 α-epoxyoestratrien-3-ol induced no epithelial reaction, while the testing of diepoxybutane proved positive for all isomers. Heating of cholesterol at 350° C for 1 hour gave a newt-positive product, while untreated cholesterol and moderately heated cholesterol (200° C for 1 hour) were negative. Commercially polymerized soyabean oil and soyabean oil heated at 350° C for ½ or 1 hour had no effect on the skin of newts. It is concluded that oxidation may be considered the most important process in the transformation of lipids leading to newt-positive and therefore carcinogen-suspected products.

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Absorption of Monoglycerides of the Higher Fatty Acids

Argyris, A. & O. Frank (Physiological Institute, Munich). Zeitschrift fur Biologie 59(41), ff. 145-164, 1912.

The problem whether fats are capable of passing the intestine epithelia during resorption remains undecided (1). It may be solved by administering substances with physicochemical properties as similar as possible to those of the triglycerides and by determining whether they appear in the chyle unchanged. Properties which primarly determine the similarity are emulsion formation and a melting point lower than the temperature of the body. These points had been emphasized by one of the authors (2) in an earlier paper. It had been shown that esters satisfying these conditions are not absorbed in the unchanged state, but are decomposed and passed into the chyle following synthesis with glycerine. Palmitic acid ethylester proved to be especially suitable for this determination, because its presence in the chyle can be detected with more assurance. Also essential for these earlier experimental results was the fact that these substances are resorbed in volumes similar to the triglycerides.

Subsequent to these investigations, attention was called to the fact that the feeding of mono- or diglycerides would probably lead to even more conclusive results, because they are even closer related to the triglycerides (they are not "organically different"). In the present investigation the authors selected the monoglycerides, because they can be detected in the chyle with more assurance than the diglycerides and they permit more definite conclusions concerning the occurrence of decomposition. In the investigation by Iwano (3), a number of the technical and analytical difficulties have already been overcome. As emphasized in the paper itself, this is their real significance. The results of the present study could therefore be derived from a relatively limited number of experiments in a satisfactory manner.

Five experiments are reported here. In the first, monoolein was given, in the second, triolein as a comparison was the first, in two others the monoglycerides of a mixture of fatty acids made of lard were administered and in the last, lard itself was given to compare it with the two preceding experiments.

Analysis included the determination of the ether extract, glycerine, the iodine number, the saponification number and the acid number.

1) The history of the problem will not be discussed here.

2) Frank, O. "The Study of the Resorption of Fats", Paper 3, Zeitschrift f. Biol. Vol. 36, p. 568.

3) Proceedings of the Society for Morphology and Physiology, Munchen, 1911

Feeding and Operation

The fat was fed to dogs following a fasting period of 2 to 3 days. In contrast to Iwano's experiments, the animals never vomitted after feeding with a bougie, not even in the unreported experiments. There is no doubt concerning the fact that vomitting in the earlier experiments was caused by contamination of the preparates. For the technique by which potential vomitting can be prevented and for the operation of the ductus thoracicus, see Iwano's paper (p. 37). The animals were always poisoned with a dose of curare which was just adequate.

A small metal tube was inserted in the ductus thoracicus at the throat together with a rinsing insert, such as previously described by O. Frank. Concentrated $MgSO_4$ solutions were used in each case for rinsing, always preventing coagulation.

Preparation of Administered Substances

a) Mono-olein

The monoglycerides were prepared in principle by the method of Guth (1). Commercial sodium oleate was finely powderized and shaken with cold alcohol. Following standing for two days in the alcohol, the mixture was filtered, washed with alcohol, dried and treated in the same manner with ether. Following the filtration and powdering, the material was dried in flowing CO_2 at 100°. The soap pretreated in this manner reacted neutrally.

After the attainment of constant weight, 50 g soap was sealed in a sealing tube filled with dry CO_2, together with 18 g monochlorhydrin. The amounts used corresponded to the molecular weight. However, the amount of sodium oleate somewhat exceeded a molecule. The substances were mixed by vigorous shaking. The tubes were then heated in a paraffin bath to between 140 and 150° with frequent shaking. There was practically no pressure noticeable upon opening the tubes.

The reaction product was poured in a separation filter and shaken with absolute ether (the latter was purified by shaking commercial ether three times with water, drying the washed ether over $CaCl_2$ and subsequent distillation).

The ether solution of the monoolein was filtered from the excess soap and cooled with a freezing mixture. Most of the soap taken up by the ether was thus precipitated. The cooling process was repeated following the concentration of the ethereal solution. The filtered solution, following treatment with animal charcoal, was shaken with a 10% solution of Na_2CO_3 and the separated solution filtered through a dry filter; the ether was then distilled by a stream of dry CO_2.

1) Zeitschr. f. Biol. Vol. 44, p. 90

Following the removal of the last traces of ether in a room under reduced pressure, a viscous yellow oil was obtained which closely resembled triolein in taste and odor. Combustion of the monoolein indicated by the appearance of a slightly alkaline reaction of the ash dissolved in water that the monoolein contained traces of soap. The substance was therefore dissolved in petroleum ether with a low boiling point (to 40°), filtered, and the petroleum ether distilled in a stream of CO_2. However, it was not possible to keep the preparation entirely free of soap.

Subsequently, the monoolein was heated to 100° in a flow of dry CO_2 and dried over H_2SO_4 in an evacuated exsiccator until constant weight was obtained. Following standing for several days, it solidified to a light yellow, semifluid mass, which proved to be crystalline upon microscopic examination, and consisted of fine needles forming bundles and concentrically arranged rosettes.

Part of the monoolein was used to determine the glycerine content, the saponification number, the iodine number and the acid number.

b) Monoglyceride Fat from the Fatty Acids of Lard

500 g lard was dissolved through heating in flasks in 500 ccm ethylalcohol of 90% per volume and impurities were filtered off. Approx. 150 g caustic potash was dissolved by heating in a dish in 100 ccm water, poured into a flask and rinsed with 500 ccm 90% per vol. of alcohol. Both solutions were heated to incipient boiling, combined, carefully shaken and reheated to boiling temperature.

The resulting alcoholic solution of the soaps was poured in a thin stream into water containing 20% H_2SO_4. The fatty acids precipitated were immediately removed. Only in this manner is it possible to obtain fatty acids free of soaps from large amounts of soap.

The fatty acids obtained in this fashion were washed with water until the water which was siphoned off no longer yielded H_2SO_4 reactions, then liquified in boiling water and allowed to solidify in the form of cakes.

The fatty acids pressed between filter papers were dissolved in alcohol. The volume of the solution was measured accurately and exactly neutralized with sodium ethylate, obtained by the action of 50 g metallic sodium on 1 liter alcohol, by first accurately titrating an aliquot of the alcoholic fatty acid solution with the sodium ethylate solution and then calculating the corresponding amount of sodium ethylate for the entire volume of the fatty acid solution. The necessary amount of sodium ethylate was gradually poured hot into the warm fatty acid solution with frequent shaking, and the mixture reheated to incipient boiling.

The alcoholic soap solution was poured into large dishes and allowed to cool. In order to prevent the formation of large, compact masses of soap including much alcohol, the mixture was agitated during cooling.

Following the decanting of the alcohol, the sodium soaps were dried at 100o in a stream of dry CO_2 powdered, washed with cold alcohol and shaken with ether. The residue was dried at 100o in a stream of CO_2 to a constant weight. The soap prepared in this manner had a neutral reaction.

Subsequently, the soaps and monochlorhydrin were heated for 5-6 hours at 145-150° in sealed tubes, as in the case of monoolein (see above). The procedure followed after this was identical with the method used for monoolein. Very high pressures were noted upon the opening of the tubes (volatile fatty acid?).

Analytical Methods

The method used for the extraction of fats from the chyle differed substantially from Iwano's procedure. Preliminary extraction with alcohol was avoided, because bodies which pass into the ether extract and cannot be removed render determinations difficult, particularly that of glycerine. The procedure followed consisted of drying the chyle mixed with the magnesium sulfate solution in a rapid drying cabinet by vigorously ventillated air at about 40°, in approx. 6-8 hours. The absence of lypolitic ferments in the chyle, which could have caused decomposition at this temperature, was ascertained by preliminary experiments. Due especially to the presence of magnesium sulfate, the dry chyle was readily reduced to powder form. Subsequently, the powder was extracted with absolute ether for three days in a Soxleth apparatus. The ether was distilled in a stream of CO_2 and the residue taken up with petroleum ether. The solution was clear as water. Following evaporation of the ether, both portions leave a residue which is light yellow, liquid and thin at ordinary temperatures. In order to remove the last traces of the petroleum ether, the material was repeatedly heated on a water bath in a stream of CO_2 and dried in an evacuated exsiccator to a constant weight in H_2SO_4. Of the analytical methods used to determine the important characteristics of the extract, only the determination of glycerine will be discussed in detail, because descriptions of the determination of the iodine number, the saponification number and the acid number in the form of simpler and better known methods can be found in Benedikt-Ulzer, 5th Edition, pp. 149, 129 and 126.

The glycerine determination was performed by the method of Benedikt-Zsigmondy as modified by Mangold (Benedikt-Ulzer, p. 196, 198). Obviously, the procedure can be used only when no substances which form oxalic acid with permangante during the oxidation are present. Presumably, the erroneous results obtained in the investigation of the alcohol-ether extract were due to this condition.

Two-third g of fat were saponified with potassium hydrate and very pure methylalcohol, the alcohol removed through evaporation, the residue dissolved in hot water and the soap dissociated with diluted HCl. The mixture was heated until the fatty acids were clearly separated. With liquid fats, it is convenient to add a small

amount of hard paraffin, in order to induce solidification of the fatty acids floating on the surface during subsequent cooling, which is assisted by placing the dish in cold water. The substance is filtered in a large flask and the dish thoroughly washed.

The subsequent procedure follows Mangold's method: the filtrate from the fatty acids which contains 0.2-0.4 g glycerine is diluted in a liter flask with approx. 300 ccm water and 10 g of potassium hydrate and enough of a 5% potassium permanganate solution added cold, with shaking, to correspond to 1 1/2 of the theoretical amount (thus, 1 part glycerine to 6.87 part potassium permanganate). Following standing for 1/2 hour at room temperature, hydrogen peroxide is added while avoiding large excesses, until the liquid above the precipitate becomes colorless. The flask is filled to the mark, thoroughly shaken, and 500 ccm of the liquid filtered through a dry filter. The filtrate is heated in a boiling flask for 1/2 hour to destroy all of the hydrogen peroxide, cooled to approx. 60° and titrated with chameleon, after sulfuric acid has been added. The maintenance for 5 minutes of the red color of the chameleon solution during titration with the latter was taken as an indication of the end of the reaction. In all determinations of glycerine, both in the monoglycerides prepared and in the chyle extracts of dogs obtained by feeding the glycerides, approximately equal amounts of the substance was analyzed, in order to receive comparable data. It was noted in the saponification preceding the glycerine determination that the soaps did not always dissolve clearly in water so that traces of nonsaponificable substances could be detected.

Experimental Record

Experiment 1. Feeding of Mono-olein:

a) Analysis of the mono-olein administered.

Subst. used:	0.7492 - N/10 KMnO4 consumed 38 cm3 = glycerine % = 23.3			average
Subst. used:	0.7332 - N/10 KMnO4 consumed 37 cm3 = glycerine % = 23.2			23.3
Subst. used:	1.5820 - N/10 KMnO4 consumed 8.90 = N/2 HCl			

Saponification number = (8.90.30.1)/1.5820 = 169.3

Subst. used:	1.6410 consumed	7.80 cm3 = sapon. No.	166.2	average
Subst. used:	0.8150 consumed N/10 KOH	1.50 = acid No.	10.3	167.8

Deduction of the acid number from the average of the saponification number yields the ester number 157.5.

Subst. used:	0.4410 consumed Iodine 24.5 cm3 = iodine No.	70.5	average	
Subst. used:	0.4780 consumed Iodine 26.4 cm3 = iodine No.	72.2	71.4	

b) Animal Experiment

The material prepared from the composition described above was used for feeding purposes.

A dog weighing 23 kg was fed the monoolein at 8 A.M., following a fast of 48 hours. Since the dog was apprehensive and refused the material, it was administered with a probe. It ate the rest after it had been wiped from the dish with a roll. The operation to expose the ductus thoracicus was performed two hours later. The animal was injected intravenously with a 1% curare solution. The collection of the chyle was begun at 10:55 A.M. A double tube was used to provide a simultaneous flow of a 25% $MgSO_4$ solution from a graduated burette. The admixture of the $MgSO_4$ solution prevented any coagulation and also greatly facilitated the drying and grinding of the chyle. The amount of the outflowing chyle was measured at 15 minute intervals for the first 3 hours and later at 1/2 hour intervals, as shown in the following table.

Zeit	Menge cm³	Stündlich cm³
10 h 55′ — 11 h 15′	26,1	—
11 h 30′	13,6	
11 h 45′	7,5	—
12 h	7,5	
12 h 15′	8,8	38
12 h 30′	8,8	—
12 h 45′	9,5	—
1 h	9,5	—
1 h 15′	11,6	[illegible]
1 h 30′	12,5	
1 h 45′	11,2	
2 h	8,1	—
2 h 15′	14,[illegible]	[illegible]
I. 10 h 55′ — 2 h 15′	14[illegible]	[illegible]
2 h 15′ — 2 h 30′	15,[illegible]	
3 h	4,[illegible]	[illegible]
3 h 30′	23,[illegible]	—
4 h	28,[illegible]	[illegible]
4 h 35′	18,9	—
5 h	27,[illegible]	[illegible]
5 h [illegible]′	17,[illegible]	
6 h	15,7	[illegible]
6 h 30′	20,0	
7 h	11[illegible]	[illegible]
7 h 30′	15[illegible]	
8 h	12,7	[illegible]
II. 2 h 15′ — 8 h	211	27

Key: 1. Time, 2. Amount, cm3, 3. Hourly, cm3

The flow of the $MgSO_4$ solution was 33 cm3 during the first, and 13 cm3 during the second period.

The obduction showed the chyle vessels of the entire small intestine to be strongly injected. The chyle was high in fat from the beginning, i.e., 3 hours after the feeding it had a milky white appearance, but reached its maximum fat content at 1:45 hours, i.e. toward the end of the 6th hour after the feeding. It remained at this level during the following hours, became more transparent toward the end, but retained its white appearance. The chyle was divided in two portions: the part collected from 1:55 to 2:15 hours, i.e. to the 6th hour after feeding, and part of the last 5:45 hours. The volume of the first portion amounted to 149 cm3 pure chyle, that of the second to 211 cm3 pure chyle.

After constant weight had been attained, the extract of the first portion weighed 2.993 = 2.01% and the extract of the second 9.093 g = 4.31%. Both extracts had an oily odor and did not crystallize after prolonged standing.

Analysis of the Chyle Fat of Portion 1:

Subst. used:	0.6872	consumed $KMnO_4$	18 cm3		= glycerine	12.1	
Subst. used:	0.6657	consumed $KMnO_4$	4.55	(KOH)	= sapon. no.	205.7	
Subst. used:	0.4992	consumed N/10 KOH	0.6		= acid no.	6.8	
Subst. used:	0.2870	consumed N/10 KOH	19.10	Iodine	= iodine no.	84.5	av
Subst. used:	0.4175	consumed N/10 KOH	27.30	Iodine	= iodine no.	83.1	83

Analysis of Portion 2

Subst. used:	0.6872	consumed $KMnO_4$	18 cm3		= glycerine	12.1
Subst. used:	0.6657	consumed $KMnO_4$	4.55	(KOH)	= sapon. no.	205.7
Subst. used:	0.4992	consumed N/10 KOH	0.6		= acid no.	6.8
Subst. used:	0.2870	consumed N/10 KOH	19.10	Iodine	= iodine no.	84.5

In the saponification of the two extracts and the decomposition of the soap by HCl, the fatty acids obtained were of semiliquid consistency at ordinary room temperature.

Experiment 2. Feeding of Tri-Olein

a) Analysis of Tri-Olein

The feeding of monoolein was followed by the administration of triolein. Commercial triolein (Kahlbaum) was brought to a constant weight and analyzed.

Subst. used:	0.6908 consumed N/10 KMnO4	17.4 cm3	=	glycerine %	11.6	avg
Subst. used:	0.6108 consumed N/10 KMnO4	15.8 cm3	=	glycerine %	11.9	11.
Subst. used:	0.3623 consumed alcoh. KOH	2.45	=	sapon. no.	203.5	
Subst. used:	0.3723 consumed N/10 KOH	0.2	=	acid no.	3.0	
Subst. used:	0.3205 consumed iodine	22.75	=	iodine no.	90.1	

b) Animal Experiment

A dog weighing 22 kg was fed 44 g triolein at 8 A.M. It had been fasting for 48 hours. Following the injection of 15 cm3 curare, the chyle was collected at 11:05.

	Zeit	Menge cem	Stündlich cem (3)
	11 h 5′ — 11 h 15′	32	—
	11 h 30′	28,8	—
	11 h 45′	29,1	—
	12 h	19,0	119
	12 h 15′	20,6	—
	12 h 30′	17,7	—
	12 h 45′	18,7	—
	1 h	17,1	74
	1 h 15′	3,2	—
	1 h 30′	17,1	—
	1 h 45′	16,4	—
	2 h	17,1	50
I.	11 h 5′ — 2 h	243	83
	2 h — 2 h 15′	15,7	—
	2 h 30′	14,3	—
	2 h 45′	17,5	—
	3 h	17,9	67
	3 h 15′	12,4	—
	4 h	37,8	59
	4 h 45′	15,6	—
	5 h	[illegible]	24
	5 h 30′	13,8	[illegible]
II.	2 h — 5 h 30′	1[illegible],2	[illegible]

Key: 1. Time, 2. Amount, 3. Hourly

The flow of the MgSO4 solution was 52 cm3 to Portion I and 26 cm3 to Portion II.

At the obduction, the chyle vessels of certain parts in the center of the small intestine were still injected, while those located in the upper and lower part of the small intestine were empty. Here again, the chyle was milky white from the beginning, i.e. 3 hours after the feeding, but became low in fat in the 6th hour after feeding

and thinly serous in the 8th hour. It was divided into two portions Portion I: 243 cm3 of pure chyle collected to the 6th hour after feeding, Portion II: 153 cm3, collected from the 6th hour after feeding to 9 1/2 hours. Both portions were dried and extracted in the same manner. Portion I yielded an extract of 2.1098 g = 0.87% and Portion II 0.6057 g = 1.38%. Both were light yellow in color, thinly viscous at ordinary temperatures and no crystallization occurred during extended standing periods. Both extracts had the odor of the triolein preparate.

Analysis of the chyle fat yielded the following data:

Subst. used:	0.6202 consumed KMnO	16.0 cm3 = glycerine %	11.9
Subst. used:	0.3417 consumed alcoh. KOH solution	2.3 cm3 = sapon.	202.2
Subst. used:	0.3101 consumed N/10 KOH	0.3 cm3 = acid no.	5.1
Subst. used:	0.1845 consumed N/10 KOH	12.45 cm3 = iodine no.	85.7

In the determination of glycerine, the preceding saponification indicated that the resultant soaps were completely and clearly soluble in water and that the fatty acid floating on the liquid following the decomposition of the soaps was entirely liquid at ordinary temperatures.

Experiment 3. Feeding the Monoglycerides of Fatty Acids from Lard

a) Analysis of the Monoglyceride used

For the analysis of the monoglyceride fat, which formed a light yellow mass, solid at ordinary temperatures and crystallizing during slow evaporation of the petroleum ether in needles larger than those of monoolein with the same arrangement in bundles and rosettes, a small portion was heated on a water bath in a stream of dry CO2 and dried to a constant weight in an evacuated exsiccator over H2SO4.

Analysis of the Monoglyceride Fat Administered:

Subst. used:	0.6527 consumed $KMnO_4$	28.8 cm3 = glycerine %	20.3	avg.
Subst. used:	0.6353 consumed $KMnO_4$	27.8 cm3 = glycerine %	20.1	20.2
Subst. used:	0.3885 consumed alcoh. KOH	2.15 cm3 = sapon. no.	166.6	avg.
Subst. used:	0.2235 consumed alcoh. KOH	1.95 cm3 = sapon. no.	165.1	165.8
Subst. used:	0.2680 consumed N/10 KOH	0.2 cm3 = acid no.	5.0	avg.
Subst. used:	0.2680 consumed N/10 KOH	0.25 cm3 = acid no.	5.2	5.1

By deducting the acid number from the saponification number = ester number = 160.7.

Subst. used:	0.1080 consumed iodine	3.3 cm3 = iodine no.	38.8	avg.
Subst. used:	0.2250 consumed iodine	6.9 cm3 = iodine no.	38.9	38.9

b) Animal Experiment

Of the preparation with the above composition, a dog weighing 23 kg was given 125 g with 1 g of a meat extract and 100 cm3 water; the dog had been fasting for 48 hours. A bougie was used; the dog ate the rest, which was wiped from the dish with a roll. No vomiting occurred. Three hours following the feeding, collection of the chyle began. The animal was pretreated with an intravenous injection of 16 cm3 1% curare solution. The chyle was high in fat from the start, had the appearance of milk, however, it attained its maximum fat content, as with triglyceride feeding, from the 5th to the 7th hour. The chyle was collected for 9 1/2 hours. Its volume amounted to 320 cm3 pure chyle, as seen in the following tables:

Zeit	Menge ccm	Stündlich ccm
11 h 15′ — 11 h 30′	19,0	—
11 h 45′	15,9	—
12 h	11,9	—
12 h 15′	12,4	59,2
12 h 30′	11,5	—
12 h 45′	10,1	—
1 h	10,3	—
1 h 15′	11,6	43,5
1 h 30′	11,0	—
1 h 45′	10,1	—
2 h	8,0	—
2 h 15′	10,8	39,9
2 h 30′	10,6	—
2 h 45′	9,7	—
3 h 5′	11,3	—
3 h 20′	8,9	37,4
1. 11 h 15′ — 3 h 20′	183	45

Key: 1. Time, 2. Volume, cm3, 3. Hourly, cm3

Zeit	Menge ccm	Stündlich ccm
3 h 20′ — 3 h 35′	8,1	—
3 h 50′	9,6	—
4 h 5′	7,6	33,7
4 h [illegible]	11,8	—
4 h 35′	8,3	—
4 h 50′	5,4	—
5 h 5′	9,6	35,1
5 h 20′	6,9	—
5 h 35′	6,4	—
6 h	8,6	23,9
6 h 30′	12,2	—
7 h 30′	19,5	—
8 h	9,2	20,5
8 h 30	11,0	—
8 h 45′	4,6	20,8
Σ 3 h 20′ — 8 h 45′	138,8	25,6

Key: 1. Time, 2. Volume, cm3, 3. Hourly, cm3

In the first period 38 cm3, in the second 13 cm3 $MgSO_4$ were added to the chyle.

The chyle was divided into two portions: the chyle of the first 4 hours (corresponding to the 7th hour after feeding) and the chyle of the remaining time (from the 7th to the 12th hour after feeding). The chyle vessels presented the appearance of milky white, full lines in the subsequent obduction.

Both parts of the chyle obtained were dried in the manner described above, the dry residue powdered, divided into two portions and extracted in a Soxleth apparatus with absolute ether for two days. The extract of both portions was treated in a manner similar to the extracts from the monoolein and triolein feedings. Both extracts were semisolid at ordinary temperatures, of buttery consistency and of a lower melting point than the extract from fats fed to the animals. During slow evaporation of petroleum ether, the ether extracts of the first portion formed a light yellow residue, while the residue of the second portion was nearly white.

Microscopic investigation showed both residues to consist of crystals in the form of needles, which formed both bundles and rosettes through concentric arrangement of the needles.

The total amount of the extract after heating on a water bath in a CO2 stream and drying in an evacuated desiccator over H2SO4 to a constant weight = 4.1790 g = 2.3% for Portion I and 2.677 g = 1.9% of Portion II.

Analysis of Portion I

Subst. used:	0.6103 consumed $KMnO_4$	15.2	cm3 =	glycerine %	11.5
Subst. used:	0.3680 consumed alcoh. KOH	2.4	cm3 =	sapon. no.	196.4
Subst. used:	0.2620 consumed N/10 KOH	0.4	cm3 =	acid no.	8.6
Subst. used:	0.2170 consumed iodine	8.30	cm3 =	iodine no.	48.3
Subst. used:	0.2295 consumed iodine	8.85	cm3 =	iodine no.	48.9

Analysis of Portion II

Subst. used:	06103 consumed $KMnO_4$	15.2	cm3 =	glycerine %	11.5
Subst. used:	0.3680 consumed alcoh. KOH	2.4	cm3 =	sapon. no.	196.4
Subst. used:	0.2620 consumed N/10 KOH	0.4	cm3 =	acid no.	8.6
Subst. used:	0.2170 consumed iodine	8.30	cm3 =	iodine no.	48.3

Experiment 4. The Feeding of Monoglycerides of the Fatty Acids of Lard

A dog weighing 16 kg was fed 125 g monoglyceride fat for 9 1/2 hours. Three hours later the chyle was collected. Twelve cm3 of curare was injected. The 260 cm3 of chyle collected was divided into 3 parts. I. The first 5 hours following the feeding; II. the following 2 3/4 hours; and III. the last 3 hours. The macroscopic and microscopic appearance of the fat obtained was similar to those of the preceding experiments. Here, Portion I was nearly white, while II and III were light yellow (judged by appearance, Portion II was higher in fat than I. Portion I was lost due to an accident and yielded only 0.5438 g of the extract; Portion II 2.1860 g = 7.8% and III. 4.3%. The progress of chyle collection is shown in the following table:

Zeit	Menge ccm	Stündlich ccm
12 h — 12 h 15′	12,4	—
12 h 30′	13,1	—
12 h 45′	15,6	—
1 h	8,9	—
1 h 15′	6,9	—
1 h 30′	3,5	—
1 h 45′	5,1	—
2 h	6,6	—
2 h 15′	5,8	—
I 12 h — 2 h 15′	77,6	35
2 h 15′ — 2 h 30′	8,3	—
2 h 45′	8,9	—
3 h	6,5	—
3 h 15′	4,7	—
3 h 30′	4,7	—
3 h 45′	5,8	—
4 h	7,8	—
4 h 15′	8,8	—
4 h 35′	6,8	—
4 h 50′	14,9	—
5 h 5′	9.1	—
II 2 h 15′ — 5 h 5′	86,3	28
5 h 5′ — 5 h 15′	6,1	—
5 h 45′	9,6	—
6 h 15′	9,3	—
6 h 45′	10,1	—
7 h 15′	19,9	—
7 h 45′	20,0	—
8 h 5′	7,2	—
III 5 h 5′ — 8 h 5′	82,2	27

Key: 1. Time, 2. Amount, 3. Hourly

During the first period 9 cm3, during the second 7, and during the final period 9 cm3 of the $MgSO_4$ solution were added to the chyle.

Only the fat extracted from chyle portions II and III were analyzed, because Portion I was insufficient for analysis.

Analysis of Portion II

Analysis of Portion III

Analyse der II. Portion:

Angew. Subst.	0,6045	verbr.	$KMnO_4$	15,2 ccm	=	Glyz. %	11,6
» »	0,3535	»	alkohol. KOH	2,4 »	=	Versz.	204,4
» »	0,2495	»	N/10 KOH	0,4 »	=	Säurez.	7,9
» »	0,2261	»	Jodl.	8,85 »	=	Jodz.	49,7

Analyse der III. Portion:

Angew. Subst.	0,4293	verbr.	$KMnO_4$	11,6 ccm	=	Glyz. %	12,4
» »	0,2087	»	alkohol. KOH	0,33 »	=	Säurez.	10,7
» »	0,1618	»	Jodl.	6,2 »	=	Jodz.	48,6

Key: 1. Substance used, 2. consumed, 3. glycerine, 4. alcoholic KOH, 5. saponification number, 6. acid number, 7. iodine number

Experiment 5. Feeding of Lard

a) Analysis of Lard.

As a control of the analysis values of the chyle extract following the feeding of monoglyceride fat, ordinary lard was given to a dog. Part of it was dissolved in petroleum ether, filtered, dried to a constant weight and analyzed. In order to obtain comparable values amounts identical with those used with monoglyceride fat and the corresponding chyle fat, however, in such cases the glycerine content will be somewhat higher, as shown by repeated experiments.

(1)		(2)				(3)		
Angew.	Subst.	0,6786	verbr.	$KMnO_4$	17,4 ccm	=	Glyz. % 11,8	11,8
»	»	0,7307	»	»	18,6 »	=	» % 11,7	
»	»	0,3090	»	alkohol. KOH	2,15 »	=	Versz. 209,4	
»	»	0,4150	»	N/10 KOH	0,15 »	=	Säurez. 2,0	
»	»	0,2750	»	Jodl.	11,05 »	=	Jodz. 51,0	51,3
»	»	0,3260	»	»	17,25 »	=	» 51,6	

(4)

Key: 1. Substance used, 2. consumed, 3. glycerine, 4. saponification number, 5. acid number, 6. iodine number

b) Animal Experiment

A dog, weighing 23 kg, was fed 200 g lard, 1 g meat extract and 100 cm3 water at 7:45 A.M. following a fast of 2 days. Three hours later the chyle was collected, as shown in the following table:

Zeit	Menge ccm	Stündlich ccm
11 h 5′ — 11 h 20′	25,3	—
11 h 5[illegible]′	33,1	—
12 h	10,0	—
12 h 15′	12,4	—
12 h 30′	9,2	—
12 h 45′	8,0	—
1 h	9,9	—
1 h 15′	7,4	—
1 h 30′	8,1	—
1 h 45′	7,4	—
2 h	6,3	—
2 h 15′	7,1	—
2 h 30′	6,9	—
2 h 45′	6,8	—
3 h 5′	9,3	—
I. 11 h 5′ — 3 h 5′	167,2	42
3 h 5′ — 3 h 30′	6,1	—
4 h	2,9	—
4 h 30′	2,4	—
4 h 45′	2,6	—
5 h	1,0	—
5 h 30′	2,0	—
II. 3 h 5′ — 5 h 30′	17,0	7

Key: 1. Time, 2. Volume, 3. Hourly

During the first period 41 cm3, during the second period cm3 of the $MgSO_4$ solution were added to the chyle.

The amount of the chyle was 184 cm3, it was divided into two parts: I: the chyle collected in the first 8 3/4 hours after feeding and II: the chyle collected in the last 2 1/2 hours. The total ether extract of Portion I after attaining constant weight amounted to 2. 71 = 1.6% and of Portion II 1.112 = 6.5%. Both extracts were solid at ordinary temperatures, but of soft consistency and weakly yellow in color.

Analysis of Portions I and II

Analyse der I. und II. Portion:

Subst.	0.6173	verbr.	$KMnO_4$	16,8 ccm	= Glyz. %	12,5
	0.3670	»	alkohol. KOH	2,5 »	= Verse.	203,0
	0.2801	»	N/10 KOH	0,4 »	= Säurez.	8,0
	0.2763	»	Jodl.	10,95 »	= Jodz.	52,0

Key: 1. Substance used; 2. consumed; 3 Glycerine, 4. Saponification number; 5. acid number, 6. Iodine number

Analysis of Portion III

Analyse der III. Portion:

Angew. Subst.	0,5827	verbr.	$KMnO_4$	15,6 ccm	= Glyz. %	12,6
» »	0,2816	»	alkohol. KOH	1,9 »	= Verse.	203,8
» »	0,1497	»	Jodl.	5,65 »	= Jodz.	51,3

Key: 1. Substance used; 2. consumed; 3. Glycerine, 4. Saponification number; 5. Acid number, 6. Iodine number

RESULTS

Review of Experimental Results

Verfüttert	Glyzerin	Verseifungszahl	Säurezahl	Jodzahl	
I. Mono-Olein					
bestimmt*) . .	23,3	167,8	10,3	157,[illegible]	[illegible]
theoretisch*) .	25,8	—	—	157,[illegible]	[illegible]
Chylus I . . .	12,1	205,7	[illegible]	19[illegible]	[illegible]
Chylus II . . .	10,5	202,3	7,9	19[illegible]	[illegible]
II. Tri-Olein					
bestimmt*) . .	11,8	203,5	3,9	[illegible]	[illegible]
theoretisch*) .	10,4	—	—	[illegible]	[illegible]
Chylus	11,9	202,2	5,1	19[illegible]	[illegible]
III. Monoglyzerid					
bestimmt*) . .	20,2	165,8	5,1	[illegible]	[illegible]
theoretisch*) .	26,0	—	—	[illegible]	[illegible]
Chylus I . . .	11,5	196,4	8,6	[illegible]	[illegible]
Chylus II . . .	10,4	194,1	7,9	[illegible]	[illegible]
IV. Monoglyzerid					
Chylus II . . .	11,6	204,4	7,9	[illegible]	[illegible]
Chylus III . .	12,4	—	10,7	[illegible]	[illegible]
V. Schweinefett					
bestimmt*) . .	11,8	209,4	2,0	19[illegible]	[illegible]
theoretisch*) .	11,0	—	—	[illegible]	[illegible]
Chylus I . . .	12,5	205,0	8,0	[illegible]	[illegible]
Chylus II . . .	12,6	208,8	—	[illegible]	[illegible]

Key: 1. Item Administered, 2. Glycerine, 3. Saponification number, 4. Acid number, 5. Ester number, 6. Iodine number, 7. determined, 8. theoretical, 9. Chyle, 10. Monoglyceride, 11. lard.

* Composition of the substances administered.

The experiments show that monoglycerides of fatty acids are absorbed to the same degree as triglycerides and that fat-like substances occur in the chyle. Apparently, the extent of resorption is greater with respect to volume and rate than after the feeding of fatty acids without glycerine, even though the numerical data presented is insufficient. Maximum absorption of fats occurs three or more hours following the intake. The time of its occurrence is evidently a function of volume. A determination of whether the resorption of monoglycerides differs in its course from that of neutral fats would require new experiments with both classes of materials.

A minor part of the fat occurring in the chyle consists of free fatty acids. Their part in the total ether extract varies from 2.5% in the triolein experiment to 5.4% in one of the monoglyceride experiments, but it is not possible to establish a definite relationship between the type of substance administered and the fatty acid content. In any case, the experimental results of the authors differ from Munks statement to the estent that it has not been found that following the feeding of oleic acid esters (amyl ester) the amount of free oleic acid is increased in chyle fat. In contrast to this, the fatty acid content following the feeding of lard was relatively high.

The principal result of the experiments is the fact that monoglycerides do not appear as such in the chyle, but are transformed into triglycerides. This is confirmed by the glycerine content and the ester number of the ether extract. Both show that no significant amounts of monoglycerides appear in the chyle, but triglycerides as in the feeding of triglycerides. This is demonstrated in an especially clear manner by the experiments with the feeding of monoglycerides and triglycerides, which otherwise proceed under identical conditions. The analytical data are identical for both ether abstracts.

The monoglycerides were thus synthetized into triglycerides. The synthesis must have been preceeded by an extensive decomposition of monoglycerides. It cannot be entirely the result of the deposition of fatty acids on the monoglycerides, because the fatty acids required for this synthesis must originate in the decomposition of the monoglycerides; two-thirds of the fatty acids appearing in the chyle must thus be the product of the decomposition of monoglycerides. The further behavior of the glycerine must be determined by additional experiments. Conclusions based on extensive decomposition of the fat administered are rendered particularly persuasive by the use of monoglycerides in place of diglycerides as the feed material.

The occurrence of other chemical changes -- in addition to the fundamental ones cited -- is shown by the iodine numbers, which are different for the feed fat and the fat in the chyle. Thus, the iodine number of the chyle fat in the triolein experiment is lower than that of the triolein administered. This change may be considered, in accordance with an earlier opinion by Frank, as part of the conversion of the fat consumed into body fat.

The view maintained by earlier physiologists that fat is absorbed in a corpuscular manner, i.e. without change in chemistry, again lost some of its foundations through the present experiments. It probably was based on the conviction that the corpuscular absorption of the finely emulsified fat represents a simple physiological process, and on the findings that fat is found behind the wall of the intestine in a chemical and physical state similar to that in the lumen of the intestine. Under no circumstances must corpuscular absorption be interpreted as a simple process. Certainly, physiological affinity or chemotactical phenomena, etc., must play a part. The forces of absorption would then be just as unknown as in the case of total decomposition. The fact that chyle contains triglyceride after the feeding of the substance does not indicate whether it was preceded by decomposition or not, since J. Munk and Minkowski have shown that synthesis takes place in the wall of the intestine. This assumption again leaves unexplained the fact that significant amounts of fatty acids and soaps are found in the lumen of the small intestine after the feeding of fatty acids. The slight amounts of fatty acids and soaps are not enough to form an emulsion. In contrast to this, the present experiments support the view derived by Altmann, Krehl et al. from histological investigations and by O. Frank from ester experiments, i.e. that "all fats must be decomposed in the intestine before they can be reabosorbed".* If any corpuscular absorption of fat occurs at all, its volume must be substantially less than the mode described above.

* Zeitschrift f. Biol., 1898, Vol. 36, p. 593. Pfluger promoted this theory specifically mentioning the latter investigation.

Psychopharmacologia (Berl.) 16, 290—304 (1970)

Comparisons of 1,3-Butanediol and Glycerol on Several Behavioral Measures*

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Received January 8, 1969
Final Version: October 6, 1969

Summary. Experiments employing rats as *S*s were performed to determine the behavioral effects of a prototypic synthetic nutrient, 1,3-butanediol (BD), and to compare these effects with those of glycerol. The following results were obtained: a) When administered intragastrically to semi-deprived and non-deprived rats, moderate levels of both BD and glycerol depressed voluntary running activity more than did isocaloric and isovolumetric control substances. b) For both compounds this depression increased monotonically with increasing dose level, but the effects of BD became relatively more profound than those of glycerol as dose size increased. c) Both compounds monotonically depressed food intake as a function of dose level in tests conducted 3 hr after intubation. d) The depression of food intake also occurred in restrained *S*s, thus was not simply an artifact of depressed activity. e) Increasing doses of BD monotonically depressed water intake for a 3-hr period following intubation, but *S*s under BD could be induced to drink extensively by administering hypertonic salt solutions. f) Increasing glycerol loads had no effect on water intake when running was permitted but increased water intake when running was not permitted. g) At the highest dose level tested BD profoundly disrupted equilibrium, but glycerol had no effect. These findings suggest that BD may act as a CNS depressant or muscle relaxant but that glycerol most probably is neither.

Key-Words: Glycols — Adverse Effects — Hunger — Thirst — Equilibrium.

Isgrig and Ayres (1968) have described some behavioral effects of two glycols currently under experimental examination as calorically dense synthetic nutrients. Though energy dense, these compounds, 1,2-propanediol (PD) and particularly 1,3-butanediol (BD) may be of limited nutritional value, if, as suspected, they are shown to be either CNS depressants (Dymza and Stoewsand, 1966) and /or muscle relaxants (Sprince *et al.*, 1966). Consistent with both hypotheses, Isgrig and Ayres found that oral

* This research was conducted at the Pioneering Research Laboratory, United States Army Natick Laboratories, Natick, Massachusetts. The authors wish to thank William K. Calhoun of the Nutrition Division, Food Laboratories, United States Army Natick Laboratories, who provided research facilities and encouragement during the course of this research. Acknowledgment is also due to Miss Mary Bergeron who helped collect the data.

administration of BD and PD depressed voluntary activity in rats more than isocaloric loads of sucrose and almost as much as isocaloric loads of ethanol. A similar depressant action of glycerol was also observed; however, glycerol did not disrupt performance of an equilibrium task as did BD and ethanol.

Two obstacles to a clear interpretation of these findings are apparent: First, *all* the compounds studied depressed activity below levels observed under sucrose, making it difficult to evaluate sucrose as a normal base line. It is possible, for instance, that sucrose may have actually increased activity.

Second, since activity was dependent on the hunger cycle employed, anything affecting hunger might affect activity; and, since glycerol did not apparently affect equilibrium as did ethanol and BD, its activity depression might have resulted entirely from satiety, nausea, or some other interference with hunger motivation.

Experiment I was designed, therefore, to evaluate the sucrose control used in previous work, to re-examine the effects of glycerol, and to compare it with BD on other measures in the hope of distinguishing their behavioral effects. Food and water intake were the measures selected because of their relationship to the presumed motivational artifact.

Experiment I

Method

Eight male Sprague-Dawley-derived rats with a mean weight of 430 g on the first day of testing were housed in eight activity wheels with attached housing chambers.

Prior to testing, *S*s were adapted for one week to a hunger-activity cycle which continued throughout the experiment as follows: From noon to 3:00 p.m. *S*s were locked out of their wheels with Purina Lab Chow available in the housing chamber. During the remainder of the day *S*s had access to the wheels but not to food. Water was always available.

Following the week of adaptation to the hunger-activity cycle, *S*s were shut out of the wheels at approximately 8:45 a.m. each morning, weighed, and given one of the compounds by gastric intubation as described elsewhere (Isgrig and Ayres, 1968) or were given a sham intubation. Beginning at 9:00 a.m. the doors to the wheels were opened, and activity was recorded hourly for a 3-hr period. Other measures included water intake during the activity test, food intake during a $^1/_2$-hr period following the activity test, and food intake during the subsequent $2^1/_2$ hr.

The order of administration of the compounds was counterbalanced so that each *S* received a different treatment each day, five days per week

for three weeks and in a different order each week. Unless otherwise noted these general procedures were followed in all of the activity studies described in this paper.

As in previous work, the dose level of BD was set at 3.5 g/kg so as to be calorically comparable to a debilitating ethanol load used by Dewey and Leung (1965). Sucrose and glycerol loads were equated in terms of calories with BD: both glycerol and sucrose were given on a 5.2 g/kg basis. Thus all three doses yielded 1.83 kcal/ml; and all solutions, including water, were administered on an 11.43 ml/kg basis.

Treatment of Activity Data. To minimize the effects of the large individual differences in base line activity levels and to obtain a more sensitive measure of the *relative* activity under different compounds, each animal's hourly activity scores were expressed as a percentage of that animal's total recorded activity during the entire experiment. In the present study activity was recorded for a total of 45 hr, 9 hr under each drug condition. If in these 45 hr an animal made 1000 revolutions (summing across all drug conditions) and if he made 10 revolutions under glycerol in a given hour, his glycerol score for that hour would be 1 %. In contrast to measures which express effects as ratios or percentages of a "base line" or "control" condition, this measure allows the possibility of more than one control in a single experiment. In the present experiment, for example, one purpose is to examine the adequacy of the sucrose control used in previous work, and sucrose is therefore compared with two other controls. In addition, the measure has the advantage of requiring no firm *a priori* commitment as to which condition is *the* control. This feature was particularly felicitous in Experiment II (Isgrig and Ayres, 1968) where glycerol was initially intended as a control but turned out to have an effect comparable to the strongest "experimental" treatment.

Results

Fig. 1 shows the percent activity data for the five treatments over the 3-hr-following intubation. The figure shows that glycerol and BD clearly depressed activity below levels shown under the three control conditions. An analysis of variance of these data revealed a significant effect for Drugs ($F = 4.87$, $df = 4/28$, $p < .01$) and Periods, i.e., time since intubation ($F = 5.20$, $df = 2/14$, $p < .05$)[1], but not for the Drugs × Periods Interaction ($p > .10$). A Duncan's Multiple Range Test (MRT) at the .05 level[2] confirmed that both BD and glycerol depressed overall

1 In all studies reported here the Periods effect reflects an increase in activity as a function of time since intubation. Unfortunately, it is impossible to determine how much of this increase is due to a decreasing Drug effect over time and how much is due to increasing deprivation effects and approach to the normal feeding time.

2 All range tests in this paper were conducted at the .05 level of confidence.

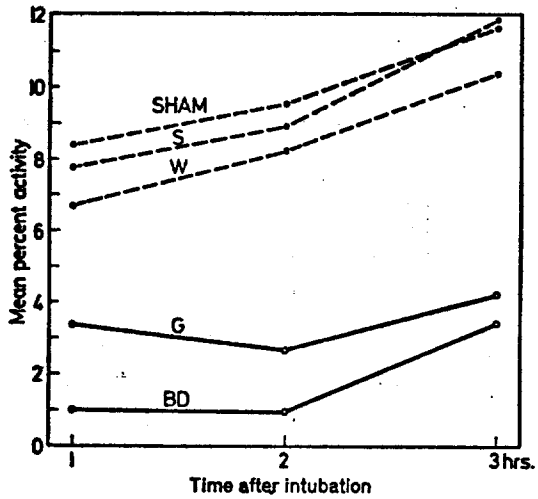

Fig. 1. Percentage activity under glycerol (*G*), 1,3-butanediol (*BD*), and three control conditions during a 3-hr period following intubation

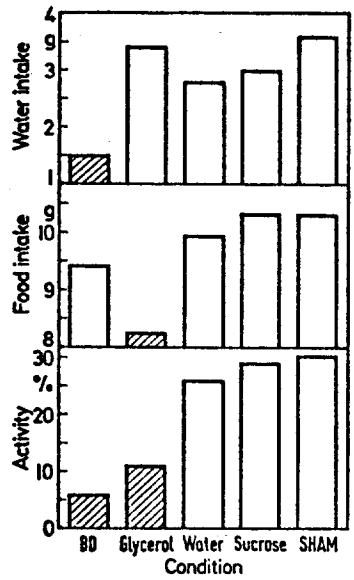

Fig. 2. Effects of glycerol, 1,3-butanediol, and three control conditions on food and water intake and voluntary activity. Cross-hatched columns differ from open columns at the .05 level of confidence

activity significantly below the levels shown by the control conditions. No other differences were significant.

Fig. 2 compares food intake during the first feeding period, and water intake during the activity period with the relative overall activity observed under each condition. Analyses of variance for both the water data and the food data from the first feeding period indicated significant Drug effects ($F = 16.86$, $df = 4/28$, $p < .01$ for water and $F = 18.05$, $df = 4/28$, $p < .01$ for food). A MRT indicated that water intake was depressed more by BD than by any other condition and that food intake was depressed more by glycerol than by any other condition. The data from the second feeding period also were subjected to anaylsis of variance and a MRT which indicated that *Ss* ate more under glycerol than under BD and sham conditions. No other differences were reliable. The results from the second feeding period appear to reflect the somewhat trivial fact that relatively heavy eaters in the first period were relatively light eaters in the second period. Since the second period scores are presumably greatly affected by the post-ingestion effects of the first period, it seems likely that first period scores better reflect real differences between compounds; consequently, the results of the second period will not be discussed further.

Discussion

As in the earlier research cited, BD and glycerol depressed voluntary activity well below control levels. Sucrose, which previously had been used as a caloric control, did not differ here from the non-caloric controls. It appears, therefore, that sucrose provided an adequate neutral base line in the previous work.

The suspicion that the depressant action of glycerol on activity might be due to an interference with hunger motivation is strengthened by the observation that glycerol, even 3 hr after intubation, significantly depressed food intake but did not affect water intake during the activity test.

The depression of water intake under BD without significant depression of food intake was, however, somewhat unexpected. A CNS depressant or muscle relaxant might be expected to depress both measures. One possible explanation for these findings is that the food measure was not taken until 3 hr *after* intubation while water intake was recorded *during* these 3 hr. Thus, the dose of BD used may have been begun to wear off at the end of the third hour. It is unlikely that the depression of water intake seen under BD is due to the conversion of BD to water, because BD depressed water intake more than an isovolumetric load of water which itself did not differ from the sham condition. It is also not probable that the effects on activity seen here are solely the result of the feeding schedules employed. In a similar experiment, 15 rats weighing an average of 513 g were tested on the voluntary activity measure 6 hr per day for

three weeks with food continuously available. Glycerol at 5.25 g/kg and BD at 3.5 g/kg both reliably reduced voluntary activity (as did corn oil at 10.3 g/kg) when compared to an H_2O control. Differences between BD and glycerol were not reliable. It should be mentioned that these *S*s had previously experienced the hunger-activity cycle for a number of weeks prior to running in the *ad lib.* study, and that previous attempts to run the study with naive *Ss* were unsuccessful because of low base line activity levels. It might also be mentioned that once the base line levels had been increased by the hunger cycle, they remained high and stable during the course of the three week *ad lib.* period.

Experiment II

In the remaining portions of this paper are presented the results of a series of dose-effect studies of BD and glycerol using as dependent measures voluntary activity, food intake, water intake, and urine output. These studies were undertaken first to determine the extent to which the results of Experiment I and our previous work were dependent upon the specific dose levels employed and secondly to reveal other possible behavioral differences between the compounds.

In some of the studies to be reported, *S*s were intentionally restrained in order to estimate the contribution of activity itself to the food and water intake measures. In addition, since the dependent measures employed were presumably sensitive to the effects of calories *per se* and since calories and dose levels covary when compounds of high caloric density are administered, we went to some lengths to attempt to rule out possible caloric effects. In a preliminary experiment, for example, 15 male rats were given daily sucrose loads of 30, 22.5, 15, 7.5, and 0 kcal/kg (7.66 to 0 g/kg in 10 ml H_2O) over a period of two weeks. Although the caloric range examined covered roughly 75% of that in the dose-effect studies to be reported, there were no significant effects on running activity or on food and water intake, and no trends were apparent. Together with the similarity of results produced by the caloric and non-caloric control conditions in Experiment I, these results tend to minimize the simple effects of caloric variation on the criterion measures.

Despite the assurance of these results, a preliminary BD-dose-effect study was conducted in which corn oil was used as a filler to equate the caloric values of various BD loads. That is, corn oil was added to the lower doses of BD to make up for their smaller caloric yields. The results of this study will be described together with those from the succeeding study which used pure aqueous solutions of BD on some of the same *S*s. In the latter case the intention was to explore BD effects in the absence of supplements which might impede absorption or even influence metabolism.

Method

In the fat-filler study, eight male Charles River Sprague-Dawley rats averaging 432 g were habituated to the apparatus and procedures over a period of weeks. The apparatus, procedures and treatment of data were identical to those of Experiment I with the exception that food and water intake were measured only in the third week, and the second-feeding-period measure was not obtained. BD was administered at levels of 7.00, 5.25, 3.50, 1.75, and 0 g/kg. Corn oil was added to the last four solutions to produce a constant caloric value across the dose range of 42 kcal/kg. Distilled water and a small amount of lecithin (20 g), used as an emulsifier, were blended to achieve a constant dose volume of 11.43 ml/kg. Each dose level was presented to each *S* over a five-day period according to a counterbalanced program.

In the succeeding study run without a caloric filler the same eight rats were re-used along with another seven which had been used in the preliminary sucrose study mentioned above, plus one additional animal making a total of 16. These were randomly assigned to an *active* and a *restrained* condition. The active group was run exactly as above (but without the caloric filler), and the restrained group was barred from the wheel during the 3 hr before feeding. In addition to the other measures, urine was collected during the 3-hr-following stomach loading.

Results

Fig. 3, Panel A compares the BD dose effects on activity obtained in the two studies. An analysis of variance for the fat-filler condition showed significant effects of Doses ($F = 38.56$, $df = 4/28$, $p < .01$), Periods, i.e., time since intubation ($F = 10.30$, $df = 2/14$, $p < .01$) and Doses × Periods ($F = 2.46$, $df = 8/56$, $p < .05$). A MRT indicated significant differences between 3.5 and 7.0; 1.75 and 3.5; and between 0 and 1.75.

The comparable test of the compound without the fat filler showed Doses ($F = 21.50$, $df = 4/24$, $p < .01$) and Periods ($F = 11.38$, $df = 2/12$ $p < .01$) to be significant, but the Doses × Periods Interaction was not reliable. A MRT for these data showed precisely the same differences among dose levels as were obtained in the fat-filler study. It is clear that the dose effects of BD on activity are almost identical with or without the isocaloric filler, with the possible exception that the filler produces different patterns over time which may be related to absorption rates.

Fig. 3, Panel B depicts the food intake data from the fat-filler, and the active and restrained no-filler conditions. Again the results show remarkable constancy of effect within and between conditions. In the fat-filler data the main effect of Doses is significant ($F = 3.17$, $df = 4/28$, $p < .05$), with the 7.00-dose being depressed in relation to all others in

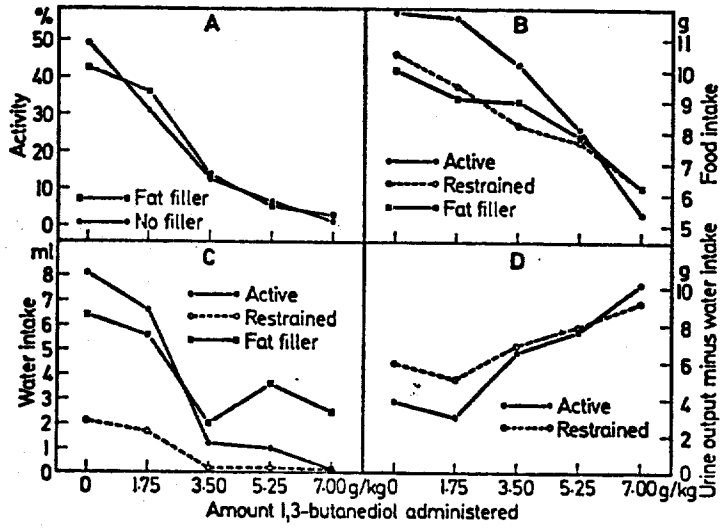

Fig. 3 A—D. Effects of graded doses of 1,3-butanediol on A Voluntary activity measured for 3 hr after intubation, B Food intake measured in a $^1/_2$-hr period 3 hr after intubation. C Water intake measured for 3 hr after intubation. D Urine output (minus water intake) measured for 3 hr after intubation

the MRT. The overall difference between the active and restrained conditions was not reliable; nor was the interaction between restraint condition and dose level.

Fig. 3, Panel C, shows the water intake data collected during the 3-hr-following intubation, which for the fat-filler and active animals was a period of wheel running. In the fat-filler condition a significant overall F was not obtained, perhaps because of the small number of observations in this sample (the data were collected only during the third week), but the 0.00-condition was significantly different from the 7.00-condition ($t = 2.71$, $p < .05$) using the t-test suggested by Lindquist (1953, p. 99) for examining monotonic functions.

In the no-filler study the active group showed a substantial monotonic effect for Dose ($F = 3.16$, $df = 4/24$, $p < .05$), but within the restrained group no differences could be demonstrated, apparently because of the low overall water intake. Unfortunately, re-examination of data previously collected on the same *S*s revealed that there had been a large sampling error, in that prior to the experiment the animals selected for the active group drank significantly more than those in the restrained group. For this reason we do not know whether the difference in base line drinking between the two groups is spurious or is a result of their different activity levels.

Fig. 3, Panel D presents the urine output data from the no-filler study as surplus urine in excess of water intake (urine output minus water intake). This presentation seemed desirable because the raw data for urine output plotted a "U" shaped curve which appeared to reflect the differences in water intake already observed, i.e., the low dosage groups drank more water and consequently produced more urine than the mid range doses, which in turn produced less urine than the high-dose conditions. As seen from the figure, the compensation for water intake differences reveals essentially monotonic increases in urine output with increases in dose. Analysis of variance of these data yielded significant Dose effects for both active ($F = 9.45$, $df = 4/24$, $p < .01$) and restrained ($F = 11.12$, $df = 4/28$, $p < .01$) groups.

Discussion

It is clear from these data that BD produces a dose-proportioned depression of voluntary activity across a wide range of doses. It likewise seems evident that this depression is not primarily due to caloric satiety in view of the similarity of results from the fat-filler and no-filler studies.

The food intake data show almost precisely the same effects as do the activity data, and the similarity of the data from the active and restrained groups indicates clearly that under high doses of BD animals do not eat less simply because they run less.

The water intake and excretion data are difficult to evaluate. In the *active* group receiving aqueous solutions of BD, and, apparently, in the fat-filler group, there is a dose-related reduction in water intake which parallels food-and-activity depression. This much corroborates the findings of Experiment I. Unfortunately, the restrained group, which was intended specifically to reduce the confounding of activity, showed a near-zero water intake across all dose levels. On the basis of this evidence, it is impossible to say whether 1) the monotonic depression of water intake was a result of monotonic activity depression, or 2) both were the results of a common depressant drug effect, or 3) BD has a thirst-specific effect in addition to its general depressant effects.

The results of two other experiments seem relevant to these issues: In one, water-deprived rats were trained to bar-press for water on a VI-1 min schedule of reinforcement and, 30 min after intubation, were given a 15-min test under BD loads of 7.0, 3.5, or 0 g/kg (the latter being a pure water load). Significantly fewer responses were made under 7.0 than under 3.5 g/kg BD which itself did not differ from the water load. The obtained difference between the two BD loads in this case cannot be simply an artifact of reduced running activity (as was possible in the preceding experiments); and it seems unlikely that it could be an artifact

of reduced general activity in the Skinner box, because activity in the Skinner box is much more restricted than it is in the running wheel. These results, then, tend to rule out alternative (1).

In a second experiment post-intubation water intake measures were obtained from eight rats housed and tested in metabolic cages. The compounds administered were formed by adding two levels of salt to two levels of BD to produce solutions either two or four times isotonic saline (.18 or .36 g/10 ml), while BD was given at either 2.5 or 5.0 g/kg in 10 ml/kg volumes. Each of the four solutions thus formed (low salt-low, BD, etc.) was administered twice to each *S*. Water intake was measured during the hr-following intubation with food withheld during the measure. An analysis of variance of the water intake scores revealed no differences between BD loads but significant differences between salt loads ($F = 18.19$, $df = 1/7$, $p < .01$). While this result may not permit a clear choice between alternatives (2) and (3), it does show that whatever the reason for BD-produced depression of water intake, rats given BD can respond to an appropriate thirst challenge.

The diuretic effects of BD seems quite clear in Experiment II, although the subtractive procedure for adjusting the intake base line may be somewhat unconventional. We believe that this is a genuine effect of a sort commonly seen in other polyols. It is unclear what physiological mechanisms may be responsible; one possibility may be a tendency to excrete large portions of the compound unchanged, but this has not been specifically tested.

Experiment III

The parametric investigation of glycerol effects was undertaken for essentially the same reasons as those for the BD studies. Glycerol in aqueous solutions had produced strong depression of activity and food intake at 5.25 g/kg, although it did not affect water intake. These had been surprising findings since glycerol is ordinarily reported to be free of toxic effects (Hanke, 1953) and was expected to behave much like sucrose when intubated.

Unlike the preceding investigations of BD effects, the glycerol tests were conducted on active and restrained animals at different times and under somewhat different circumstances.

Method

In the active tests, *S*s were 14 male Charles River, Sprague-Dawley rats, of which 13 had participated in the earlier BD experiments. The apparatus, procedures and treatment of data were similar to those of preceding experiments. Measures were made of activity, food and water

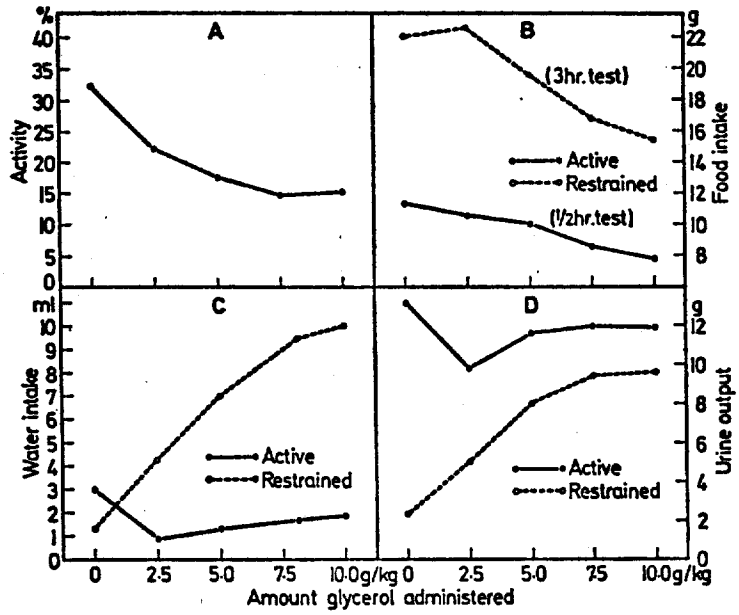

Fig. 4 A—D. Effects of graded doses of glycerol on A Voluntary activity measured for 3 hr after intubation. B Food intake measured 3 hr after intubation. C Water intake measured for 3 hr after intubation. D Urine output measured for 3 hr after intubation

intake, and urine output over a two-week period during which each dose—10.0, 7.5, 5.0, 2.5, and 0 g/kg in H_2O at 10 ml/kg—was administered twice.

In the non-active, or restrained, tests nine male Holtzman rats were housed in metabolic cages without access to activity wheels. Following an adaptation period of two weeks, aqueous glycerol solutions were administered for two weeks as in the activity tests. Water intake was measured in the 3-hr-following intubation (preceding feeding) as was urine output. Food intake was measured, however, over a 3-hr period (which followed intubation by 3 hr); and the material fed was a 30% fat, 20% protein, agar gel diet incorporating approximately 25% water.

Results and Discussion

Fig. 4, Panel A shows that increasing glycerol doses produced progressive reductions in activity ($F = 21.24$, $df = 4/52$, $p < .01$). A MRT indicated that the 0 dose level differed from all others and that the 2.5 level differed from the 7.5 and 10.0 levels. No other differences were reliable.

The effects of graded doses of glycerol on food intake in both active and inactive animals are shown in Fig. 4, Panel B. The Dose effect was highly significant in both the active condition ($F = 20.83$, $df = 4/52$, $p < .01$) and the restrained condition ($F = 7.28$, $df = 4/32$, $p < .01$).

Water intake for the active group (Fig. 4, Panel C) was generally low and no dose effect was apparent. In the restrained group, however, there was a very striking dose effect with water intake increasing dramatically with dose ($F = 14.49$, $df = 4/32$, $p < .01$); and a MRT confirmed reliable differences between 0.00 and 2.5, 2.5 and 7.5, and 5.0 and 10.0 dose levels.

We have also seen an elevation of drinking with increasing glycerol loads in an operant conditioning situation. The same solutions used in Experiment III were administered to nine Long-Evans rats trained to bar-press on a VI 1 min schedule for water. In 15-min tests given 30 min after intubation, highly significant differences were found among the dose levels ($F = 4.02$, $df = 4/32$, $p < .01$). A MRT demonstrated reliably *greater* levels of bar-pressing (higher intake) under the 5.0 and 7.5 g/kg doses than under either the 0 or 10.0 g/kg doses, which were almost identical.

With the exception of the drop at 10.0 g/kg, this result is very similar to that for *ad lib.* water intake in restrained *S*s. At the same time the drop at 10.0 g/kg suggests some of the same depression seen in voluntary activity. The difference between the *ad lib.* and work situations may result from the difference in expenditure of effort.

The urine output measured during the 3-hr, post-intubation period is shown in Fig. 4, Panel D. In the active group there were no statistically reliable differences among dose levels. In the non-active group, on the other hand, output shows an increase with dose ($F = 19.8$, $df = 4/32$, $p < .01$). In either instance the urine curve is quite similar to the water intake curve in almost every respect. If water intake is subtracted from urine output (not shown) both plots become almost flat but with the active group producing urine at a higher rate than its water intake, and the restrained group excreting almost exactly its water intake.

It is known from other research (Gissell, 1933) that over 25% of a large oral dose of glycerol may be excreted unchanged. Therefore, a portion of the urine collected under the higher loads must have been glycerol. Increased loads and increased glycerol excretion are also accompanied by general increases in urine volume and excess water loss. This diuretic effect may in turn have been the cause of the increased drinking noted above.

General Discussion

Several lines of evidence suggest that BD and glycerol produce their similar effects on voluntary activity through different mechanisms. First,

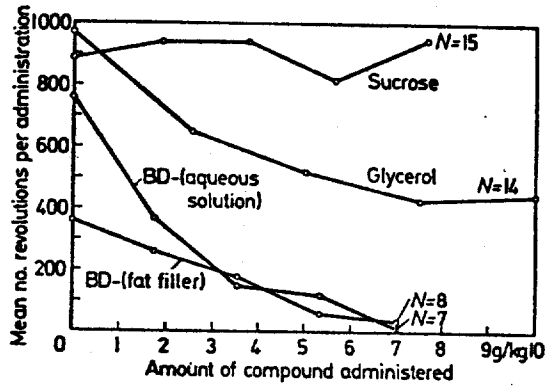

Fig. 5. Effects of graded doses of sucrose, glycerol, 1,3-butanediol, and 1,3-butanediol supplemented with corn oil on voluntary activity

the similarity in the extent to which these compounds depressed activity in Experiment I and in previous work seems to be somewhat specific to the particular dose levels employed in those studies. In spite of the fact that both BD and glycerol monotonically depress activity with increasing dose level, the effects of glycerol seem to asymptote at a moderate level, i.e., at the high dose levels the effects of BD become more profound than those of glycerol. Fig. 5 illustrates this difference by comparing raw data means from a number of separate studies. Perhaps most striking is the fact that both of the BD curves approach the zero level at high dose concentrations while activity remains fairly high under comparable glycerol loads.

Secondly, the effect of glycerol on water intake appears to differ markedly from that seen with BD. Whether BD has any specific anti-thirst properties or not, in high doses it reduces water intake to a considerable extent. Glycerol, as seen in the restrained *ad lib.* animals and in the operant-responding animals, tends to increase water consumption. The fact that this effect was not seen in active *S*s can probably be explained as a summation of competing thirst-inducing and activity-reducing effects, since activity, which is reduced by glycerol, generally seems to be accompanied by increased water intake.

Thirdly, and perhaps most striking, are the different effects the *high* doses of these compounds have on equilibrium. These effects are documented in the following experiment.

Experiment IV

The large difference in the effects of high doses of BD and glycerol on activity (just noted in Fig. 5) prompted a replication of Isgrig and Ayres'

(1968) Experiment III but with doubled doses of all the compounds previously used except ethanol.

Method

Ten naive, male Charles River Sprague-Dawley rats weighing 284 g, on the average, were trained over several days to balance for 3 min periods on a wooden dowel rotating at 6 rpm. The dowel was 4 cm in diameter, 40 cm long, and was suspended between the two sides of a relay rack. Each 3-min trial was timed by a standard timer which was stopped when *S* fell from the bar and was restarted when *S* was replaced. Thus each *S* spent a full 3 min on the bar on each trial. Over a 3-week period, sucrose (5.25 g/kg), glycerol (10.50 g/kg), BD (7.0 g/kg), PD (8.4 g/kg), and ethanol (3.0 g/kg) were administered three times each on a counterbalanced program. Subjects were given a 3-min trial $^1/_2$, 1, and 2 hr after intubation.

Results and Discussion

The mean number of falls per subject per trial were 10.1, 3.5, 2.2, 0.6, and 0.3 for BD, ethanol, PD, glycerol, and sucrose, respectively. The Drug effect was highly reliable ($F = 20.98$, $df = 4/36$, $p < .01$), and a MRT indicated that BD produced more falls than any other compound, followed by ethanol and PD, which differed from sucrose and glycerol but not from each other. There was no difference between glycerol and sucrose. Thus even the largest glycerol dose tested, did not produce perceptible disruption of equilibrium, while under the high BD load *S*s were hardly able to stand.

The foregoing evidence suggests strongly that when administered in a pure form, BD (and probably PD) acts as a CNS depressant or strong muscle relaxant. Glycerol, however, which at first appeared to resemble BD in its effects, seems to produce them through some sort of motivational impairment including possibly nausea or malaise. Against this interpretation are the subjective reports from humans (Hanke, 1953) of no unpleasant effects with glycerol doses near 2.5 g/kg—a dose which in the present study significantly affected activity, water intake, and urine output.

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Emulsifiers. II. Periodic Acid Analysis for 1-Monoglycerides

By F. J. BAUR and E. DISTLER (Procter and Gamble Co., Winton Hill Technical Center, 6000 Center Hill Rd., Cincinnati, Ohio 45224)

Two periodic acid analytical methods have been compared for the determination of 1-monoglycerides. A revised Miner procedure is apparently faster and more precise than the official AOCS method, Cd 11-57. The AOCS procedure is recommended for adoption as official, first action. A joint AOAC-AOCS multi-methods study should be conducted to compare the Miner and AOCS procedures.

Polyhydric alcohols containing adjacent hydroxyl groups react with periodic acid in acid solution to form aldehydes. This reaction was the basis for the first quantitative method for the determination of 1-monoglyceride (1).

A number of modifications were proposed in following years, and in 1953 the AOCS Fat Analysis Committee created a monoglyceride subcommittee to investigate them. The investigation was completed in 1957 with the recommendation (2) that a slightly modified Pohle and Mehlenbacher procedure (3) be adopted as a tentative AOCS method. It was accepted by the AOCS Uniform Methods Committee and published in that Society's methods in 1957. In 1960 it was adopted as official.

The 1957 data, presented in Table 1, showed that the precision of the method is a function of 1-monoglyceride present in the sample. The average standard deviation ranged from 0.2% at the shortening level of 2–3% to 1.1% for a distilled concentrate of about 90% monoglyceride.

AOCS Smalley Committee data over the past four years are in good agreement with these early data. These results are summarized as follows:

% Monoglyceride Present, approx.	*Smalley Laboratories (ca 40), σ*
3.5–6.0	0.17
10–25	0.31

The precision of the periodic approach for monoglycerides is comparable to the column chromatographic procedure. Recent collaborative studies on the column technique showed 0.1–0.2(σ) for monoglycerides at levels of 1.8–10.5% (4) and 0.7–1.2 for 40–90% levels (5). The periodic technique is, however, considerably faster, requiring less than one-half the time.

A revision of the Miner procedure (6) is reputed to be faster and more precise than the official AOCS periodic acid procedure. It involves a separate determination of

Table 1. Precision of method based on original AOCS collaborative study in 1957 (95% probability limits)

% Monoglyceride Present (approx.)	σ	Diff. between Single Detn by Same Analyst on Different Days	Diff. between Single Detn in Different Labs
3%	0.18	0.27	0.51
40%	0.4	0.6	1.2
90%	1.1	2.0	3.2

glycerol as opposed to the aqueous removal practiced in the AOCS method. Precision (σ) in the order of 0.05 at 3.5–6.0% level, 0.20 at 10–25%, to 0.51 at > 40% level has been claimed for the Miner procedure. A cross comparison of the two procedures on two samples using a relatively inexperienced analyst was therefore run. These results are tabulated below.

AOCS (10 Determinations)

Sample	Range	Av.	σ
1	1.81–1.95	1.88	0.039
2	3.15–3.27	3.22	0.048

Miner (6 Determinations)

Sample	Range	Av.	σ
1	1.42–1.47	1.44	0.016
2	2.94–3.05	2.99	0.037

The revised Miner procedure proved to be more precise; however, an unexpected question was raised—accuracy. The column chromatographic procedure (4) on these samples gave the following results.

Sample	Mono-	Di-	Tri-
1	1.7	4.5	94.1
2	3.1	7.3	89.4

The monocuts, via TLC, were substantially free of di- and triglycerides (about 1% present). Since the column procedure will pick up 2-monoglycerides, the modified Miner procedure may be more accurate; however, this would have to be confirmed.

The two methods are described below.

METHODS

AOCS Cd 11-57

Principles

1-Monoglycerides are detd from HIO_4 consumed in the oxidation of adjacent hydroxyl groups. 2-Monoglycerides are not oxidized by HIO_4. Method is applicable to fats, oils, monoglycerides, and blends; not applicable to samples contg $CHCl_3$-sol. substances with 2 or more adjacent hydroxyl groups.

Reagents

(a) *Periodic acid soln.*—Reagent grade (available from G. Frederick Smith Chemical Co., Box 23344, Columbus, Ohio 43223, or equiv.). Test as follows: To 0.5–0.6 g glycerol in 50 ml H_2O add 50 ml HIO_4 soln from pipet. Prep. blank contg 50 ml H_2O. Let stand 30 min. and titr. as in *Determination*. Titer of soln contg glycerol/titer of blank = 0.75–0.76 if reagent is satisfactory.

Dissolve 5.4 g HIO_4 in 100 ml H_2O, add 1900 ml HOAc, and mix thoroly. Store in g-s. bottle in dark.

(b) *Starch indicator soln.*—Make homogeneous paste of 1.0 g sol. starch with H_2O. Add to 100 ml boiling H_2O, stir rapidly, and cool. Salicylic acid, 0.125 g/100 ml, may be added as preservative. Soln keeps longer if stored in refrigerator. Color of 2 ml starch soln dild with 100 ml H_2O contg 0.05 ml 0.1*N* I must be discharged by 0.05 ml 0.1*N* $Na_2S_2O_3$. Discard if end point from blue to colorless is no longer sharp.

(c) *Chloroform.*—Reagent or USP. Blanks on HIO_4 soln with and without $CHCl_3$ must check within 0.5 ml.

Preparation of Samples

(Do not subject samples to excessive temps or monoglyceride content may be reduced.)

(a) *Solids in flake form.*—Mix without melting.

(b) *Solids not in flake form.*—Melt at not > 10° above m.p., mix thoroly, and take sample. Do not test samples contg so much free glycerol that it seps on solidification.

(c) *Semi-solids and liquids.*—Proceed as in (b).

This paper was presented as part of F. J. Baur's Associate Referee report at the Seventy-ninth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11-14, 1965, at Washington, D.C. See also *This Journal*, 49, 812-816 (1966).

Determination

Weigh accurately 0.3–10 g sample calcd from equation: g = 30/% monoglyceride in sample (10 g for 3% or less), dissolve in $CHCl_3$, and transfer to 100 ml g-s. vol. flask. Dil. to vol. with $CHCl_3$ and mix. Transfer entire soln to 500 ml g-s. erlenmeyer (do not rinse) and add 100 ml H_2O. Stopper, and shake vigorously 1 min. Let stand until layers sep. and $CHCl_3$ layer is clear or only slightly cloudy (1–3 hr). If emulsions form, causing poor sepn, repeat detn using 100 ml HOAc (5 + 95) instead of H_2O.

Pipet 50 ml HIO_4 soln into series of 400 ml beakers. Add 50 ml $CHCl_3$ to 2 and 50 ml H_2O to third as blanks (*Reagents*, (c)). Pipet 50 ml $CHCl_3$ sample soln to fourth, avoiding any aq. phase, and shake gently. (Solns must be cooled to below 95°F, if necessary.) Cover with watch glass and let stand 30 min.

Add 20 ml 15% KI soln, mix by gentle shaking, and let stand at least 1 min. but not > 5 min., and not in strong sunlight. Add 100 ml H_2O, and titr. with stdzd 0.1*N* $Na_2S_2O_3$, stirring continuously with mechanical stirrer. After I color disappears from aq. layer, add 2 ml starch soln and continue titrn to disappearance of blue from aq. layer. Vigorous stirring is necessary to remove I from $CHCl_3$ layer.

If titer of sample is < 0.8 titer of blanks, repeat detn, using smaller aliquot of $CHCl_3$ soln or smaller sample wt, to assure adequate excess of HIO_4. If titer of blank minus titer of sample is < 4 ml, repeat detn, doubling sample wt to a max. of 10 g.

% Monoglyceride as monostearin = $(B - S) \times N \times 17.927/W$, where B = titer of $CHCl_3$ blank, S = titer of sample, N = normality of $Na_2S_2O_3$, W = g sample in $CHCl_3$ aliquot, and 17.927 = molecular wt monostearin/20.

If content is to be calcd to monoglyceride other than monostearin, substitute for 17.927 molecular wt of other monoglyceride/20. Alternatively, det. molecular wt as follows: Sep. fatty acids as in first 2 paragraphs of 26.015. Weigh accurately ca 2 g fatty acids into 250 ml erlenmeyer and add 20–30 ml hot alcohol neutralized to faint pink of phthln. (SDA formulae 30 or 3A are satisfactory.) Add 0.5 ml phthln and titr. immediately while shaking with 0.5*N* NaOH to pink that persists 30 sec. Calc. acid no. (mg *KOH* required to neutralize fatty acids in 1 g sample) = ml NaOH × normality × 56.1/g sample. Av. molecular wt of fatty acids = 56104/acid no. = *M*. Molecular wt monoglyceride = (M + 92.09) − 18.02.

Duplicate detns made on same day by 1 analyst should not differ (95% confidence limits) by more than ca 0.1, 0.5, and 1.2% alpha-monoglyceride at 3, 40, and 90% levels; single detns and av. of duplicate detns made in 2 different laboratories should not differ by more than ca 0.5, 1.2, and 3.2%, and 0.5, 1.1, and 3.1%, resp., at same levels.

Revised Miner Procedure

Principle

This method is based on the periodic oxidation of vicinal hydroxyl groups. Compounds with =O, —OH, —NHR and —NH_2 attached to adjacent carbon atoms will be oxidized by periodic acid; thus phospholipids can interfere. Such substances are absent from the usual fat samples to be analyzed.

Samples to be analyzed are dissolved in $CHCl_3$ containing 5% dimethylformamide (to assure solution of the glycerine) and aliquots are oxidized with an excess of periodic acid. A periodic acid-water solution extracts and oxidizes free glycerine and a periodic acid-methanol solution oxidizes monoglyceride plus glycerine. The amount of unused periodic acid is determined iodometrically by reacting with potassium iodide and titrating with 0.05*N* standard arsenite solution.

Calculation of the monoglyceride content is based on the amount of periodic acid consumed in the oxidation of the monoglyceride plus glycerine corrected for the amount consumed in the oxidation of the free glycerine alone.

Reagents and Apparatus

(a) *Periodic acid stock solution.*—Dissolve 12.0 g H_5IO_6 (G. F. Smith Chemical Co., reagent grade) in 100 ml distilled water. Store at room temperature in brown glass-stoppered bottle.

(b) *Periodic acid in 95% methanol.*—Mix 25 ml stock reagent (a) with 475 ml methanol (ACS grade). Store at room temperature in a brown glass-stoppered bottle and prepare fresh every 3–5 days.

(c) *Periodic acid in water.*—Mix 50 ml stock reagent (a) with 950 ml distilled water.

(d) *Potassium iodide.*—Dissolve 75 g KI (ACS grade) and 50 g $NaHCO_3$ in distilled water.

(e) *Sodium arsenite.*—0.05*N* (sec. 42.005, *Official Methods of Analysis*, 10th Ed.).

(f) *Starch indicator.*—Make homogeneous paste of 0.50 g potato starch with 5 ml distilled

water. Add to 200 ml boiling distilled water and boil for 15 min., stirring constantly. Cool and store in a glass-stoppered bottle. For best results, prepare fresh daily.

(g) *Chloroform.*—ACS, must pass USP test for purity. Iodine absorption should be < 0.1 ml 0.1*N* $Na_2S_2O_3$/30 ml solvent.

(h) *N,N-Dimethylformamide (DMF).*—25% solution in $CHCl_3$ (g). *Caution:* This material is toxic. Avoid contact with skin or inhalation of vapors.

(i) *Hot plate.* — Thermolyne-Model HP 1915B, or equivalent.

Preparation of Samples

See AOCS Cd 11-57.

Determinations

Weigh sample to be analyzed into a 100 ml beaker, calculating the amount required by the following formula:

$$S = K/(M + 8G) = 50/(M + 8G)$$

where S = g sample required ($= 4W = 2W_2$); M = % monoglyceride expected; G = % free glycerine expected; K = a constant which determines the % excess reagent [$100\ T/(B_1 - T_1)$]; W_1 = aliquot wt for monoglyceride; W_2 = aliquot wt for free glycerine; B_1 = blank titration for monoglyceride plus glycerine; B_2 = blank titration for free glycerine; T_1 = sample titration for monoglyceride plus glycerine; and T_2 = sample titration of free glycerine.

For 20% excess, K = 70; 50% excess, K = 50 (preferred); and 100% excess, K = 42. The maximum for S should be 40 g/100 ml or, if preferred, 10 g can be weighed directly into the reaction vessel.

A. Procedure for monoglyceride plus glycerine.—Dissolve weighed sample in 20 ml 25% dimethylformamide (DMF) (h). Use $CHCl_3$ (g) to transfer quantitatively to a 100 ml volumetric flask and dilute to volume. Aliquots from this volumetric can be used for analysis of both the monoglyceride plus glycerine and the free glycerine.

Pipet 25 ml aliquot into 500 ml Erlenmeyer flask and record the sample wt in aliquot as W_1. Prepare 2 blanks in similar flasks by adding 5 ml 25% DMF and 20 ml $CHCl_3$ to each.

From a pipet, add exactly 25 ml periodic acid-methanol (b) to the 25 ml aliquot and to each blank. (For samples low in monoglyceride, such as triglyceride fat or oil, the volume of reagent (b) may be reduced from 25 ml to 10, 5, or even 3 ml; use the same amount of reagent for the blanks.) Add 2–3 glass beads to each sample and heat just to boiling on hot plate, swirling several times during heating. (Do *not* heat the blanks.) Let stand for 30 min. to cool, protected from sunlight or other strong illumination. Add 200 ml distilled water to each blank and sample, and swirl several times. Let sample stand 5 min. (up to 45 min. is permissible). The blanks may be analyzed immediately. Add 40 ml KI (d) from graduated cylinder and swirl to mix. Let stand for 1 min., then titrate with sodium arsenite (e). After iodine color fades to pale yellow, add 2–3 ml starch indicator (f) and continue titration until starch-iodine color disappears. Record average titration of the two blanks as B_1 and the sample as T_1. When more than one sample is run on the same day with the same reagents, the average titration of two blanks which check within 0.1 ml can be used in all calculations. If two blanks do not check within 0.1 ml, a third blank must be run. The excess periodic acid (b) must be at least 20%, but not over 100%. If $100\ T_1/(B_1 - T_1)$ is less than 20%, repeat with a smaller sample; if the excess is much over 100%, repeat with a larger sample or use less periodic acid reagent. To complete the periodic acid oxidation, a minimum of 20% excess is required. If excess periodic acid-methanol is much greater than 100%, high apparent monoglyceride may result.

B. Procedure for free glycerine.—For determination, free glycerine must be extracted completely from the DMF layer into a water layer. If the glycerine is not in the water layer when the periodic acid-water reagent is added, or during the standing time, results may be low.

Pipet 50 ml aliquot of weighed sample from (*A*) into a 500 ml Erlenmeyer flask. Record sample wt in aliquot as W_2. Prepare two blanks in similar flasks by adding 10 ml 25% DMF and 40 ml $CHCl_3$.

Add ca 100 ml distilled water to the sample and blanks. Swirl for ca 1 min., reversing direction several times. With a pipet add exactly 25 ml periodic acid-water (c) to each flask. Let flasks stand at room temperature for 30 min., swirling vigorously for at least 30 sec. at the beginning and every 5 min. during this period. Add 40 ml KI (d) with a graduated cylinder, swirl, and let stand 1 min. Titrate with 0.05*N* sodium arsenite (e) to a starch-iodine end point as described in (*A*), including values for blank titrations. Record sample titration as T_2 and the average of the blank titration as B_2.

Any excess over 20% is permissible for the periodic acid-water (c).

Calculation

$$\% \text{ monoglyceride} = \frac{[(B_1 - T_1) - \frac{W_1}{W_2}(B_2 - T_2)] \times N \times M \times 100}{W_1 \times 2 \times 1000}$$

For this study, the monoglyceride content of all samples can be calculated as monostearin. Using exactly 0.05*N* arsenite reagent, 358 as the molecular weight of monostearin, and a sample weight of the free glycerine equal to twice the amount used for the monoglyceride plus glycerine, the following formula can then be applied for the calculation:

$$\% \text{ monoglyceride (as monostearin)} = [(B_1 - T_1) - \tfrac{1}{2}(B_2 - T_2) \times 0.895]/W_1$$

Recommendations

In view of its speed and proven utility, it is recommended that the official AOCS procedure for 1-monoglycerides, Cd 11-57, be adopted as official, first action. It is further recommended that a joint AOAC-AOCS committee be formed to conduct a multi-methods study comparing the AOCS and revised Miner procedures.

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The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee C, and were adopted by the Association. See *This Journal*, 49, 167–172 (1966).

Specific Determination of Mono-, Di- and Triglycerides in Model Mixtures and in Soybean Oil

G. Berner

Summary

A method for the specific determination of mono-, di- and triglycerides is described. After the thin-layer chromatographic separation of the glyceride mixture, the mono-, di- and triglycerides are determined enzymatically after saponification. The accuracy of the method was tested with mixtures of glycerol-mono-, -di- and -tristearate. The content of mono- and diglycerides of some soyabean oil samples was determined.

The method proved suitable for the detection of larger contents of mono- and diglycerides as well as for the analysis of trace amounts of mono- and diglycerides besides triglycerides. Without a special enrichment process it is possible to analyse as low amounts as 6 µg ($6 \cdot 10^{-3}$%) mono- and 10 µg ($1 \cdot 10^{-3}$%) diglycerides in the oil.

Introduction

According to our experiences, a quantitative analysis of mixtures of mono-, di-, and triglycerides of higher fatty acids by gas chromatography is next to impossible. A densotimetric measurement of the density was obtained after thin-layer chromatographic seperation and the substance zones, which were carbonized with sulfuric acid, yield values, (1, 2) which depended on the strength of the structure of the substance examined; with the possibility of interference from other fatty substances (1). Therefore, the glyceride zones separated by thin-layer chromatography should be isolated and subjected to enzymatic determination of glyceride (3 a, b).

Method - model investigation

We used glycerine monostearate, glycerine distearate[1], and glycerine tristearate[1] as sample mixtures. Free glycerine was not enzymatically evident in these substances.

Extractions from soybean oils were deslimed, neutralized, and steamed on a small scale under exclusion of air[2](4).

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Experimental

About 50-100 mg of the samples were dissolved in 10.0 ml of chloroform or dichloromethane before analysis. Thin-layer chromatographic separation was carried out with 0.5 ml of this solution. For the chromatography we used the prepared plates F254 of Merck Co. and as the liquid phase petroleum ether (40-60°C)/ether (65+35). As indicator reagent we used a 1% aqueous solution of Ultraphor WT4(1). After evaporation of the liquid phase, the zones of mono-, di-, and triglycerides were distinguishable as blue spots in uv-light at 366 nm. The substance zones were scraped off and extracted with dichloromethane in a Soxhlet apparatus. The solvent was evaporated from the extract and the trailings were saponified with 2.5 ml of ethanolic 0.5 n - KOH at a waterbath temperature of 70°C. It was verified that the saponification was quantitatively complete after 30 minutes. After cooling the saponification mixture, 10µl of ethanolic 0.5% phenolphthalein solution was added and then 2.5 n-perchloric acid solution was added until color change of the indicator was complete. The solution was diluted with 3 ml of buffer solution and the reaction vessel placed in an ice bath for 30 minutes. The reaction mixture was filtered into a 10 ml graduated flask, the sediment washed with buffer solution, and the solution diluted to 10 ml with double distilled water. The enzymatic determination (3) of glycerine was carried out with an aliquot of this solution.

Evaluation

The average molecular weight (MW) of the fatty acids of soybean oils was 262 (6); accordingly we used 356 for monoglyeride and 620 for diglyceride and thereby calculated the proportion of glyceride (3b).

$$\frac{\Delta E \cdot \text{End volume} \cdot F \cdot MW}{3.3} = \mu g \text{ glyceride/sample weight}$$

End volume: 2.68 ml F = dilution factor

Results and Discussion

The accuracy of the enzymatic determination of bound glycerine is known; the average deviations of the sample weight amounted to, from 15 analytical values, -1.8 and +1.0%, at a concentration of 0.043 - 0.279 μmol/kuvette preparation (3b).

In spite of the additional thin-layer chromatographic separation, the average deviation here for monoglyceride, from 10 analytical values amounted to -1.8 and +1.1%, for diglyceride -2.9 and +4.5%, and from 10 values, for triglyceride -4.0 and +2.7%. Table 1 contains the results. The portions of the glycerides in the substance mixtures are indicated here as sample weights.

In Table II the mono- and diglyceride proportions for the soybean oil extractions are presented. Considering the concentration derived from the enzymatic determination, as low as 5×10^{-3}% monoglyceride or 1×10^{-2}% diglyceride could be quantitatively determined without a previous enrichment.

Table II. Mono- and Diglyceride Concentration in Soybean Oil

Sample	Remarks	Monoglyceride content %	Diglyceride content %
I	Soybean oil extraction	0.11	1.10
II	deslimed and boiled with soda in a water glass	0.10	1.14
III	bleached	0.06	1.25
IV	attenuated	0.07	1.05

I. These samples were kindly placed at our disposal by Dr. Menz, Unilever Research Lab. ; glycerin distearate was purified according to Quinlin and Weiser (5).
2. This work was performed by Dr. Eicke, Unilever Research Lab., who also kindly provided the samples.
3. For the samples with a value less than 0.1% of mono- and/or diglyceride, the sample weight and/or the corresponding quantity used on the thin-layer plate must be raised.
4. BASF, Ludwigshafen. Control tests show that Ultraphor WT does not disturb the enzymatic analysis.

Table I. Determination of mono-, di-, and triglycerides in model mixtures after a thin-layer chromatographic seperation.

(1) Nr. des Substanzgemisches	(2) Substanzgemisch								
	(3) Einwaage	(4) gef.[a]	(5) Abweichung	Einwaage	gef.[a]	Abweichung	Einwaage	gef.[a]	Abweichung
	mg	mg	%	mg	mg	%	mg	mg	%
	Glycerin-monostearat			*Glycerin-distearat*			*Glycerin-tristearat*		
1	9,1	9,03	—0,8	10,1	10,25	+1,5	11,9	11,00	—7,5
	9,1	9,08	—0,2	10,1	10,20	+1,0	11,9	12,38	+4,2
				10,1	10,36	+2,6	11,9	12,31	+4,0
2	10,6	10,19	—3,8	12,7	12,33	—2,9	10,4	10,50	+0,7
	10,6	10,42	—1,7	12,7	13,10	+3,1	10,4	10,60	+2,3
	10,6	10,66	+0,6	12,7	13,76	+8,3			
	Glycerin-monostearat			*Glycerin-distearat*			*Glycerin-trioleat*		
3	8,93	8,99	+0,7	10,68	11,25	+5,3	19,14	19,65	+2,7
	8,93	8,94	+0,1	10,68	11,08	+3,7	19,14	19,65	+2,7
	8,93	8,99	+0,7	10,68	11,17	+4,5	19,14	18,70	—2,3
	8,93	9,23	+3,3	10,68	11,59	+8,5			
4	23,24	22,44	—3,4				23,51	23,01	—2,1

[a] These values were derived from different thin-layer chromatographic seperations and are averages of a double determination of the same saponification mixture.

Key 1. Number of the substance mixture
2. Substance mixture
3. Sample weight
4. Found (experimental value)
5. Deviation

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Alkali Isomerization—Gas Chromatography With the Microreactor Apparatus[1]

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ABSTRACT

A simple and rapid method of simultaneously conjugating polyunsaturation and forming methyl esters from triglyceride oils, other esters and fatty acids has been developed by using the microreactor apparatus (MRA). These reactions occur while a mixture of tetramethylammonium hydroxide and sample of fat is being injected into a gas chromatograph from the MRA reaction chamber. The speed of this microanalysis is limited only by peak elution and peak integration times. Analyses of soybean oil and six partially hydrogenated fats have been compared with those previously obtained by the alkali isomerization spectrometric method. The amount of conjugation that occurs during the reaction is a measure of the linoleic acid in the oil. This procedure should prove useful for rapid microscale analysis of conjugatable dienes in a variety of samples, including edible fat products.

INTRODUCTION

While esterifying fatty acids with tetramethylammonium hydroxide (TMAH) (1) in the microreactor apparatus (MRA) (2) for subsequent gas liquid chromatography (GLC), it was observed that linoleic acid gave methyl ester peaks corresponding to conjugated dienes. This observation forms the basis for developing the new MRA-alkali isomerization-GLC procedure of determining linoleic acid, other conjugatable dienes and nonconjugatable dienes in triglyceride esters, simple esters and fatty acid mixtures. Compared to the conventional alkali isomerization spectrometric method (3) there are the advantages of ease, microscale capability, speed and versatility. However, this new procedure is not suitable for samples containing large amounts of triene and is not proposed as a replacement for the more accurate alkali isomerization spectrometric method. It is suggested as a technique for the analyst having a gas chromatograph and needing to determine linoleic acid in oil which contains a small amount of triene.

In brief, the procedure consists in drawing TMAH and a sample of fat into a micro syringe, introducing the solution into the MRA and injecting the reaction mixture from the MRA into the injection port of a gas chromatograph at 250-300 C with helium gas flow. Two ways of calculating conjugatable dienes are employed, one which has indigenous methyl palmitate as internal standard and the other which measures the areas under the conjugated diene and nonconjugated diene peaks.

EXPERIMENTAL PROCEDURES

Materials

The refined soybean oil analyzed was purchased commercially. Soybean fatty acid and soybean methyl ester macrosamples were made by saponifying, acidifying and esterifying. Also analyzed were six partially hydrogenated fats previously studied by the alkali isomerization spectrometric method and subsequently stored in a zero degree room (4).

cis,cis-9,15 Methyl octadecadienoate and a mixture of *cis,cis*-9,12 and *cis,cis*-12,15 methyl octadecadienoates were purified and separated as described (5).

Linoleic acid, methyl linoleate and methyl palmitate from The Hormel Institute, Austin, Minn., showed 99.9+% purity as determined by gas chromatography.

TMAH was purchased from Aldrich Chemical Co., Milwaukee, Wis., as a 20% solution in methanol.

Analytical Procedure

The microalkali isomerization was performed by drawing 20 μl of TMAH solution, 1-6 μl of lipid sample and an additional 20 μl of TMAH solution into a 100 μl syringe, consecutively. Sample and TMAH were mixed by injecting into the MRA (2) reaction chamber. An MRA reaction temperature of 250-300 C and an injection time of 3 min were used to transfer the sample to the gas chromatograph from the MRA.

A 10% mixture of methyl palmitate (internal standard) in methyl linoleate was used to determine the amount of conjugation in pure methyl linoleate.

Transesterification and esterification reactions to obtain a chromatogram of the methyl esters were performed in the MRA as previously described (6,7).

A gas chromatograph with thermoconductivity detector (Aerograph Model 350) was used for all runs with the injector port at 250 C, the column temperature programmed from 60 to 190 C at 10 deg/min, a 10 ft x 1/4 in. column filled with 10% EGSS-X on 100/120 mesh Chromosorb W (Applied Science Lab., State College, Pa.), a flow rate of 60 cc/min helium and X1 attenuation. Total time for GLC operations ranged from 40 to 60 min.

The *trans,trans* peak from the conjugation of linoleic acid (see Results and Discussion) was collected by placing the glass reaction chamber of the MRA at the collector port of the gas chromatograph. Ozonization pyrolysis of the collected sample to determine double bond positions was then performed in the MRA as described earlier (8).

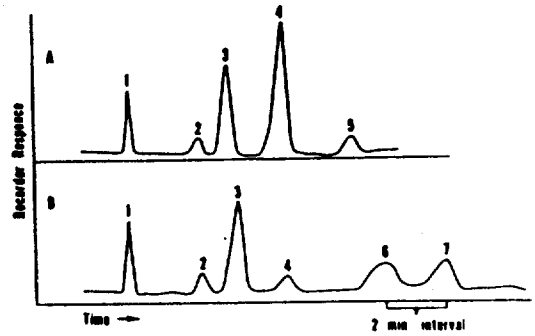

FIG. 1. Typical chromatograms before (A) and after (B) isomerizing in the microreactor apparatus with tetramethylammonium hydroxide. Portion of recording shown is after reaching temperature of 190 C. Gas chromatographic conditions given in text. Significant peaks are the methyl esters of 1) palmitate, 2) stearate, 3) monoene, 4) diene, 5) triene, 6) conjugated *cis,trans* diene and 7) conjugated *trans,trans* diene.

[1]Presented at the Fall ISF-AOCS Meeting, Chicago, September 1970.

[2]No. Market. Nutr. Res. Div., ARS, USDA.

TABLE I

Alkali Isomerization Gas Chromatographic (AI GLC) and Alkali Isomerization Spectrometric (AI S) Analyses on Six Partially Hydrogenated Fats

Sample	Total diene, % Gas chromatography		Linoleic acid, % AI GLC[a]		AI S	Conjugation, % AI GLC[a]	
	Literature,[b]	Present	1	2	Literature,[b]	1	2
	a	b	c	d	e	f	g
Liquid oil A	37.4	36.4	33.7	31.9	32.2	92.5	87.6
Liquid oil B	41.8	43.7	37.8	34.4	38.5	86.4	79.0
Shortening C	27.4	24.3	22.1	21.3	24.1	91.1	87.6
Shortening D	11.8	4.1	3.6	3.1	6.6	88.4	75.6
Shortening E	25.5	24.6	22.8	22.4	19.7	92.3	91.0
Shortening F	10.4	9.0	8.4	8.0	7.0	92.6	89.1

[a]Method of calculating: 1, Methyl palmitate peak used as a standard (method 1 in text). 2, Conjugated and nonconjugated peaks used (method 2 in text).

[b]Reference 4.

Calculations

An electronic integrator system (Infotronics, Houston, Tex.) allowed for base line correcting and determining areas under peaks.

Each reaction was carried out in triplicate and the average integral was used for calculating per cent conjugations.

Conjugation was calculated by two methods. Method 1 required two chromatograms of methyl esters, one before (Fig. 1A) and one after (Fig. 1B) isomerization. A ratio of methyl palmitate (internal standard) to diene calculated from Figure 1A was used to obtain the unknown total diene integral of Figure 1B. The measured unconjugated diene peak integral from Figure 1B completes the necessary information for calculating the amount of conjugation.

Referring to Figure 1A,B and indicating peak numbers as subscripts for the peak areas, the calculations of method 1 are:

$$X/B_1 = A_4/A_1$$

or,

$$X = A_4B_1/A_1$$

$$\% \text{ Conjugation} = 100 - (B_4/X)100$$

Substituting for X and dividing:

$$\% \text{ Conjugation} = 100 - (A_1B_4/A_4B_1)100$$

X is the total diene peak integral after conjugation.

Method 2 required only the chromatogram of methyl esters after isomerization. Integrals of the conjugated and nonconjugated peaks provided the basis for calculating the amount of conjugation.

Again, referring to Figure 1 and indicating peak numbers for subscripts, the calculations for method 2 become:

$$\% \text{ Conjugated diene} = 100 \times (B_6 + B_7)/(B_4 + B_6 + B_7)$$

RESULTS AND DISCUSSION

Figures 1A and 1B are the chromatograms resulting from a typical experiment. Figure 1A is the methyl ester chromatogram obtained by microtransesterification (6) in the MRA and Figure 1B is the chromatogram after conjugating and transesterifying the soybean oil with TMAH. It is obvious in Figure 1B that the diene peak (A_4) has practically disappeared, a small unconjugated peak (B_4) remains and conjugated peaks of *cis,trans* or *trans,cis* (B_6) and *trans.trans* (B_7) are formed.

acid and conjugation of dienes in six partially hydrogenated fats obtained by alkali isomerication GLC and alkali isomerization spectrometric techniques (9). All samples (columns a and b) showed minor differences in amount of total diene except for shortening D in which the diene was the lowest. Differences are also small in amount of linoleic acid content due to methods of calculating conjugation (columns c and d) and due to analytical techniques (columns c and/or d vs. e).

The third section of Table I shows that the amount of conjugation varies with the method of calculation. A larger percentage of conjugation is always noted when the palmitate peak is used as the standard (method 1 under Calculations, column f). The lower amount of conjugation (method 2 under Calculations, column g) is in agreement with Jackson et al. (9) for the alkali isomerization spectrometric method. These authors noted that at elevated reaction temperatures (234 C) the conjugated diene peaks were as much as 8% lower than total diene, and they speculated that conjugated dienes were polymerizing. Another factor favoring calculation by method 1 is the possibility of nonconjugated triene adding to the conjugated diene peaks. In the partially hydrogenated fats listed in Table I, the triene was small and varied from 0.1% to 3.1% when MRA was used for transesterification and analysis.

Although the evidence appears to recommend method 1 calculation, as mentioned previously, it requires two chromatograms of methyl esters to be run, one before isomerization, one after. The results with pure linoleic acid and a 10% mixture of methyl palmitate in methyl linoleate of 96.6% and 95.5% conjugation, respectively, favor method 2 calculation since less experimental time is involved. Therefore, the choice of method for calculating results must be based on prior knowledge of the sample, time allotted for determinations and desired agreement with alkali isomerization spectrometric technique.

The effectiveness of TMAH isomerization for a variety of fatty material was demonstrated with refined soybean oil, soybean fatty acids and soybean methyl esters; they gave values of 95.2%, 96.1% and 96.3% conjugation, respectively.

The chromatogram resulting from the reaction of TMAH and a diene mixture of *cis,cis*-9,15, *cis,cis*-9,12 and *cis,cis*-12,15 methyl octadecadienoates showed a peak for *cis,cis*-9,15 methyl octadecadienoate and two conjugated diene peaks: *cis,trans* or *trans,cis* and *trans,trans*. Conjugation of the *cis,cis*-12,15-diene illustrates isomerization of pentadiene structures at positions other than 9,12 of linoleic acid. The nonreaction of the *cis,cis*-9,15-diene is also found with the alkali isomerization spectrometric method (10).

Figure 2 shows the results of ozonizing the collected

acid. The large number of peaks indicate that the double bond has been scattered throughout the molecule by TMAH at the elevated temperatures. However, scattering of the double bond does not interfere with use of TMAH isomerization for determining total conjugation or linoleic acid content of specific vegetable oils.

TMAH isomerization can also be performed in the injector port of the gas chromatograph at a temperature of 250 C. The injector port reaction is probably the method of choice if an ester analysis has already been performed (method 1) or a standard peak comparison is not necessary (method 2). A definite advantage of the MRA is the rapid transesterification of oils to obtain the reference chromatogram for calculations. Other advantages of MRA over the injection port technique includes those inherent to the MRA, such as its capability of handling microsamples isolated from thin layer chromatographic plates or collected from preparative GLC by direct condensation.

Compared to the conventional alkali isomerization spectrometric method the MRA isomerization GLC procedure handles microsamples easily through its isomerization and GLC analysis steps and has no dilution steps required for spectrophotometry. Like the more accurate and more precise alkali isomerization spectrometric method, the MRA GLC procedure can be performed on a variety of materials–triglycerides, monoesters and fatty acids–with equal ease. This procedure should find important applications in the analysis of hydrogenated fats, surveys of new oilseed crops, production of conjugated fatty acids and similar areas of research and analysis where knowledge of the double bond structure of dienoic fatty acids is required.

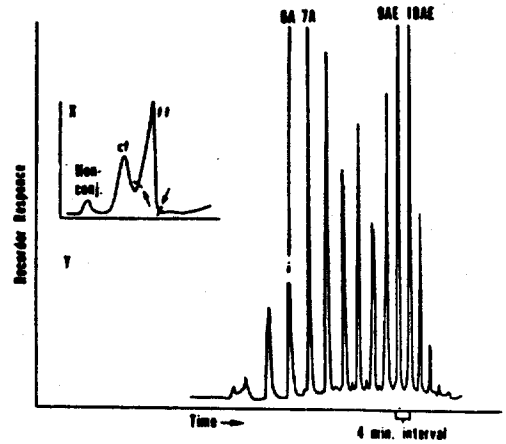

FIG. 2. Ozonization pyrolysis of the collected *trans,trans* conjugated diene peak from the tetramethylammonium hydroxide microreactor apparatus isomerization of linoleic acid. X, insert showing chromatographic result of linoleic acid isomerization. Start and stop collection points noted by arrows on *trans,trans* diene peak. Gas liquid chromatography (GLC) conditions given in text. Y, Aldehyde (A). Aldehydic ester (AE). GLC conditions: flame ionization detector, 4 ft x 1/8 in. column packed with 10% OV-17 on 80/100 Chromosorb WHP (Supelco Inc., Bellefonte, Pa.), 38 cc/min helium flow, temperature programmed from -40 to 280 C.

ACKNOWLEDGMENTS

A.E. Johnston performed the ozonization pyrolysis to determine double bonds. C.R. Scholfield made us aware of the TMAH reagent and gave advice during the course of the work.

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[Received February 11, 1971]

Lipids
5(7): 601-606, 1970

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Quantitative Determination of Glycerol, Mono- and Diglycerides by Gas Liquid Chromatography[1]

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ABSTRACT

Standard chemical procedures for the determination of glycerol, mono- and diglycerides in food products are lengthy, particularly when more than one component has to be determined in the same sample. Gas chromatographic techniques are applicable to these compounds when they are converted into their trimethylsilyl derivatives. A simple, rapid and quantitative gas chromatographic procedure has now been developed which is based on the addition of an internal standard (cholesteryl acetate) to samples which are subsequently silylated. This procedure has been applied to the direct determination of glycerol, mono- and diglycerides in emulsifiers, shortenings and other samples.

INTRODUCTION

Mono- and diglycerides (and mixtures thereof) are common emulsifiers used in shortenings, margarines and other foods. One of the most common procedures for the determination of monoglycerides in these mixtures is based on periodic acid titration (1) which determines the alpha monoglycerides by oxidation of adjacent hydroxyl groups. Beta monoglycerides are not oxidized by periodic acid because the hydroxyl groups are not adjacent. This procedure is an official AOCS Method of Analysis (2). It is quite lengthy and it is further lengthened when glycerol and monoglycerides are to be determined in the same sample.

On occasion, silicic acid column chromatography (3,4) is employed, followed by the gravimetric determination of the fractions eluted. This procedure is even lengthier than the periodate titration and considerable difficulties can be encountered in determining completeness of elution from the column and the weighing of small residues from large volumes of eluates. Both procedures are time consuming and neither satisfied our goal for a quick, simple and quantitative procedure that would determine glycerol, mono- and diglycerides simultaneously in the same sample.

Since 1965, a number of papers on the analysis of monoglycerides as trimethylsilyl (TMS) derivatives by gas liquid chromatography (GLC) have been published (5-14). Most of these reports have dealt with the determination of chain lengths and isomer distributions of fractions first separated by silicic acid column chromatography. Apart from a preliminary reference in a recent paper by Distler and Baur (15), the direct gas chromatographic analyses of mixtures such as the commercial products we will discuss have not been reported. We have now developed a simple, rapid and quantitative procedure for the direct GLC analysis of glycerol, mono- and diglycerides in emulsifiers, shortenings and other samples.

EXPERIMENTAL PROCEDURES

Materials

Hexamethyldisilazane (HMDS) and trimethylchlorosilane (TCS) were obtained from Supelco and glycerol, 99.45% pure, from Lever Brothers Company. Mono-, di- and triglycerides, 99+% pure, were obtained from the Hormel Institute and Supelco. Monoglycerides (Hormel) consisted of about 90% α-isomer; diglycerices (Hormel) consisted of a mixture of 1,3- and 1,2-isomers.

Pure 1,2- and 1,3-dimyristin (Supelco) were

TABLE I

Area-Weight Correction Factors (f) Relative to Cholesteryl Acetate

Sample	(f)	Standard deviation (N=25)
Glycerol	0.50	0.020
Monomyristin	0.84	0.022
Monopalmitin	0.90	0.017
Monostearin	0.85	0.020
Monoolein	0.87	0.043
Cholesteryl acetate	1.00	---
Dimyristin	1.01	0.018
Dipalmitin	1.06	0.022
Distearin	1.06	0.028
Diolein	1.06	0.031
Trimyristin	1.27	---
Tripalmitin	1.31	---

[1]Presented at the AOCS Spring Meeting, San Francisco, California, April 1969.

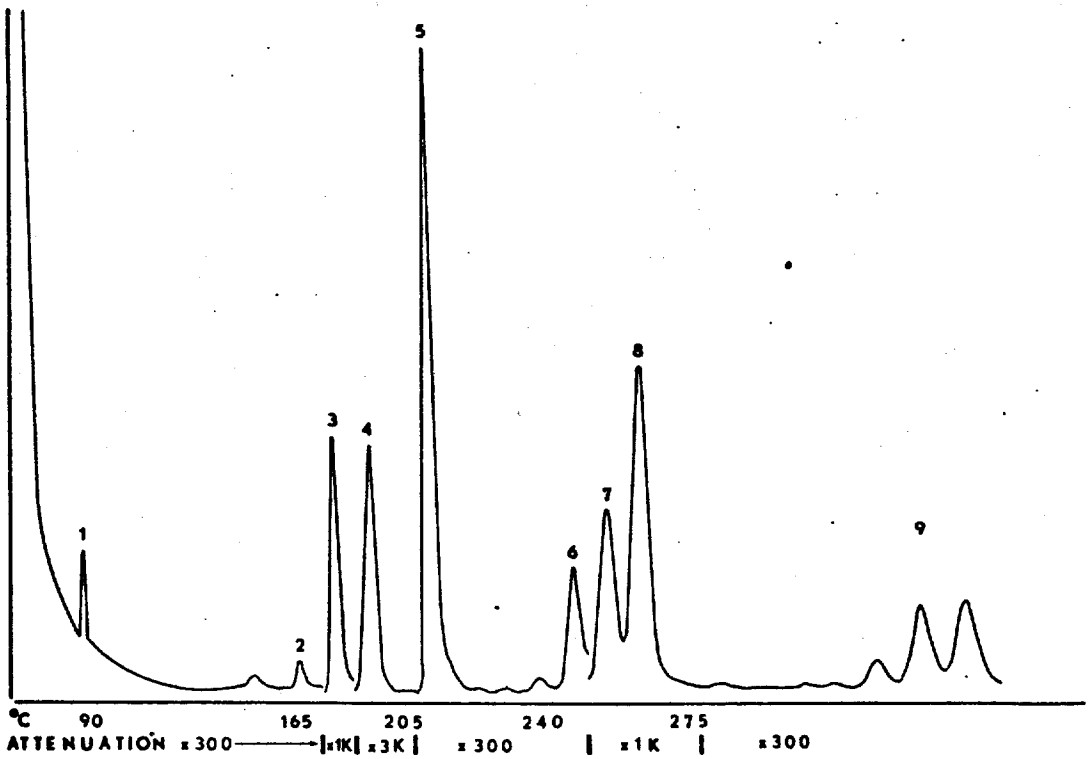

FIG. 1. Chromatogram of emulsifier A after silylation. 1: Glycerol; 2-4: C_{14}, C_{16} and C_{18} monoglycerides; 5: cholesteryl acetate; 6-8: C_{32}, C_{34} and C_{36} diglycerides; 9: triglycerides.

also run independently to determine their retention temperatures. Cholesteryl acetate, 99+% pure was obtained from Hormel. All these standards were used as received.

Gas Chromatography

A Barber Colman Model 5000 gas chromatograph, equipped with hydrogen flame detector and temperature programmer was used in this study. We used a glass column 1 ft x 4 mm i.d. (Glenco) packed with 3% OV-1 on Chromosorb W, 80-100 mesh. The columns were first cured overnight at 350 C before placing them in the chromatograph. These columns had an efficiency of 13,400 plates, as calculated from the cholesteryl acetate peak. The flash heater temperature was about 325 C; the detector was 300 C. Carrier flow rate was measured at 75 ml/min. The temperature program began at 50 C with a simultaneous sample injection: temperature rate, 12 C/min to 300 C. On reaching the end of the temperature program, the column was kept at this temperature until the triglycerides were eluted. Glycerol was eluted at 90 C; monoglycerides between 165 and 205 C; cholesteryl acetate, the internal standard, at about 210 C; and diglycerides between 240 and 275 C.

TABLE II

Analysis of a Known Mixture[a]

Sample	Amount taken	Peak area	(f)	Amount found
	mg	percent		mg
Glycerol	0.35	2.3	0.50	0.27
Monostearin	7.5	35.7	0.85	7.2
Cholesteryl acetate	7.0	30.2	1.00	(7.00)[b]
Dimyristin	7.5	31.8	1.01	7.4

[a]Milligrams found = f (area of component/area of internal standard) mg I.S.
[b]Assumed.

TABLE III

Precision of Analysis of Three Replicate Samples of Emulsifier A (Wt %)

Sample	GLC Procedure 1	2	3	Average	Silicic acid	Periodic acid
Glycerol	0.15	0.16	0.15	0.15	1.5	0.12
Monoglycerides	41.1	40.7	41.4	41.0	44.2	46.9
Diglycerides	41.6	41.2	42.2	41.6	43.7	NA[a]
Triglycerides	6.5	4.4	NA[a]	5.5	10.8	---

[a]Not available.

The silicone rubber septa used in our columns had a tendency to bleed. The material which bled from the septa in the high temperature flash heater area apparently condensed on the upper part of the column which is generally kept at lower temperatures. We noticed that the first run of the day, after the column had been standing overnight at 50 C, gave rise to a group of peaks resembling a homologous series of compounds, particularly between 170 and 230 C. These peaks were eluted when a column was taken through the temperature cycle even when there was no injection. Therefore at the beginning, we always began the workday with a blank run. We attempted to overcome this problem by using septa claimed by a supplier to be specially prepared to reduce bleeding. Use of these septa complicated the situation; they did not hold well to the glass wall of the column due apparently to their composition, and unexpectedly popped up when runs were performed, resulting in a shower of packing and the loss of the column. Finally we totally eliminated the problem by heating the septa overnight in a 280 C muffle furnace.

Detector Response Factors (f)

Calibration mixtures containing different ratios of glycerol, mono-, di and triglycerides vs. cholesteryl acetate were prepared by weight, silylated and chromatographed. For each component, the weight ratio (weight of component/weight of cholesteryl acetate) and area ratio (peak area of component/peak area of cholesteryl acetate) were calculated. The Detector Response Factors (f) were determined by dividing each weight ratio by the corresponding area ratio.

Procedure

The sample is accurately weighed in a 1 dr (approximately 4 cc) vial and 1 ml of internal standard solution, as defined later, is added. For emulsifiers, about 60-80 mg are weighed; for shortenings, between 180 and 200 mg. In both cases, a solution of 200 mg of cholesteryl acetate in 25 ml of pyridine is used as internal standard. For margarines and other products where the level of monoglycerides is expected to be low, about 350 mg of sample are weighed and a solution of 20 mg of cholesteryl acetate in 25 ml of pyridine is used as internal standard. A micromagnetic stirrer is placed in the vial and the sample dissolved with stirring. This process can be sped by placing the vial in a 30 ml beaker containing a few milliliters of lukewarm water. In a matter of seconds the sample is dissolved. Then, and in this order, 0.2 ml of HMDS and 0.1 ml of TCS are added with continuous stirring for 5 extra minutes. The

TABLE IV

Analysis of Emulsifiers by Two Procedures

Emulsifier	Glycerol GLC	Glycerol Periodic acid[a] Lab. 1	Glycerol Periodic acid[a] Lab. 2	Monoglycerides GLC	Monoglycerides Periodic acid[a] Lab. 1	Monoglycerides Periodic acid[a] Lab. 2
08[b]	0.80	0.73	—	90	93	---
18	0.10	0.06	0.12	42	47	45
20	0.13	0.08	0.11	48	47	44
25	0.22	0.22	0.34	43	42	47
26	0.10	0.08	0.12	41	46	44

[a]See Reference 2.

[b]This emulsifier was run in triplicate: glycerol 0.79, 0.81, 0.85; monoglycerides 91.0, 90.6, 89.3.

TABLE V

Recoveries Obtained on Adding Pure Glycerides and an Emulsifier to a Shortening

Sample	Added, mg	Found, mg	Recovery, percent
Pure standards			
Monostearin	7.9	8.1	102
Dimyristin	9.3	8.8	95
Commercial emulsifier			
Monoglycerides	9.5	9.0	95
Diglycerides	9.6	9.5	99
Average			97

micromagnetic stirrer is retrieved and a portion of the supernatant is injected into the gas chromatograph.

RESULTS

Nine calibration mixtures, each composed of four or five components, were prepared by weight, and Detector Response Factors (f) were determined. Table I shows the combined results obtained from all the runs for each compound assayed and the standard deviation of each value. These factors increase essentially with molecular weight or, better, extent of esterification.

To test the accuracy of the procedure, a sample containing glycerol, monostearin, cholesteryl acetate and dimyristin was prepared by weight. The results and calculations are shown in Table II. The first column shows the actual amounts of the four components taken. The next column gives the peak areas obtained and the last column, the amount found.

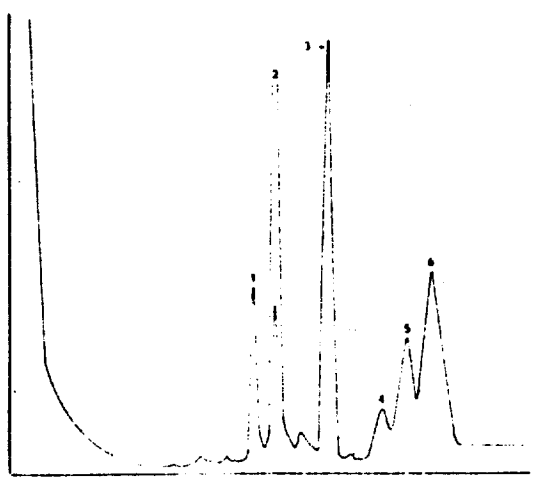

FIG. 2. Chromatogram of a commercial shortening after silylation. 1-2: C_{16} and C_{18} monoglycerides; 3: cholesteryl acetate; 4-6: C_{32}, C_{34} and C_{36} diglycerides.

Figure 1 is a chromatogram obtained from a commercial emulsifier A. All peaks were identified by comparison of their retention temperatures with those of known pure standards. For the middle peak in the diglyceride area (7), we did not have a standard with the same retention temperature. However, the diglyceride fraction of this emulsifier was isolated by silicic acid chromatography. Based on fatty acid analyses of this fraction, we assigned a C_{16}-C_{18} diglyceride structure (C_{34}) to the middle peak.

In our columns, resolution occurred according to chain length and not according to position isomers. This point is illustrated for instance in the peak of C_{18} monoglycerides which represents monostearin, monoolein and monolinolein, with the fatty acids in positions 1 or 2. The resolution of the 1- and 2-monoglyceride isomers, or the 1,2- from the 1,3-diglycerides, known to take place in longer columns, does not take place in our 1 ft columns. The particular column and conditions used in this investigation were selected to provide this separation according to chain length only. Separations other than these were not desirable since they would give rise to GLC curves more complicated and difficult to interpret.

The retention temperatures indicate that cholesteryl acetate is a very good internal standard. Its elution temperature is such that it is eluted immediately after the monoglycerides and a few degrees ahead of the diglycerides. Other internal standards could be used if other emulsifier mixtures are to be quantitated. For instance methyl stearate or stearyl alcohol could be used to advantage if only glycerol and monoglycerides are to be quantitated. Their retention temperatures are such that they peak at about 150-160 C, between the glycerol and monoglyceride peaks.

Emulsifier A was assayed in triplicate in order to obtain an estimate of the precision of the method. Table III shows the results obtained by our procedure. The individual values obtained are listed along with the

TABLE VI

Level of Monoglycerides (%) in Other Samples as Determined by Two Procedures

Sample	GLC	Periodic acid[a] Lab. 1	Lab. 2	Literature
Commercial shortening	2.60[b]	2.8	2.9	
Refined soybean oil	0.13	NA[d]	NA[d]	0.12-0.47[c]
Margarine	0.19	NA[d]	NA[d]	

[a]See Reference 2.
[b]Average of three determinations.
[c]See Reference 16.
[d]Not available.

average. Also included in the table are the results obtained on the same emulsifier when silicic acid chromatography (3) and periodic acid titration (2) procedures were used.

The procedure was applied to five other commercial emulsifiers. The results obtained are shown in Table IV. All these emulsifiers were analyzed for glycerol and monoglycerides by the GLC and the periodic acid procedures. Two operators performed the periodic acid titrations at two different locations.

Figure 2 shows the chromatograms obtained when the procedure was applied to a commercial shortening. In addition to the C_{16} and C_{18} monoglycerides and the corresponding diglycerides, a number of small unidentified components are observed. Some of these overlap into the internal standard region but that can be corrected by making runs without internal standard. In this case, the correction was so small that it could be ignored.

Table V shows the results obtained when a recovery study was carried out. Known weights of pure glycerides were added to a blank shortening, containing very low levels of mono- and diglycerides. The level of addition was approximate to that found in commercial shortenings. In this experiment, recoveries were of the order of 95 to 102%. In another recovery experiment, a known weight of an emulsifier, previously analyzed, was added to the blank shortening. Again, the recoveries were good running from 95% to 99%.

Table VI shows the results obtained with shortenings when analyzed by the GLC and the periodic acid titration method. A refined soybean oil and a soybean oil-based margarine are also included in this table. No periodate results were available for the soybean oil or margarine samples but both results reported here are in the expected range of values.

Figure 3 is the chromatogram of the monoglyceride region of the soybean oil sample. A higher sensitivity was used in the instrument which accounts partially, for the higher baseline. The dotted outline indicates where the internal standard is eluted. A very similar chromatogram was obtained from a soybean oil-based margarine. The high level of other (unidentified) components eluted allows for only semiquantitative estimates of monoglycerides in such samples.

DISCUSSION

Two approaches can be used to obtain quantitative data by gas chromatography on samples such as we have described. The first is to quantitatively isolate a fraction by some

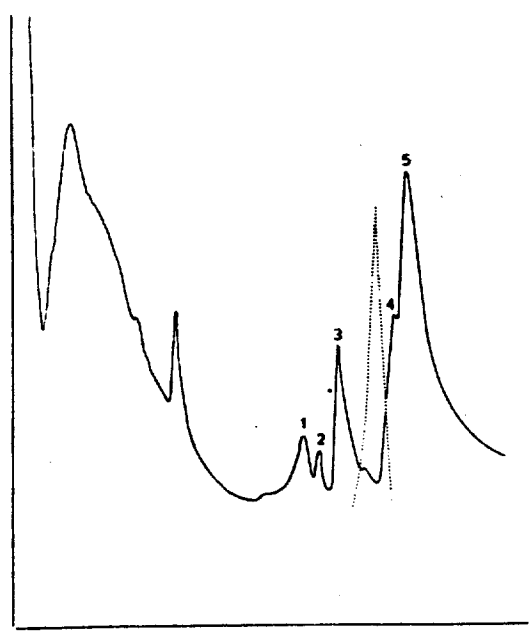

FIG. 3. Chromatogram of soybean oil after silylation. 1-3: monoglycerides; 4-5: diglycerides; dotted line: internal standard.

method such as silicic acid chromatography or thin layer chromatography (TLC) and then perform qualitative analysis on this fraction by gas chromatography. This approach has been used by other workers (6,7,9,11,13,14). It is, however, too lengthy for routine determinations of mono- and diglycerides in industrial samples.

The second approach, which we have followed, is to add a known quantity of an internal standard to the sample and compare the peak areas of the components of interest with that of the internal standard. Consequently, the flame detector response factors, relative to cholesteryl acetate, of glycerol, mono- and diglycerides were determined.

Their absolute values will have little value to other laboratories since it is generally recognized that calibration factors are dependent upon the sensitivity of the instrument and operating conditions and will, therefore, have to be calculated under the conditions prevailing in each laboratory.

The response factors obtained in our experimental conditions showed an increase in value according to the degree of substitution of the hydroxyl groups. Glycerol has the lowest value, indicating that the detector had a high response to this compound. The monoglycerides showed values in between glycerol and that of cholesteryl acetate. The slight intragroup differences according to chain length and unsaturation are probably not significant.

Excellent precision and recovery of glycerol, mono- and diglycerides were achieved. However, it will be noted that the GLC analysis of Emulsifier A only accounted for 88% of the sample. We believe that most of this discrepancy is due to errors in measuring the triglyceride levels, caused in part by volatilization problems on injection and in part by excessive column bleed at the relatively high temperatures required to elute these components. We therefore do not recommend this procedure for determining triglycerides levels.

In our work with margarines and oils, where the monoglyceride level is quite low, large injections were attempted to obtain a readable chromatogram. These large injection volumes and weights caused overloading of our short columns and the peaks were slanted and nonsymmetrical. We consequently reduced the volume of injection in order to avoid this tailing and increased the sensitivity of the instrument. Another difficulty with these samples was that a number of unknown peaks were noticed in the area where the internal peak should be eluted. Other components overlapped into the monoglyceride region. Consequently, under these circumstances only semiquantitative estimates of monoglycerides or emulsifier levels can be performed with these samples.

A simple and rapid procedure for the determination of glycerol, mono- and diglycerides in emulsifiers and shortenings has been developed. The data obtained showed good accuracy for samples where the monoglyceride level was at least 2%. Values obtained when the procedure was applied to oils and margarines do not have such a good accuracy. However, even in these cases, the procedure can be used for rough estimates of monoglycerides levels in these products.

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[Received January 12, 1970]

Arzneimittel-Forsch.
4:643-646, 1954

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Basic effects of the glycols and their relevance to toxicity.

1. Report*)

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1. Introduction.

The multivalent, straight-chained alcohols, the bivalent compounds of which, i.e. those showing two OH-groups, also defined as glycols, became increasingly important in recent years. This fact is due to the easier availability of numerous glycols which can therefore be produced less expensively; it is also due to the expansion of the field of application for glycols as solvents.

In his lecture on "Solvents and dissolving intermediaries" LOESER (10) disclosed that for example, the production of ethyl-glycol in 1937 was negligible as compared to 13 years later when the production of ethyl glycol in the U.S.A. amounted to 235.000 tons per year. In 1951 the capacity of the U.S.A. rose to 340.000 tons. Conditions in Europe are presumably similar, although specific conditions of the war- and post-war years make it difficult to evaluate production figures. In 1951 Western Germany supplied about 2/3 of the of the total quantity produced in Germany during 1940, which amounted to 30.000 tons of ethyl-glycol. The capacity continued to increase in 1951 and 1952; it should be considered in this context that at that time, ethyl-glycol was used for peaceful purposes only. As for the other glycols: basic facts are presumably similar to those determined for ethylene glycol; however, the total quantity of all other glycols was considerably less than the produced ethylene glycol quantity; it amounted to approximately 2/3 in the united economic field (Common Market area) in 1951.

From the large number of the available glycols only a comparatively few compounds are used in practice. Apart from price and availability factors, this is no doubt primarily connected with the characteristics to be utilized, which (characteristics) are not found to the same extent in all glycols.

*) This lecturer's thesis is published in 3 reports; the literature list is found at the end of the 3rd report.

Characteristics which are regarded as especially valuable are: hygroscopicity, reduced freezing point, lubricity, viscosity and dissolving capacity. As a result of the above, the glycols can be utilized in airplane coolers or as liquid coolant respectively, as brake fluid in oil pressure brakes, as softener for cellophane manufacturing, cork pressure disks, paper, as media for preventing the misting and icing of glass panes and also as dissolving agents for the manufacturing of dyes, varnishes,etc. Ethylene glycol and diethylene glycol are especially adequate for these purposes. 1,2 prolylene glycol is mainly used as a dissolving agent, but also as lubricant and for maintaining flexibility, for drugs and cosmetics, principally in the U.S.A. Recently the 1,3 butylene glycol became relevant for similar purposes as well.

While reference is made only to those application fields in which the multivalent alcohols can be utilized as such, i.e. without chemical modification, a remarkable variety of possibilities results for their profitable and meaningful utilization.

If compounds were mentioned here which contain chemically built-in glycols, then their number would continue to rise. For example: besides the simple ethers and esters of the multivalent alcohols which were not referred to so far, the tweens(83,84) should likewise be mentioned, i.e. the compounds of ethereally linked acethylene glycol chains with sorbite and mannite and a higher fatty acid; muscle relaxants similar to guaiacol- or creosol glycerine ethers respectively,(commercial names: Myoc in (56 - 58, 107), Myanesin (69,70),cystostatica of the Myleran type (55,62) ($CH_2SO_2OCH_2CH_2OSO_2CH_3$, i.e.a derivative of the 1,4 butanediol). Finally, reference should be made here as well to the efforts aimed at making purine bodies water-soluble with the aid of glycols. (7-oxyethiltheorphylline= "Cordalin",(see 10). However, only a brief reference is made to these substances, despite occasionally observed coincidence with the known effects of multivalent alcohols, because their action is quite specific.

It does not seem surprising, considering the wide-spread use of multivalent alcohols, primarily in technology, that clinical medicine was likewise prompted to deal with part of these substances

because some poisoning occurred. Characteristically, however, these cases did not involve industrial toxicity in the stricter sense of the term which develops to a factually expected extent when handling multivalent alcohols; instead, the toxic symptoms in question resulted from misuse. The characteristic alcohol-effect, an intrinsic property of the glycols, causing intoxication, acts as a temptation to consume various technical products. Therefore the most widely used glycol, the ethylene glycol, is held to be responsible for numerous serious poisoning cases. But it must be taken into consideration thereby that the glycol at issue probably often was not a pure glycol, nor even a low-grade contaminated glycol, but a mixture of various substances, including glycols, among others. Poisoning cases occurred even after the medically erroneous or imprudent use of glycols. It is remarkable that only a few of the listed bivalent alcohols caused serious intoxications, namely: ethylene glycol (18 - 21, 23 - 26, 28 - 29, 32 - 33), diethylene glycol (17,22,31,36,37), 1,4 butylene glycol (27,30), while others had no such effect (1,2 propylene glycol, 1,3 butylene glycol). The above circumstances might be partially explained by the especially frequent use of ethylene- and diethylene glycols, and by the resulting easy access to the same; in part, however, action variations no doubt are largely to be held responsible as well.

Despite the fact that the poisoning symptoms evident in humans after the intake of larger glycol quantities show some specific aspects, a profound coma is the most characteristic symptom, according to general (medical) opinion. However, differences exist regarding the course of the intoxication: on the one hand, the comparatively speedy start of dizziness, soon followed by death after gradual onset of unconsciousness; on the other hand, dizziness followed by a period of restored consciousness; after a stage of aneurysm in the course of the next few days, death in a deep coma follows, or--in a few cases--full recovery takes place. Accordingly, an acute, a sub-acute as well as a chronic course of the poisoning undoubtedly exist, at least in cases of ethylene glycol intoxication (see 34), a larger number of which cases has been described to date. Furthermore, it results, with some degree of certainty, from poisoning reports and section protocols, as well as from the success of certain therapeutic measures that kidney

damage is involved in cases of sub-acute intoxication. The following facts argue in favor of the above: the aneurysm symptom, death occuring during uremia, the occasional therapeutic success with kidney capsulation, and finally, the quite regular pathological-anatomical diagnosis of an epithelial degeneration in the kidney tubules. Specifically, the pathogenesis remains largely uncertain and obscure.

Not only the poisoning cases, but also the question: to what fields could the use of multivalent alcohols be extended, besides their purepy technical application--demand that the origin of their toxic effect be clarified. Only when the action mechanism of the multi-valent alcohols becomes known will it be possible to make positive statements on those quantities and uses,etc., which can be evaluated as harmless. Only then can it be decided whether or not their use for public health purposes is justifiable.

II. Basic effects of the glycols.

Numerous works are available since the turn of the century dealing with the effect of bivalent alcohols (48,49,51,66 - 69,87,88,94, 95, 104, 105), in particular with the effect of ethylene glycol (64,65,73,96,97), 1,2 propylene glycol (45,89,101,108,110), diethylene glycol (91), as well as - later - with the effect of 1,3 butylene glycol (41,52,71,82,90). Without discussing details, the following joint result can be found: the multivalent alcohols, as do the univalent alcohols in larger quantities, have a distinct narcotic effect; they cause urine surge and after parenteral supply--and only then--result in hemoglobinuria. The only exception with the glycogen to be discussed here is the lack of a hemoglobinuria after (the intake of) 1,3 buthylene glycol (40).

With the above-mentioned exception, these three basic effects are characteristic for all three glycols, but the predominance of one or another effect varies. An attempt will be made to trace the specific effect of the individual glycols according to their characteristics and basic action. While it seems obvious to consider various derivatives as an explanation for the various glycol effects, evidence to confirm this supposition regarding toxicity is still missing, despite years of research.

While oxalic acid can undoubtedly form in the organism, for example: from ethylene glycol (50,75,100), it is equally certain that the formed oxalic acid quantity is too small for toxicity (50,75,109). The other multivalent alcohols, with the exception of glycerine, do not form oxalic acid; this eliminates said simple explanation for the toxicity of glycogen in the form of oxalic acid. The above presumably applies likewise to other derivatives of the multivalent alcohols as well.

The action analysis*) shown below is implemented with the bivalent alcohols ethilene glycol (ethanediol, CH_2OH . CH_2OH), diethylene glycol (CH_2OH . CH_2. O . CH_2.CH_2OH) and with butylene glycol (L,3-butanediol, CH_3. CHOH . CH . CH_2CH). The trivalent alcohol glycerine served as control substance (anhydrous 1,2,3-propanediol, CH_2OH . CHOH .CH2OH). This well-known triol, occurring in natural fats, was used for comparison, not only because of its usability which corresponds in numerous cases to the usability of the glycols, but also because much experience was gained in humans with said substance. The fundamental reasoning thereby was: the inclusion of glycerine (see 77,98), considered practically non-toxic for humans, into the animals tests should be valuable for the determination and classification of test findings obtained with glycols, or concerning their transferability respectively.

a) The narcotic effect.

It was mentioned earlier that the most comprehensive symptom of glycol poisoning is the narcosis. It occurs during tests with rats as well, after the administration of ethylene glycol, diethylene glycol, 1,3 butanediol, and even of glycerine. Dizziness and sluggishness, together with insecurity in motion, is observable in animals, depending on the dose, frequently after 20 to 60 hours already, but always within a few hours. Reactions to stimulation become weaker and clumsier; dizziness intensifies later, to a condition of unconsciousness; the animals lie on their bellies or sides and remain mostly for several hours in this condition.

*) Test animals: more than 700 rats (mostly ♂); individual cages, warm stable, at least 22°C. Food in general: standard biscuits, baked from oat flake, corn meal, powdered milk, dry yeast, and salt mixture, n. McCollum, and cod-liver oil, tap water ad libidum.

Excited conditions and convulsions do not necessarily result from any of the investigated substances, but are neverthless found in sporadic cases, with ethylene glycol as well as with diethylene glycol and glycerine. Death occurs during increasingly deep narcosis for animals which succumb to said acute poisoning. Already several hours before their death the test animals feel cold to the touch; the ears and the visible mucous membranes become pale; respiration and heartbeat finally stop (central paralysis).

The fate of the test animals--life or death--within a certain period of time, mostly within 24 hours, is the subject of the acute toxicity determination of a substance, from which the average lethal dose is calculated.

The average lethal dose (= LD_{50}) is understood thereby as the quantity of the substance which kills 50% of the test animals within the scheduled period of time. In practice said value is computed as follows: increasing doses of the compound in question are administered to groups of 5 to 10 animals each, whereby according to KARBER a number of intermediary groups are inserted between a group within which all animals are destroyed and the group in which all animals survive. It seems understandable that the reliability of the indication "average lethal dose" increases simultaneously with the increasing number of animals.

Table 1 shows a comprehensive compilation of the average lethal doses (determined according to KARBER(78), calculated according to BURN (47) for various multivalent alcohols; the observation time is 24 hours.

Table 1: LD_{50}(24 hours) of various multivalent aclcohols for the mouse and rat (cm^3/kg body weight):

Tab. 1: LD_{50} (24 h) verschiedener mehrwertiger Alkohole bei Maus und Ratte (cm^3/kg K.-gew.)

1 Substanz	2 LD_{50} Maus p.o.	Maus sc.	3 Ratte p.o.	Ratte sc.
3 Acthylenglykol $CH_2OH \cdot CH_2OH$	13,79	—	—	—
4 1.2-Propylenglykol $CH_3 \cdot CHOH \cdot CH_2OH$	30,76	—	—	—
5 1,3-Butylenglykol $CH_3 \cdot CHOH \cdot CH_2 \cdot CH_2OH$	23,31	16,51	29,42	20,06
6 Diacthylenglykol $CH_2OH \cdot CH_2 \cdot O \cdot CH_2CH_2OH$	25,23	20,16	23,70	18,06
7 Glycerin (wasserfrei) $CH_2OH \cdot CHOH \cdot CH_2OH$	20,71	—	—	—
8 Glycerin DAB VI*)	30,21	10,78	46,72	16,69
9 1,2,4-Butantriol $CH_2OH \cdot CH_2 \cdot CHOH \cdot CH_2OH$	23,31	12,64	—	11,37

1. Substance 2. Mouse 3. rat
4. ethylene glycol
5. 1,2 prolylene glycol
6. 1,3 butylene glycol
7. glycerine (anhydrous)
8. glycerine DAB VI*)
9. 1,2.4 butane triol

*) Glycerine DAB VI is an 84% to 87%, on the average 85% watery solution of glycerine.
The data originate from the works of L. FISCHER, R. KOPF, A. LOESER and G. MEYER (52), G. BORNMANN, A. LOESER and G. MEYER (42) and LOESER and collaborators respectively (91).

While the hypothetical postulate of the acute intoxication when using various multivalent alcohols is largely coincidental, important differences become evident in the further course of the poisoning, within those animals which survive 24 hours. While the question whether or not the test animals will survive is decided (with 1,3 butane diol and likewise with glycerin use) during the narcosis stage (which can last, however, in sporadic cases somehwat longer than 24 hours, but which generally terminates within said period of time), an observation period of 24 hours concerning the fate of rats poisoned with ethylene glycol or with diethylene glycol will yield decisive results only in case of death. The toxicity determination for rats observed for 10 days after a single oral administration of diethylene glycol shall serve as an example (Table 2). The basic result is immediately evident; the dyethylene volume which is tolerated by the test animals during the

first 24 hours can still have a lethal effect during the next few days. Simultaneously with increasing doses the number of animals succumbing on the first day after the poisoning increases; the zone of a dose which is lethal within 24 hours is reached only with very large doses.

Table 2: Course of the toxic effect after a single oral administration of various diethylene glycol quantities to rats.

Tab. 2: Ablauf der toxischen Wirkung nach einmaliger oraler Zufuhr unterschiedlicher Mengen von Diaethylenglykol bei Ratten

1 Dosis cm³ je kg K.-Gew.	2 Anzahl der eingegangenen Tiere am 3 1. Tag	2. Tag	3. Tag	4. Tag	4 Anzahl der überlebenden Tiere
15	0	2	7	1	0
18	0	7	3	—	0
21	3	5	2	—	0
24	0	5	4	—	1
27	4	6	—	—	0
30	3	6	1	—	0
33	4	4	2	—	0
36	10	—	—	—	0

1. Dose, cm^3 per kg of body weight
2. Number of animals which succumbed on
3. . . . day
4. Number of surviving animals

A somewhat similar statement applies to ethylene glycol as well. Of 15 rats which had received 6,0 cm^3 ethylene glycol per kg of their body weight, only 4 animals subbumbed on the first day; but 10 animals succumbed between the 2nd and 9th day following the administration of the glycol, while 1 rat survived.

Despite the large number of toxicity determinations carried out during recent years, no corresponding data could be obtained when using 1,3 butylene glycol or glycerine. The lethal effect occurs occasionally after somewhat more than 24 hours, i.e. on the 2nd day. But on the one hand, this applies to a very few animals only, while on the other hand the course of the intoxication is always quite continuous. Death occurs during the deepest narcosis, immediately coinciding with the standard intoxication pattern.
At variance from the above, in case of the animals which received ethylene glycol and diethylene glycol a period of apparent relative well-being occurs between the described acute aspects of the poisoning and the debility and numbness which precede death.

A lethal poisoning can in fact occur with diethylene glycol without previous evidence of anything more serious than a low-grade "narcosis".

It seems justified according to the above statements to assume a two-stage acute intoxication for ethylene and diethylene glycol and to attribute an early and a late toxicity to these two substances, while the toxic effect of glycerine and 1,3 butanediol occurs in a single stage, in the form of an early toxicity only. No doubt can pervail,according to the intoxication pattern and also on the basis of findings in poisoned persons, concerning the type of said early toxicity; it is the characteristic narcosis death, i.e. a central paralysis.

It should be considered hereby that a period could and presumably does exist between the narcotic dose(according to the narcosis doctrine)and the dose causing central paralysis, the length of which (period) varies. In any case, investigations concerning the sedative effect of comparatively small glycol quantities (43), tested for anti-convulsive action (for example: the suppression of Cardiazol-convulsions) speak in favor of the centrally attenuating effect of 1,4 butanediol, even when administered in smaller doses as compared to other glycol doses, while a centrally paralysing toxic effect occurs only after the administration of very large quantities.

It can be stated in general, without going into these particulars; when a multivalent alcohol has a predominantly central paralysis effect, the toxicity pattern is decided at the moment when the highest concentration of the tested substance predominates in the organism, possibly also in a specific tissue. This interpretation is certainly a reasonable assumption, if it is taken into consideration that the substances in question are administered only once, that their excretion begins soon--in less than 2 hours--and terminates, for the most part, within 24 hours. The resorption of the undiluted alcohols, however, presumably starts after a certain delay, after which it occurs with comparative speed.

The above fact results, for example from the finding that after a subcutaneous application of undiluted 1,3 butanediol approx. 30 minutes will elapse until the mentioned anti-convulsive effect can be verified.

Early toxicity and its course can be explained with the known facts concerning the resorption and excretion of multivalent alcohols. This interpretation, however, is not sufficient to explain the delayed toxic action (late toxicity); it can hardly be due to the presence of ethylene glycol or diethyl glycol, and can therefore not be ascribed to the narcotic effect. A detectable elimination, however, still takes place on the 2nd day after the administration of said glycols; subsequently, it can be assumed that the organism will be largely free from glycols. The course of the intoxications as well as the facts known concerning the resorption and elimination of the glycols therefore indicate that late toxicity demands an interpretation which differs from the interpretation of early toxicity; it will presumably have to be specified as an impairment which became, to a considerable extent, independent from the presence of the active substance at issue-- derivatives are not taken into consideration, for reasons already known. .

Two different methods are principally used for the detection of chronic toxicity, i.e. in case of toxic effects resulting from the repeated, long-term administration of the test substances. With the first method, the animals receive certain qunatities of the substance under investigation in intervals of one or several days, administered with the esophagus probe (Procedure I); with the second method, the test substances are admixed to the drinking water (Procedure II). The two methods differ insofar as in the first case, the test animals receive standard drinking water after the administration of the test substance, while in the second case each intake of drinking water coincides with a forcible administration of the test substance. Accordingly, extreme and unusual conditions pervail here.

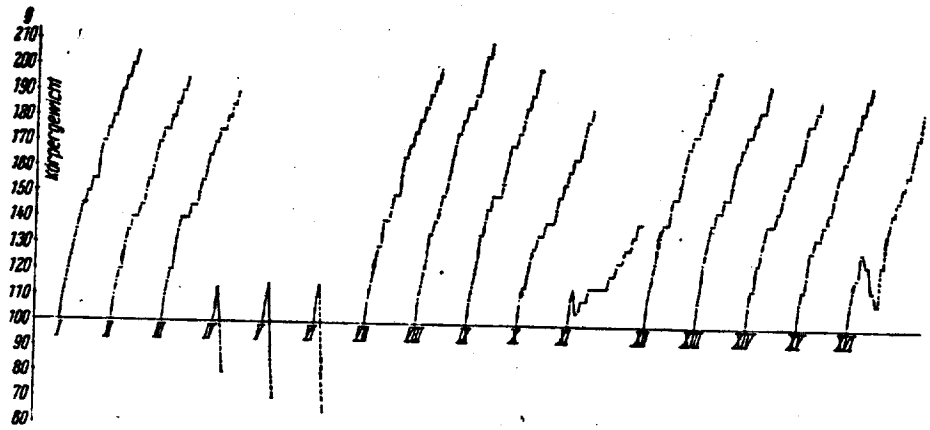
It is a metter of course that experiences gained during the examination of acute toxicity are taken into consideration when suggesting such tests, i.e. experiences suggesting that single doses should be definitely below the toxicity level, and on the other hand, that within certain periods of time, a total volume corresponding to an acutely toxic dose of the tested substance be administered.

After the fractioned administration of such quantities during the tests according to Procedure I no definitely narcotic symptoms can be found either with 1,3 butanediol or with diethylene glycol.

However, after supplying ethylene glycol or diethylene glycol with the drinking water (Procedure II), a rapid loss of weight, numbness and lethal poisoning of the animals occur, provided that more than 2% of said glycols was added to the drinking water. 1,3 butylene glycol, even with a 10% content, has no effect; nor does glycerine. It is possible that the 20% solutions of these multivalent alcohols also have a certain effect, because the weight gain of the animals shows some--temporary--impairment, and some animals (1 or 2 respectively, in each case) perish. Ill. 1 displays the body weight behavior for such "drinking water animals".

In the course of the drinking water tests 12 rats in each case (6♂, 6♀) were offered watery 1-, 2-, 5-, 10- or 20% solutions of diethylene, glycol, or 1,3 butylene glycol or glycerine respectively, as drinking water. Furthermore, an additional test was carried out to determine the effect of drinking water with a 5% ethylene glycol content.

The aspect of the poisoning case which emerges in the diethylene glycol-animals in the course of 3 to 5 days is characterized by the increasing decrepitude of the rats; they hardly react to stimulation any more, but up to the terminal stage they appear to be weakened rather than under a narcotic effect. The weight loss which is noticeable already on the 2nd day and which becomes increasingly evident later, cannot be explained, or at least not entirely explained by nutritional deficiency; it can hardly be ascribed to a below-threshold narcotic effect.



Ill. 1.: Effect of diethylene glycol, 1,3 butylene glycol or glycerine respectively, in drinking water on the body weight of rats (diluting agent: aqua dist; control: aqua dist; test period: 96 days. Weighing: twice weekly, in intervals of 3 to 4 days. Each point of the curves corresponds to the average body weight of 12 rats (6♂,6♀). Shading of the curve indicates, that the animal group is no longer complete. I = control(aqua dist); II - VI = watery diethylene glycol solution, percentage: 1,2,5,10 and 20% respectively; VII - XI = watery 1,3 butylene glycol solution, percentage: 1,2,5,10 and 20% respectively; XII - XVI = watery glycerine solution, percentage: 1,2, 5,10 and 20% respectively. Start of the test substance supply after an average body weight of 115 g is reached.

1. Body weight.

Finally, a third factor must be considered: if it is assumed that the results of acute tests and their interpretation are correct, that accordingly the LD_{50} explains the narcotic effect, then the diethylene glycol, the 1,3 butanediol and the glycerine which have a practically the same acute LD_{50}, should act identically, or at least in a very similar way in the drinking water, to justify the supposition which ascribes the narcotic effect to the findings obtained here. The above, however, is not the case; therefore an effect at variance from the narcotic action must be presupposed on the basis of this reasoning.

It can be stated, when summarizing the results obtained to date, that the narcotic or centrally paralyzing effect which is a fundamental property of all multivalent alcohols under investigation, is unquestionably decisive concerning early toxicity in acute poisoning cases. Said early toxic effect generally reaches and exceeds its maximum within approximately 24 hours. In this context--and presupposing the oral administration of the multivalent alcohols--LD_{50}(24 hours) is the criterion for the narcotic or centrally paralyzing effect respectively, of the alcohols under consideration.

Following a parenteral application, the (time) limit seems to advance slightly, since on the one hand the early toxic effect occurs faster with this method; on the other hand,however, an eventually present late toxic effect becomes likewise evident earlier. No precise data concerning the chronological limit can be supplied. It should be noted that the toxic effect rate of parenterally administered multivalent alcohols is higher as well.

Neverthless, the narcotic or centrally paralyzing action respectively, is obviously without substantial importance for a late toxic effect to be specified for certain glycols; nor is it essential for the chronic toxicity to be detected in the same glycols (ethylene glycol, diethylene glycol) under the specific conditions of the drinking test. Considering the quantities used, such an effect could be considered possible primarily when the alcohols accumulate in the organism. No clue is found for the above.

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From the pharmocological institute of the University of
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CONCERNING THE EFFECTS OF BI- AND TRIVALENT ALCOHOLS ON THE INTESTINES

by Gerhard Bornmann, Rudolph Kopf & Gerda Meyer

with one illustration

(December 23, 1949)

During the course of research on the pharmacology and toxicology of polyvalent alcohols, one can establish, among other things, that bivalent and trivalent alcohols cause certain special effects besides the known characteristic effects of the genre. Thus it can be shown that certain bivalent and trivalent alcohols, after a subcutaneous injection, cause hemoglobinuria, while others don't; it can be shown that some quickly lead to deadly toxification[4], even in small quantities, while others can be tolerated even in large doses.[1,3,5] The same distinctions were shown in experiments in which the effect of some alcohols with 2 or 3 OH groups were tested in situ on the isolated rectum as well as on the intestine itself of different animals.

RESULTS

The experiment used the bivalent alcohols ethylglycol, 1,2 propylenglycol, 1,3 butylglycol, 1,4 butylglycol and 2,3 butylglycol; and the trivalent alcohols 1,2,3 propantriol (glycerine) and 1,2,4 butantriol.

1. Isolated intestine.

A freshly extracted piece of a rat's rectum, 2-3 cm. in length, was placed in a solution of tyrodel and kept for two hours in a refrigerator at a temperature of +4°C. Then it was placed in an intestinal bath of 40 or 50 cm.[3],

kept at a temperature of 37°C. Its motor activity was registered and evaluated in the usual way, according to the method of Magnus.

Under the influence of the bivalent alcohols tested by us, the intestinal movement changed as follows. The intestine, after the addition of the substance, either showed an immediate spastic contraction (ethylglycol, 1,2 propylenglycol) or it relaxed (1,3 and 1,4 butylglycol) or, after a transitory contraction, it relaxed (2,3 butylglycol). After preliminary experimentation had shown that a concentration of the bath fluid of 1:50 was particularly suitable, we chose this concentration to use with all the substances examined, whereby, if necessary, the concentration could be increased to a recognizable effect.

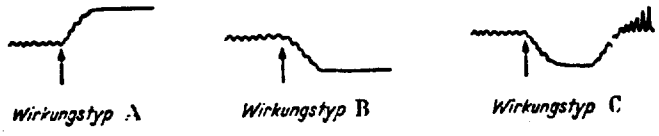
In particular, the results were as follows. The addition of ethylglycol or 1,2 propylenglycolled to a more or less pronounced contraction, which did not revert spontaneously, but only after being rinsed. 1,2 propylenglycol (0.33 cm^3 to 50 cm^3) worked surely and clearly, while stronger concentrations of ethylglycol were necessary to produce the same effects.

Under the influence of 1,3 and 1,4 butylglycols (0.33 cm^3 to 50 cm^3) the movements of the intestine became progressively weaker and the tone decreased. With a concentration of 1:80 the described changes were more pronounced, and the intestine relaxed. 2,3 butylglycol (0.25 cm^3 to 40 cm^3) had very little influence on the tone and motor activity of the intestine. Stronger concentrations, after a transitory increase in the tone, caused a decrease in the tone and the cessation of all motion.

Of the trivalent alcohols, 1,2,3 propantriol (0.33 cm^3 to 50 cm^3) caused the movement of the isolated rectum to cease and caused the intestine to become somewhat more relaxed; it then spontaneously reversed these changes with an occasionally stronger intestinal motor activity.[3,6] The 1,2,4 butantriol (0.33 cm^3 to 50 cm^3) produced similar effects: the butantriol, after a transitory decrease of tone and a cessation of the movements of the intestine -- with a uniformly weaker concentration -- often brought the intestine to increased activity.

On the basis of this observation, we have attempted to represent schematically the effects of the tested alcohols on the isolated rectum (Illustration 1).

Illustration 1: Tone and motility of an isolated rectum after the introduction of (↑) different polyvalent alcohols shown in schematic representation. Ethyl and 1,2 propylenglycol: type A; 1,3 and 1,4 butylglycol: type B; and 1,2,4 butantriol: type C.



Type A is meant to show the influence of ethyl and 1,2 propylenglycol, type B the influence of 1,3 and 1,4 butylglycol. In our research, 2,3 butylglycol produced results similar to 1,3 and 1,4 butylglycol; the only difference is that before the decrease in tone, there is a more or less pronounced, quickly passing, contraction of the intestine. The effect of the trivalent alcohols, 1,2,3 propantriol and 1,2,4 butantriol is characterized by type C.

2. Laxative effects on the entire animal.

An increased activity of the lower colon manifested itself in the entire animal in this way: the processed feces were excreted. In systematic, orderly investigations the polyvalent alcohols that were selected at the beginning were administered rectally, and their effects on different animals examined.

Our method was as follows. After withdrawing the feces from the rectum of rats and guinea pigs, we gave them a strong enema (5-7 cm) of the substance to be tested (0.5 cm^3 to rats, 1.0 cm^3 to the guinea pigs). For the duration of the examination, the rats were urethrally anesthetized, and fixed on their backs. We observed the unanesthetized guinea pigs in individual cages. The dogs received 5.0 cm^3 of the substance to be tested; it was administered rectally with a probe (12 - 15 cm long). They were not anesthetized.

Of the bivalent alcohols in the above described process, only one showed a laxative effect. The alcohols had different effects according to the different kinds of animals: ethyl and 1,2 propylenglycol showed a laxative effect on rats and dogs; with the guinea pigs a laxative effect could be seen only occasionally.

In contrast, 1,3 and 2,3 butylglycols were ineffective with dogs and rats; with the guinea pigs, without causing toxic symptoms, these alcohols cause, in individual cases, the evacuation of the intestine. Similarly, 1,4 butylglycol was ineffective in the tests on the rats, but worked with the guinea pigs.

In our opinion, these findings must be evaluated with the greatest caution, because the guinea pigs, as a result of the high toxicity of 1,4 butylenglycol, were already drugged when the laxative effect began to appear, and later showed signs of severe intoxication. We are probably quite correct in assuming that the emptying of the intestines in this case was the result of progressive toxification. On the basis of the general toxic effect of 1,4 butylenglycol, we undertook no experiments on the dogs with this substance. 1,2 butylenglycol had no effect, as our preliminary experiments showed.

Trivalent alcohols 1,2,3, propantriol (glycerine DAB VI was used) and 1,2,4 butantriol had a laxative effect on rats, dogs and guinea pigs, and worked even when the rectum was obviously empty and the feces had to be removed from a higher section of the intestine.

In Table I we have collated the results of our investigation into the laxative effect of polyvalent alcohols, rectally administered. From this table one can deduce that all bivalent alcohols stemming from butan obviously do not have a laxative effect, while the lower bivalent alcohols cause an emptying of the intestines. From these observations, a certain regularity of the effects on the intestine can be obviously deduced; for example one can think of bringing the laxative effects of bivalent alcohols into connection with the length of their carbon chain. In our opin ion such a process would be premature until further relationships of this kind were tested.

Certainly it would be a mistake to look upon the established common aspects of butylglycol, with reference to its effect on the intestines, as given with respect to other pharmacological questions. For example, the examination of the toxicity of this relationship has already shown

that 1,3 butylenglycol is quite tolerable, while 2,3 butylenglycol and especially 1,4 butylenglycol are considerably toxic.

Table 1: Laxative effect of polyvalent alcohols.

Comparative substance: physiological table salt.

Substance	Quantity of substance dispensed per animal	Experimental animal	Number of animals	Laxative effect
Äthylenglykol	0.5	Ratte	10	+
	5.0	Hund	3	+
	1.0	Meerschweinchen	10	fraglich #
1.2-Propylenglykol	0.5	Ratte	10	+
	5.0	Hund	3	+
	1.0	Meerschweinchen	8	fraglich #
1.3-Butylenglykol	0.5	Ratte	10	—
	5.0	Hund	3	—
	1.0	Meerschweinchen	10	fraglich #
1.4-Butylenglykol	0.5	Ratte	10	—
	1.0	Meerschweinchen	5	+ *
2.3-Butylenglykol	0.5	Ratte	10	—
	5.0	Hund	3	—
	1.0	Meerschweinchen	5	fraglich #
1.2.3-Propantriol (Glycerin DAB VI)	0.5	Ratte	10	+
	5.0	Hund	3	+
	1.0	Meerschweinchen	13	+
1.2.4-Butantriol	0.5	Ratte	10	+
	5.0	Hund	3	+
	1.0	Meerschweinchen	10	+
0.9%ige NaCl-Lösung	0.5	Ratte	10	—
	5.0	Hund	3	—
	1.0	Meerschweinchen	5	—

* Agonale Defäkation.

= doubtful

Summary

The investigation of the effect of different polyvalent alcohols on the intestines show that:

1. Of the bivalent alcohols, some (in reference to their acute toxicity, they are less tolerable that glycerine, ethylglycol) have a laxative effect; 2,3 butylenglycol and 1,4 butylenglycol do not have a laxative effect.

2. Of the bivalent alcohols, some (in reference to their acute toxicity, they are just as tolerable as glycerine, 1,2 propylenglycol) have a laxative effect; 1,3 butylenglycol do not have a laxative effect.
The laxative effect of the named bivalent alcohols is only certain with omnivores (dogs, rats), whereas it is not certain with herbivores (guinea pigs).

In contrast to the investigated bivalent alcohols, the trivalent alcohols 1,2,3 propantriol (glycerine) and 1,2,4 butantriol, whose toxicity is not essentially to be distinguished from one another, certainly have a laxative effect upon rats, dogs and guinea pigs.

With the same distinctions as on the whole animal, the polyvalent alcohols that were investigated have an effect upon the isolated rectum of a rat in the arrangement according to Magnus.

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Bioassay of a Hemostatic Factor from Peanuts

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Received February 8, 1960

Extreme vasoconstriction is induced on the transection of arteries of the cheek pouch of bilaterally adrenalectomized male hamsters that have ingested an absolute alcohol extract of defatted peanuts. This is compared with slight or moderate vasoconstriction observed with control bilaterally adrenalectomized male hamsters. Apparently the vasoconstrictor from peanuts is released from storage in the blood platelets during their viscous metamorphosis in the formation of the platelet plug. The active factor is also myotonic to smooth muscles.

INTRODUCTION

The existence of a hemostatic factor in peanuts is indicated in the report by Boudreaux and Frampton (1) of remissions of clinical symptoms of hemophilia in several AHF hemophiliacs when they consumed peanut products during hemophilia attacks. Additional evidence for the presence of a hemostatic factor in peanuts has developed in the course of a search for a bioassay for the peanut constituent which is responsible for this apparent hemostasis.

METHODS AND RESULTS

Methods

The preliminary studies of the effects of ingested peanut products on blood vessels indicated that use could be made of the cheek pouch of the golden hamster [*Mesocricetus auratus* (Waterhouse)] in an investigation of hemostasis induced by peanut products. The cheek pouch can be exposed and the effects of severing blood vessels in the pouch membrane may be observed under the microscope. Male golden hamsters of the Louisiana State University strain were used.

The animals were anesthetized with Nembutal for observations, and laid on a paraffined balsa wood platform, one end of which was hollowed out to form a shallow moat. A half-inch square Plexiglass window was built into the bottom of the moat [a modification of the technique of Lutz *et al.* (2)]. This permitted the transillumination of the exposed cheek pouch. The head of the hamster under study was laid on the edge of the depressed portion of the platform, and the exposed cheek pouch was spread out over the window and anchored to the bottom of the moat where the exposed cheek pouch could be washed with Ringer's solution while observations were made on the blood vessels with the aid of a stereoscopic microscope.

Most of the investigations reported here were carried out with an extract prepared from lightly roasted peanuts. The skin-free cotyledons and germs were flaked. The oil was extracted from the flaked material with commercial hexane. The resulting meal (moisture content of about 10%) was then exhaustively extracted with 95% ethyl alcohol at room temperature. The residue was discarded. The alcohol content was reduced by evaporation at room temperature and low-temperature vacuum distillation. The final drying of the extract was by lyophilization. This powder, which

[1] One of the Laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

represented about 3.5% of the whole peanuts, is referred to in this paper as the primary extract.

Where the primary extract was used, it was dissolved in water (100 mg./ml.) and administered orally with the aid of a blunted No. 18 hypodermic needle and syringe at the rate of 50 mg./day/100 g. body weight in three doses 8 hr. apart. The hamsters were held vertically and drank from the tip of the needle.

Vessel bleeding time for one pouch of each of the control and experimental animals was determined after 4–5 days on the primary extract regimen. The feeding of the groups was then reversed so that, whenever possible, each animal served as his own control, and the bleeding time of the vessels in the opposite cheek pouches was determined. Approximately ten transections were made at random in each pouch.

The reduction in the bleeding time, as measured in these experiments, was too variable for usefulness in a bioassay of the hemostatic factor. There was observed, however, an enhanced vasoconstriction at the site of the transection of the blood vessels in the animals receiving the primary extract.

This enhanced vasoconstriction at the site of the transection in the primary extract-fed animals may have been partly masked by the effect of 5-hydroxytryptamine, since this myotonic amine is reported to be released from platelets during the formation of the platelet plug (3) and to induce vasoconstriction of the transected vessel. Accordingly, hamsters were bilaterally adrenalectomized, since adrenalectomy markedly reduces the 5-hydroxytryptamine content of the platelets (4). One group of the adrenalectomized hamsters received regular laboratory chow, while the other received an aqueous solution of the primary extract (at a rate of 75 mg./day/100 g. body weight) for 2 days in addition to laboratory chow. One cheek pouch from each was exposed, and the main artery was transected in each successively toward the base. Note was taken of the degree of vasoconstriction during and following the formation of the platelet plug at the end of the severed artery. The feeding of the two groups was then reversed so that, whenever possible, each animal served as his own control.

Results

Data recorded in Table I for 130 vasoconstriction responses show that vasoconstriction was much more pronounced in the animals receiving the primary extract. The symbol (−), in this table and in the succeeding table and in the figures, indicates no vasoconstriction response of a transection after the establishment of the platelet plug; (+) indicates a mild vasoconstriction; (++) indicates a moderate vasoconstriction; (+++) indicates an extreme vasoconstriction. Under this last classification, the vasoconstriction spread up the artery from the site of the cut. It was so strong that a segment of the cut vessel almost disappeared from view. The same marked enhancement of the vasoconstriction occurred whether the administration of the primary extract preceded or followed the control period.

Completely adrenalectomized hamsters died in 4–5 days. The hamsters surviving more than 7 days lived long enough for repeat experiments. The results with these animals were always the same. Those animals that survived more than 7 days were found, on autopsy, to have retained fragments of adrenal cortex. Subsequent adrenalectomies were performed with the intent of leaving a small bit of the cortex and of removing all of the medullary portion. The vasoconstriction response of these animals to the primary extract was excellent, as may be seen from the data presented in histogram form in Fig. 1. These data represent 130 transections in 16 cheek pouches.

It was observed that the response to the primary extract was most marked between 1 and 5 hr. after the last administration of the concentrate.

Experiments involving intraperitoneal injection of the primary extract were carried out with four partially adrenalectomized (demedullated) hamsters. Two injections were given to each animal at the rate of 20 mg./100 g. body weight 3 hr. apart. One cheek pouch from each was exposed under anesthesia 1–3 hr. later, and the main cheek pouch artery was transected in the manner indicated above. The opposite cheek pouch was exposed and observed for vasoconstriction 2 days later. Intraperitoneal injection produced the same effects as oral administration, as may be seen from the data for 32 transections, as recorded in the histogram in Fig. 2.

Actually, the animals respond to single injections of 20 mg./100 g. body weight, as may be seen from the data recorded in Table

TABLE I

EFFECT OF ORAL ADMINISTRATION OF PRIMARY CONCENTRATE ON VASOCONSTRICTION IN TRANSECTED CHEEK POUCH ARTERIES OF ADRENALECTOMIZED HAMSTERS

Animal No.	Number of vasoconstriction responses on							
	Treatment with 25 mg./100 g. per dose, 3 times per day, 2 days before examination				No treatment for at least 2 days before examination			
	Days since Ad-X[c]	Degree of vasoconstriction[a]			Days since Ad-X	Degree of vasoconstriction[a]		
		−	+ & ++	+++		−	+ & ++	+++
83[b]	6	0	1	3	3	6	6	0
83	28	0	2	8	—	—	—	—
84	—	—	—	—	2	2	8	0
85[b]	5	0	4	7	18	2	7	0
85	26	2	4	1	—	—	—	—
86	2	0	4	6	—	—	—	—
87[b]	2	1	7	3	6	8	0	0
87	8	0	8	2	12	7	2	0
107	3	0	4	6	—	—	—	—
109	3	4	9	0	—	—	—	—
111	3	0	2	10	—	—	—	—
123	—	—	—	—	3	2	9	1
124	3	0	7	3	—	—	—	—
126	3	0	8	2	—	—	—	—
127	—	—	—	—	3	5	5	0
128	—	—	—	—	3	3	8	0
129	—	—	—	—	3	7	3	0
130	3	0	9	1	—	—	—	—
133	3	0	3	4	—	—	—	—
134[b]	3	0	6	3	6	0	1	8
134	—	—	—	—	9	1	5	3
135	—	—	—	—	3	7	4	0
136	—	—	—	—	3	7	3	0
137	—	—	—	—	3	5	5	0
138	—	—	—	—	3	0	9	1
139	3	0	0	10	—	—	—	—
141	—	—	—	—	3	5	7	1
142[b]	6	0	1	9	3	5	5	0
143	—	—	—	—	2	0	5	5
Sum of vasoconstriction responses..		7	78	78		72	92	19

[a] The symbol (−) represents no vasoconstriction after establishment of platelet plug; (+) represents mild local constriction at site of cut; (++) represents stronger constriction near site of cut; (+++) represents extreme constriction spreading from site of cut up the artery to the point of almost disappearance of a segment of the vessel.

[b] Survived long enough to be tested a second time; adrenalectomy not complete.

[c] Adrenalectomy.

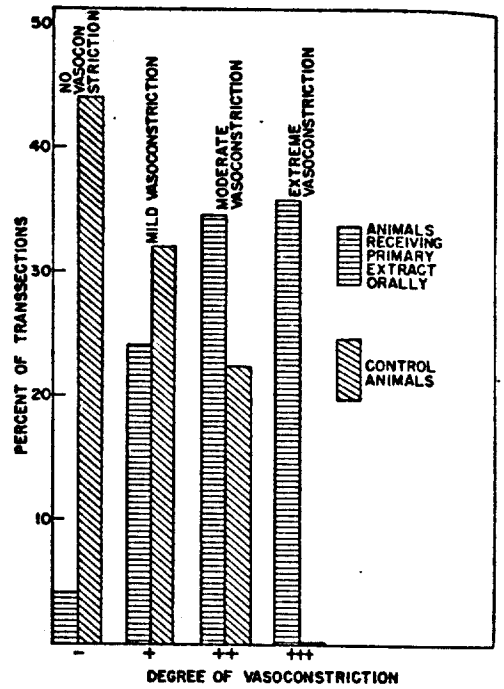

FIG. 1. Effect of oral administration of the primary extract on vasoconstriction in transected cheek pouch arteries of partially adrenalectomized (demedullated) hamsters. (−) No vasoconstriction after establishment of the platelet plug; (+) mild local constriction at the site of the cut. (++) moderate vasoconstriction at the site of the cut; (+++) extreme vasoconstriction spreading from the site of the cut up the artery, and which occurs to a degree that a segment of the vessel, as viewed in the stereoscopic microscope, almost disappears.

II where two partially adrenalectomized hamsters were used. These data were obtained in the manner indicated immediately above; one cheek pouch was exposed and examined for vasoconstriction in 2–3 hr. while the other pouch was exposed for observation 2–3 days later.

It was observed that the intraperitoneal administration of the primary extract produced a cramplike response, in which the animal would stretch and arch its back for a few seconds.

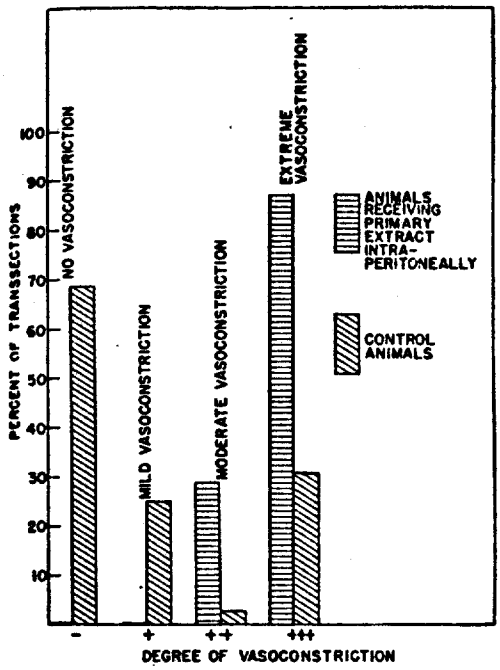

FIG. 2. Effects of intraperitoneal administration of the primary extract on the vasoconstriction in transected cheek pouch arteries of partially adrenalectomized (demedullated) hamsters. (−) No vasoconstriction after establishment of the platelet plug; (+) mild local constriction at the site of the cut; (++) moderate constriction; (+++) extreme constriction.

TABLE II

RESULTS OF INTRAPERITONEAL INJECTION OF PEANUT CONCENTRATE ON THE VASOCONSTRICTION OF CUT ARTERIES IN CHEEK POUCHES OF PARTIALLY ADRENALECTOMIZED (DEMEDULLATED) HAMSTERS

Animal No.	Hours after injection	Number of vasoconstriction responses on							
		Intraperitoneal injection, 20 mg./100 mg. per dose. Intensity of vasoconstriction				Two or more days following injection. Intensity of vasoconstriction			
		−	+	++	+++	−	+	++	+++
212	1.5	0	0	1	9	1	3	6	0
224	2.0	0	0	6	4	2	7	1	0

In view of the very strong vasoconstriction induced in the cheek pouch arteries of the partially adrenalectomized animals by the primary extract, as well as the apparent intestinal cramp produced when the active material was administered intraperitoneally, it seemed possible that the factor in the primary extract might be similar in action to 5-hydroxytryptamine and be myotonic for smooth muscles. Excised hamster duodenum muscle suspended in 75 ml. of Tyrode's solution at 39°C. contracted strongly when 0.4 ml. of a 10% aqueous solution of the primary extract was added to the bath. A typical kymograph tracing of the muscle response is shown in Fig. 3(*c*).

Included also in Fig. 3 are the kymograph tracings obtained from successive responses of a single muscle preparation to 0.4 mg. 5-hydroxytryptamine (5)(*b*), to 0.5 mg. of the absolute alcohol extract of the primary extract (*d*), and to 40 mg. of the residue remaining after the primary extract was extracted with absolute alcohol (*e*). It is apparent that the active factor is removed quantitatively from the primary extract with absolute ethyl alcohol.

DISCUSSION

It was indicated in the report by Boudreaux and Frampton (1) that the hemostatic effect of peanut flour and of the primary extract on hemophiliacs is not because of any change in the clotting mechanism, but that the hemostasis must be related to blood vessel response, following trauma. While the evidence presented in this communication

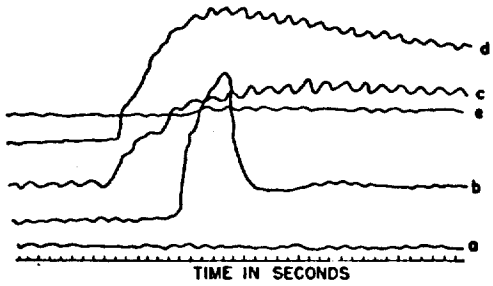

FIG. 3. Kymograph tracings obtained with a hamster duodenum muscle preparation: (*a*) resting muscle; (*b*) contraction induced by 0.4 mg. of 5-hydroxytryptamine; (*c*) contraction induced by 0.04 g. of primary extract; (*d*) contraction induced by 0.005 g. of the absolute alcohol extract of the primary extract; (*e*) contraction induced by 40 mg. of the residue from the absolute alcohol extract of the primary extract.

does not necessarily identify the vasoconstrictor-myotonic factor in peanuts with the hemostatic agent which seems to give relief in hemophilia, it is noted that preparations which give the responses reported in this paper also give relief from hemophilia symptoms.

The impression gained from the observations made in the course of this study is that the peanut factor has a physiological behavior similar to that of 5-hydroxytryptamine in being bound in the platelets and released from them, during the viscous metamorphosis of the platelets in the construction of the platelet plug, to cause the vasoconstriction.

ACKNOWLEDGMENTS

Support for that part of the research conducted at Louisiana State University was received from the New Orleans Chapter of the National Hemophilia Foundation and from the Graduate School of the University. The interest and helpful suggestions offered by Dr. B. Jackson are gratefully acknowledged.

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American Pharmaceutical Association

THE TOXICITY OF PROPYLENE GLYCOL.*

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In recent years propylene glycol has assumed a position of somewhat increasing pharmaceutical interest as a vehicle and solvent. Seidenfeld and Hanzlik (1) have reported extensive toxicity studies in which propylene glycol is compared with glycerol and ethylene glycol. Hunt (2) has discussed the use of ethylene and propylene glycols as medicinal solvents and has shown that systemically propylene glycol is much less toxic than ethylene glycol.

We have compared the acute toxicities of propylene glycol and glycerol in rats by intramuscular, subcutaneous and intravenous injection and have obtained results which show satisfactory agreement with those obtained by Seidenfeld and Hanzlik (1). In addition, the acute and chronic toxicities of propylene glycol administered orally in rabbits have been studied.

The propylene glycol used in these studies is the Alpha Propylene Glycol (1, 2 Propane Diol).

Acute Toxicity. Propylene glycol was injected in undiluted form intramuscularly and subcutaneously in rats. Parallel groups of rats were similarly injected with undiluted glycerol. With the large doses administered to establish the minimum fatal dose, all of the rats showed profound depression, analgesia and coma. With sub-lethal doses, the toxic symptoms persisted for a longer time in the glycerol-injected animals than in those injected with propylene glycol. In the larger doses, glycerol produced greater local tissue damage than was observed with propylene glycol in corresponding percentages of the M. L. D. The results of these experiments given in Table I show that propylene glycol is much less

* From the Research Laboratories, The Upjohn Company, Kalamazoo, Michigan.

toxic than glycerol. Intramuscularly, the M. L. D. for propylene glycol is 15.7 Gm. per Kg.; for glycerol, 7.6 Gm. per Kg. Subcutaneously, the M. L. D. for propylene glycol is 23.1 Gm. per Kg.; for glycerol, 15.1 Gm. per Kg.

The acture oral toxicity was determined, in rabbits, by administering a 20 per cent aqueous propylene glycol solution by stomach tube giving divided doses over a period of one hour. The toxic symptoms consist of increased respiratory rate, loss of equilibrium, profound depression, analgesia and coma. Death occurred in from 18 to 36 hours. The results given in Table I indicate that the M. L. D. of propylene glycol orally in rabbits is 20 Gm. per Kg.

TABLE I.—ACUTE TOXICITY OF PROPYLENE GLYCOL AND GLYCEROL IN RATS AND RABBITS.

Propylene Glycol. Dose per Kg. Cc.	Gm.	No. of Animals Used.	Per Cent Mortality.	Glycerol. Dose per Kg. Cc.	Gm.	No. of Animals Used.	Per Cent Mortality.
Intramuscular Injections in Rats.							
12	12.60	3	0	4	5.04	5	0
13	13.65	3	0	6	7.56	5	60
14	14.70	3	0	8	10.08	4	100
15	15.75	5	60	10	12.60	3	100
16	16.80	5	80	12	15.12	3	100
Subcutaneous Injections in Rats.							
16	16.80	2	0	8	10.08	3	0
18	18.90	3	0	12	15.12	5	60
20	21.00	5	20	14	17.64	3	100
22	23.10	5	60	16	20.16	3	100
24	25.20	5	80	18	22.68	3	100
				20	25.20	2	100
Oral Administration in Rabbits.							
15	15.75	2	0				
18	18.90	9	33				
19	19.95	7	86				
20	21.00	4	100				

The intravenous injection of propylene glycol in 20 rats indicated a minimum fatal dose of 18 cc. (18.90 Gm.) per Kg. This value shows satisfactory agreement with that given by Seidenfeld and Hanzlik of 16 cc. (16.8 Gm.) per Kg. intravenously in rats. The intravenous injection of undiluted propylene glycol produced rapid obliteration of the veins making administration very difficult. At doses of 16 to 19 cc. per Kg. intravenously, all the rats showed marked muscular tremors and later became comatose. Some of these symptoms may be due simply to the hypertoxicity of the undiluted propylene glycol.

Since the use of undiluted glycerin intravenously proved impracticable, a 50 per cent dilution was used. A series of 24 rats indicated that the minimum fatal dose for glycerol at this dilution was 6 cc. (7.56 Gm.).

Chronic Toxicity.—The toxicity of propylene glycol was studied by chronic oral administration in rabbits. Daily doses of 1 to 8 cc. (1.05 to 8.40 Gm.) per Kg. were administered by stomach tube as a 20 per cent aqueous solution of propylene glycol. The animals were weighed daily and the average body weights at successive 10-day periods are given in Table II. The experiment was continued for 50 days,

at the end of which time the animals were killed by the intravenous injection of magnesium sulfate. Comparison of the experimental animals with their controls showed no gross pathology which could be ascribed to the drug.

TABLE II.—CHRONIC ORAL TOXICITY OF PROPYLENE GLYCOL IN RABBITS.

Rabbit Number.	Dose per Kg. Daily. Cc.	Gm.	Initial Body Weight. Kg.	Average Body Weight at Successive 10-Day Periods. 1–10. Kg.	11–20. Kg.	21–30. Kg.	31–40. Kg.	41–50. Kg.
1	1	1.05	1.59	1.59	1.61	1.64	1.70	1.65
3	2	2.10	1.70	2.15	2.52	2.66	2.75	2.77
4	2	2.10	1.70	1.92	2.30	2.53	2.65	2.68
6	3	3.15	1.70	1.81	2.15	2.26	2.41	2.34
25	4	4.20	2.32	2.36	2.48	2.56	2.58	2.57
26	4	4.20	2.53	2.56	2.55	2.59	2.58	2.66
39	4	4.20	1.72	1.78	1.87	1.86	2.05	2.21
17	4	4.20	2.42	2.46	2.47	2.48	2.52	2.58
27	8	8.40	1.70	1.74	1.83	1.92	1.94	2.01
28	8	8.40	2.59	2.52	2.48	2.51	2.47	2.51
38	8	8.40	2.36	2.31	2.23	2.11	2.26	2.36
2	Control		1.81	1.88	1.86	1.88	1.89	1.83
11	"		1.81	1.89	2.13	2.45	2.64	2.75
7	"		1.81	2.00	2.40	2.59	2.69	2.68
5	"		1.70	1.92	2.30	2.20	2.69	2.70
24	"		1.98	2.12	2.31	2.45	2.60	2.79
55	"		1.94	2.04	2.06	2.00	2.00	2.07
50	"		2.00	2.16	2.17	2.24	2.32	2.54

The absence of any significant weight loss at the highest dosage and the satisfactory growth observed at the lower dosages indicate that daily doses of 4 to 8 cc. per Kg. are tolerated by the rabbit for a period of 50 days without any toxic symptoms other than slight anorexia. The highest dosage of 8 cc. per kilo represents the daily administration of 40 per cent of the fatal dose with no demonstrable cumulative effects. This lack of cumulative action in rabbits with a high percentage of the fatal dose of propylene glycol administered daily over a long period of time agrees with similar observations made in rats by Seidenfeld and Hanzlik (1) in which propylene glycol was added to the drinking water.

Local Irritation. The low systemic toxicity observed for propylene glycol would indicate it to be a solvent of choice for certain medicinal preparations. It is an excellent solvent for a number of substances which are now administered in oil solution. However, the local pain caused by the injection of undiluted propylene glycol constitutes a serious handicap to its use in hypodermic preparations. Experiments on human subjects in our laboratories have shown that the subcutaneous injection of as little as 0.1 cc. of undiluted propylene glycol produces a very marked local burning sensation which, however, disappears in 5 to 10 minutes. When injected intramuscularly the local irritation appears to be no greater than that produced by oil.

SUMMARY.

The acute toxicity of propylene glycol has been compared to that of glycerol by intramuscular and subcutaneous injections in rats. The acute and chronic oral toxicity of propylene glycol has been studied in rabbits.

The M. L. D. for propylene glycol intramuscularly and subcutaneously in rats is 15.7 and 23.1 Gm. per Kg., respectively, as compared to corresponding values of 7.6 and 15.1 Gm. per Kg., respectively, for glycerol.

In rabbits, the acutely fatal dose of propylene glycol by oral administration is 20 Gm. per Kg. Daily oral doses up to 8 cc. per Kg. administered for 50 days are tolerated by rabbits with no observed cumulative effects.

The low systemic toxicity of propylene glycol would recommend it as a solvent for certain medicinals. However, the severe, although transient, local irritation produced by its subcutaneous injection would appear to preclude its use in hypodermic preparations in undiluted form.

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The Nutritive Properties of Monoglycerides

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The fat of natural food in practically all cases occurs as triglycerides (5). Synthetic mono- and diglycerides were first prepared by Berthelot (2) in 1854 by heating fatty acids with an excess of glycerin at 200° C. Other methods for the preparation of mono- and diglycerides are described by Grün (3).

The work presented here was designed to study the comparative utilization and metabolism of synthetic and natural glycerides by the rat. Under the conditions of the experiment, mono- and triglycerides were found to be utilized in a similar manner and to have approximately equal value in the nutrition of the rat.

Usually the chemical composition of the food fat is reflected in the chemical composition of the fat stored in the animal body. However, when the food fat is insufficient, the animal converts carbohydrate material into fat and this fat is different in character from the ordinary fat occurring in feeds in that it contains a higher percentage of solid acids.

The rate of glyceride digestion in vitro was studied by Balls (1) who found that mono- and diglycerides were digested more rapidly than triglycerides by pancreatic lipase. Rosenthal and Trautwein (10) studied glyceride absorption with pancreas-extirpated dogs. They reported that monoglycerides were utilized much better than triglycerides. They suggested this might be due to the ease with which monoglycerides form emulsions or to their greater solubility in water.

Experimental

Monostearin and monolinolein were secured * and incorporated in a purified diet for rats. The α monostearin used had a melting point of 81° C. and a mixed melting point with known α monostearin of 81°-81.5° C. The sample of monolinolein was not pure, but contained about 20 per cent of monoolein. Neither sample contained diglycerides. A ration containing lard was used as a control. The composition of the rations is given in Table I. The rations containing the three types of glycerides were fed to groups of albino rats. Two male and two female rats constituted a group. The feeding of the rations extended over a period of eight weeks during which time the animals were confined to individual cages. Growth and feed consumption were measured at weekly intervals. It was the plan to feed rations having equivalent percentages of fatty acids in all cases. This entailed the incorporation of 24 per cent of monoglycerides in the ration, which was not well tolerated by the animals. Therefore, at the end of four weeks feeding the amount of monoglycerides was reduced to eight per cent. At the end of the feeding trial the animals were killed and their gastrointestinal tracts removed. Care was exercised not to lose any of the abdominal and organ fat. The remaining carcasses were frozen and ground in a food chopper, after which they were extracted three times with redistilled acetone, followed by extraction with peroxide-free ether. The combined extracts were concentrated in vacuo and reextracted with ether. The ether solution was washed and made up to volume. The extracted fat was of a reddish color. This color was removed by treating the fat with an aluminum silicate adsorbent (13). This absorbent has been shown to remove most of the phospholipids in the fat without affecting the neutral glycerides. The fats, after partial separation into solid and liquid fractions by the Twitchell lead salt method, as modified by Hilditch (4), were esterified with methyl alcohol and the methyl esters distilled and fractionated with a 36 inch rectifying column of the Podbielniak type packed with glass helices.

TABLE I

Percentage Composition of Rations

Constituent	Lard Control No. 1	Monostearin		Monolinolein	
		No. 2	No. 2A	No. 3	No. 3A
Casein [1]	20.0	20.0	20.0	20.0	20.0
Sucrose	62.2	46.2	62.2	46.2	62.2
Salts [2]	4.8	4.8	4.8	4.8	4.8
Cod Liver Oil	1.0	1.0	1.0	1.0	1.0
Dried Yeast	4.0	4.0	4.0	4.0	4.0
Lard	8.0	.....	.....	.....	.....
Monostearin	.....	24.0	8.0	.....	.....
Monolinolein	.....	.....	.....	24.0	8.0

[1] Extracted with alcohol and ether.
[2] Osborne and Mendel. J. Biol. Chem. 37, 572, 1919.

Results

The results of the feeding trial are given in Table II. During the first four weeks' feeding period when equivalent amounts of fatty acids were fed in the diet the growth produced on the monoglyceride diets was markedly slower than on the lard diet. The mechanical state of the rations containing the synthetic fats was thought to be responsible. These rations were very oily and produced large fatty excreta. Accordingly the amount of monoglycerides was reduced from 24 to eight per cent. This change brought about an immediate increase in food consumption, and growth during the second four weeks' feeding period was satisfactory and approximately equal on all of the rations. Growth over the entire eight weeks' period was slower on the ration containing monostearin than on the lard or monolinolein ration.

TABLE II

Growth of Rats on Natural and Synthetic Glycerides

Ration	Average Total Gain			Food Required Per Gram of Gain		
	1st Period 4 wks.	2nd Period 4 wks.	8 wks.	1st Period	2nd Period	8 wks. Period
	grams	*grams*	*grams*	*grams*	*grams*	*grams*
Lard	28.80	18.60	23.14	2.39	5.58	4.12
Monostearin	14.34	19.02	16.70	3.17	5.33	4.85
Monolinolein	14.88	22.40	19.60	3.04	4.20	3.63

The experiments demonstrate that synthetic monoglycerides are utilized practically as well for the growth of rats as natural triglycerides, when fed at a level of eight per cent.

Table III describes the general characteristics of the body fat of the rats fed the natural and synthetic

* These materials were furnished by Dr. A. W. Ralston, Research Division, Armour and Company, Chicago, Illinois.

glycerides. Table IV contains the fractionation data and Table V the distribution of fatty acids in the body fats calculated from the fractionation data. Reference to Table III will show that the monoglycerides

TABLE III

Characteristics of Body Fat of Rats Fed Natural and Synthetic Glycerides

Kind of Fat in Ration	Iodine Number	Saponification Equiv.	Fatty Acids		Fat in Rat Bodies
			Solid	Liquid	
			per cent	*per cent*	*per cent*
Lard	70.6	289	23.5	76.5	18.7
Monostearin	61.7	286	25.5	74.5	14.3
Monolinolein	97.1	291	17.3	82.7	13.8

TABLE IV

Fractionation Data on Body Fat of Rats Fed Synthetic and Natural Glycerides

Fraction	Solid Esters			Liquid Esters		
	Weight of Fraction gms.	Saponification Equivalent	Iodine Number	Weight of Fraction gms.	Saponification Equivalent	Iodine Number
Lard Ration						
1	1.22	259.5	0.6	0.85	254.7	47.3
2	2.17	268.9	0.5	1.07	262.3	61.7
3	4.12	269.5	0.3	2.49	270.0	62.3
4	4.95	272.1	0.5	3.90	280.0	76.3
5	4.79	276.0	0.4	6.35	293.4	99.1
6	3.03	283.0	3.1	14.40	295.5	97.2
7	3.32	310.0	8.5	4.60	299.0	125.5
Monostearin Ration						
1	0.66	257.2	0.8	0.81	242.8	36.9
2	0.89	260.0	0.4	1.04	260.1	64.6
3	3.24	270.7	0.3	4.70	260.5	70.0
4	2.56	270.7	0.3	2.40	276.0	65.4
5	1.56	272.5	0.5	4.04	290.5	85.3
6	3.05	275.3	1.1	10.90	295.2	83.6
7	3.11	307.7	3.6	8.80	298.0	97.4
Monolinolein Ration						
1	0.59	257.4	0.7	1.09	250.4	45.3
2	0.86	268.7	0.8	1.24	263.7	56.7
3	3.40	271.8	0.4	3.06	264.5	58.4
4	2.73	272.9	0.3	3.64	288.6	112.1
5	1.84	273.4	0.6	8.57	294.7	126.9
6	1.83	282.2	3.8	10.15	294.8	124.2
7	2.08	313.0	11.5	8.90	295.3	116.9
				6.77	301.0	146.2

TABLE V

Molar Percentages of Fatty Acids in Body Fat of Rats Fed Synthetic and Natural Glycerides

Acid	Lard Ration	Monostearin Ration	Monolinolein Ration
Myristic	1.5	4.2	2.3
Palmitic	25.2	27.2	18.0
Stearic	3.6	4.3	2.8
Arachidic	1.4	1.5	1.1
Tetradecenoic	1.0	3.9	2.1
Hexadecenoic	5.8	9.0	4.8
Oleic	51.9	49.1	38.4
Linoleic	7.7	0.5	29.4
Arachidonic	1.6	0.3	1.3
Total Saturated Acids	31.7	37.2	24.2
Total C_{14} Acids	2.5	8.1	4.4
Total C_{16} Acids	31.0	36.2	22.8
Total C_{18} Acids	63.2	53.9	70.6
Total C_{20} Acids	3.0	1.8	2.4

were being utilized and their acids deposited in the body. The iodine numbers and percentages of solid and liquid esters show that the ration containing monostearin produced body fat that was less unsaturated, and the one containing monolinolein produced depot fat that was more unsaturated than the fat from animals consuming the lard ration. This in itself would constitute evidence that the monoglycerides were being utilized to good advantage by the experimental animals. The percentage of fat in the animals was higher in the group fed the lard ration, perhaps because a smaller amount of fatty acids was directly available for storage in the case of the groups fed the monoglycerides. The saponification values, which were similar, indicate that the monoglycerides were stored in the body as triglycerides.

Analyses of rat body fat, obtained from animals fed normal rations, have been reported by several workers (6, 7, 8, 12). These workers showed that rat fat contains low proportions of stearic acid and relatively high amounts of palmitic acid. The unsaturated acids were chiefly oleic with some hexadecenoic acids.

In the group fed the ration containing monostearin (Table V) there was little increase in the stearic acid content of the body fat. The increase in saturation of the fat was due to proportionate increase in most all of the other saturated acids found in the fat. This might have been accomplished by the interconversion between stearic, palmitic, and other saturated acids as suggested by Schoenheimer (11) and Rittenberg (9). The fat deposited by the rats on the monostearin ration could possibly have been derived from the carbohydrates in the ration. The percentage of C_{14} acids (especially the C_{14} unsaturated acids), however, is considerably higher than percentages of C_{14} acids reported for rats fed high carbohydrate diets (8). This would indicate appreciable metabolism of stearic acid in order to change it into an acid which can be stored by the rat. The total saturated fat content of 37.2 per cent is nearly the maximum value that can be obtained. Hilditch and Longenecker (6) and Longenecker (7) found that the percentage of saturated acids was rarely more than 35 to 37 per cent.

The fat from the animals fed the ration containing monolinolein was characterized by a high amount of linoleic acid which was apparently derived directly from the monolinolein of the ration. This indicates a high tolerance of linoleic acid by the rat.

Summary

1. Monostearin, monolinolein, and lard as compared in the rations studied were found to be practically equal for the growth of rats.

2. Stearic acid from monostearin was not stored to any appreciable degree in the body fat of rats.

3. Linoleic acid from monolinolein was apparently stored directly in the body fat of rats.

4. Fatty acids fed as monoglycerides appear in the depot fat of rats as triglycerides.

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7370: 1151-1152, 1964

EFFECT OF GLYCEROL BY MOUTH ON RAISED INTRACRANIAL PRESSURE IN MAN

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AT the Second European Congress of Neurological Surgery in Rome in 1963, Cantore et al. described the successful use of glycerol for the relief of cerebral œdema in laboratory animals and in man. Because of the real need for another orally active osmotherapeutic substance to supplement urea, we decided to follow their lead and explore the application of glycerol.

Plasma Osmolality after Oral Administration of Urea or Glycerol in a Normal Subject

The first requirement was to see whether we could obtain a rise of plasma osmolality quickly enough to produce an effective osmotic gradient between brain-water and plasma. This was first measured in the laboratory in a normal subject whose plasma osmolality changes following urea had already been charted, so as to get a comparison between the two substances.

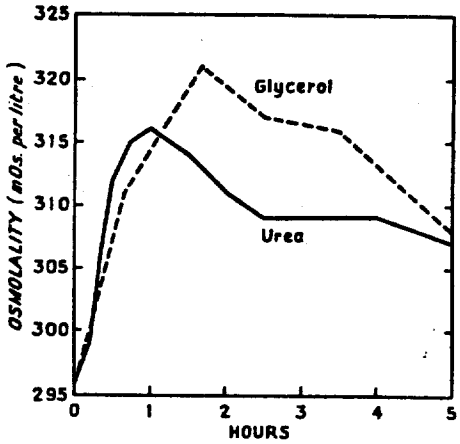

Fig. 1—Plasma osmolality after oral administration of glycerol (75 ml.) or urea (50 g.) in the same normal subject (weight 58.6 kg.).

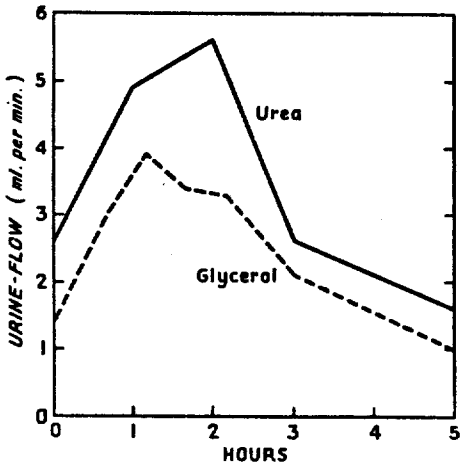

Fig. 2—Diuresis after oral administration of glycerol (75 ml.) or urea (50 g.).

75 ml. of glycerol was chosen as the dose to compare with 50 g. of urea. Actual measurement on a sample from the same batch of glycerol gave 996 milliosmols (mOs) for the 75 ml. dose. 50 g. of urea contains 833 mOs. The glycerol, mixed with an equal volume of iced lemon juice, was sipped slowly over 20 minutes. This mixture proved to be more palatable than urea, though it too produced a slight feeling of nausea immediately after the dose was finished. Headache, which developed from about 30 minutes onwards, was severe at about 2 hours, when it was relieved by lying down. After 75 ml. of glycerol, the plasma osmolality rose at about the same rate as after urea (fig. 1), but the rise continued for longer so that the maximum increase recorded (25 mOs per litre) was 25% greater than that produced by 50 g. of urea, and it remained above the urea maximum for 2 hours.

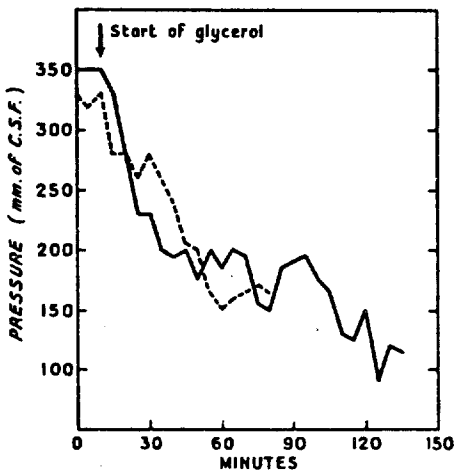

Fig. 3—Lumbar C.S.F. pressure after ingestion of 2½ oz. of glycerol in two patients with benign intracranial hypertension.

Diuretic Effect

The diuresis following 75 ml. of glycerol was less than that after 50 g. of urea (fig. 2) although the osmotic dose given was greater; 1018 ml. of urine was passed in the first 5 hours after urea, and 725 ml. after glycerol, when the state of hydration of the subject was comparable on the two occasions. This was to be

expected because some of the glycerol is metabolised, and this reduces the osmotic load presented to the kidney.

Effect on Lumbar Pressure

Fig. 3 shows serial lumbar-pressure readings in two patients with benign intracranial hypertension. Both patients were conscious and cooperative; measurements were made with a manometer and an indwelling lumbar-puncture needle, and they were discontinued when the patients felt that they had had enough. The same dose was used as in the normal subject; in each case the pressure began to fall about 10 minutes after starting to take the glycerol and before the whole dose had been ingested, and it fell fairly steadily for about an hour. In one case an opening pressure of 330 mm. of cerebrospinal fluid (C.S.F.) had fallen to 150 mm. when observations were discontinued at 1 hour; in the second case the opening pressure was 350 mm., and the lowest pressure recorded was 90 mm. 1 hour and 55 minutes after the start of the glycerol. Neither patient experienced any headache.

Penetration of Glycerol into the C.S.F.

Theoretically the administration of a hypertonic solution for the reduction of intracranial pressure may be followed by a late increase of pressure (" rebound pressure ") if the substance administered is able to enter the brain tissue or the C.S.F., and then its rapid excretion by the kidneys lowers the plasma concentration below that of C.S.F. so that the direction of the osmotic gradient is reversed. This is known to occur with urea (see Bell and Arnold 1960).

In a patient who received repeated doses of glycerol for the control of a raised pressure associated with an aqueduct stenosis, the C.S.F. glycerol was 1·3, 1·5, and 3 mg. per 100 ml. on the 2nd, 3rd, and 10th days of therapy respectively (9·2 mg. of glycerol per 100 ml. = 1 mOs. per litre). In this patient, however, a fairly normal blood C.S.F./brain relationship was still probably present, whereas in cases with infarction, traumatic damage, operative interference, or gross œdema round a tumour, glycerol may well enter the C.S.F. in greater amount. In the cell, glycerol will be used up by the normal process of metabolism so that its entry would not prove so disadvantageous as that of urea.

Clinical Application

In the past year or so we have used glycerol in the treatment of patients with raised intracranial pressure from many different causes. We have used it post-operatively, in patients with head injuries and in patients with benign intracranial hypertension. Cantore et al. (1964) refer to its use intravenously, but we have always given it by the oral route. We have found it to be effective even if used repeatedly over many days, and in these circumstances we prefer it to urea given orally. We still use urea or mannitol intravenously during operations, or for the initial treatment of a seriously ill patient.

In patients with raised intracranial pressure, we have used glycerol 75 ml. by mouth, diluted with an equal quantity of water or fruit juice, once, twice, or three times a day as indicated, and we have continued this treatment in some cases for many weeks. No toxic effects have been noticed, but patients often find the mixture nauseating, especially if it is taken too fast, and it may be necessary to administer it through a gastric tube. We regard glycerol as a useful addition to urea and mannitol in the treatment of raised intracranial pressure.

Summary

The rise of plasma osmolality in a normal subject following glycerol by mouth is compared with that following urea. The diuresis after glycerol is less than after an osmotically larger dose of urea.

The reduction of raised intracranial pressure following glycerol is illustrated in two cases. Very little glycerol was found in the cerebrospinal fluid even after some days of repeated oral administration.

One of us (M. B.) is grateful to the Medical Research Council for a personal grant and to the British Empire Cancer Campaign for a grant for technical assistance.

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Helv. Physiol. Pharmacol Acta
14: 299-303, 1956

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OBSERVATIONS ON THE DIGESTIBILITY OF PALMITIC AND STEARIC ACIDS AND OF THEIR GLYCERIC ESTERS IN THE RAT.

By M. Buensod and P. Favarger

Received July 7, 1956

It has already been demonstrated in this laboratory (1) that the digestibility of stearic acid depends upon the chemical form in which it is ingested, glycerol monostearate being much more digestible than free acid or than tristearate. This observation is in full agreement with current conceptions on the subject of the mode of absorption of fats, and confirms that the privileged form of penetration is that of partial glyceride. However, the extremely bad digestibility of stearic acid was perhaps responsible for the special position which it occupied. We therefore measured under identical conditions the apparent and true coefficients of digestibility of palmitic acid, of its mono and of its tri-glyceride, alone, or mixed with olive oil.

Experimental Part

1. D-palmitic acid was prepared according to the Van Heynigen method (2). We synthesized the 1-monopalmitine starting with acetone-glycerol, as described by Malkini and Shurbagy (3), and tripalmitine starting with the palmityle chloride (4. 5).
2. The fats are administered to the rats, mixed in the amount of 15% to a full diet (1). For certain series, the experiment lasted 5 days, after two days of orientation diet; for others, 3 days only, without orientation diet. We added Geigy carmin to the food and gathered the colored feces.
2. The analytic method followed was that of Annegers (6) and the deuterium was proportioned according to the technique used in this laboratory (7).

Results

Our results are parallel to those which were obtained with stearic acid and its derivatives (1), the palmitic acid being better absorbed that the stearic acid (Tables 1 and 2).

In both cases, the highest coefficient of digestibility is that of the mono-glyceride. Yet, the rats eat it with much difficulty and rapidly become thin.

The monoglycerides having no particular taste, we thought that their high melting point might explain this distaste and, as these compounds exist in two distinct crystalline forms having very different melting points (81° and 58° for the monostearate), we gave the rats a diet containing the monostearine with the lowest melting point: the rats showed the same distaste. On the other hand, the rats eat in a normal way monopalmitine mixed with 50% olive oil (series B_2). The true coefficient of digestibility of monopalmitine varies practically not at all, whether it is given pure, or mixed with 50% olive oil.

When tripalmitine and 50% olive oil are mixed, its coefficient of digestibility increases considerably, but the rats digest, in absolute quantities, about two times less palmitic acid as when tripalmitine alone is given to them.

Table 1. Coefficient of digestibility of D-palmitic acid in the rat.

The rats were weight at the start and at the end of the diet. In only one series (monopalmitine) was a significant change in weight observed (series B_1). The apparent coefficient of digestibility is the ratio:

$$\frac{\text{total ingested fats} - \text{total excreted fats}}{\text{total ingested fats}}$$

The true coefficient of digestibility takes only deuterated fats into account.

Fat and length of regimen	Rat no.	Weight of rats (g)	Appar. coeff. of digest. (%)	True coeff. of digest. of palmitic acid (%)	Endogenous fats mg/day
A. Palmitic acid (5 days)	AP 1	160	16.2	30.0	280
	AP 2	150	26.0	32.0	123
	AP 3	320	14.6	19.0	101
	AP 4	300	13.0	24.0	255
B_1 Monopalmitine (5 days)	MP 1	155 - 118	79.5	88.0	17
	MP 2	155 - 111	80.5	86.5	11
	MP 3	320 - 255	85.0	90.5	17
	MP 4	275 - 200	80.0	86.0	15
B_2 Monopalmitine 1/2 + olive oil 1/2 (5 days)	MP 5	140	96.0	96.5	12*
	MP 6	260	90.0	90.5	46*
	MP 7	160	95.0	94.5	17*
	MP 8	310	86.5	87.0	57*
	MP 9	260	93.0	93.5	29*
	MP10	140	90.0	90.0	22*
C_1 Tripalmitine (3 days)	TP 1	165	66.5	69.0	18
	TP 2	180	46.5	52.0	50
	TP 3	180	48.0	55.0	41
	TP 4	180	37.5	45.0	63
C_2 Tripalmitine 1/2 + olive oil 1/2 (3 days)	TP 5	150	87.5	87.0	31*
	TP 6	150	86.0	84.0	27*
D. Tripalmitine ord. 1/2 + Monopalmitine 1/2 (3 days)	MP11	150	81.0	89.0	23*
	MP12	155	75.0	86.5	20*

*The weights of endogenous fats thus designated also include the non-absorbed olive oil.

Table 2. Average coefficients of digestibility of stearic and palmitic acids. (Legend: see Table 1)

Fat administered	True coefficient of digestibility (%)	Endogenous fats mg/day
Stearic acid	22.9	122
Palmitic acid	26.3	189
Monostearine	77.6	6
Monopalmitine	87.8	15
Tristearine	37.1	84
Tripalmitine	55.5	43
Fat-less regimen	-	51

In the series tripalmitine 50% + monopalmitine 50%, both coefficients of digestibility are high; the digestibility of tripalmitine thus increases considerably in the presence of monopalmitine. Lastly, our prior observations concerning the excretion of endogenous fats are confirmed: the endogenous fats are increased in the presence of free fatty acids and reduced in the presence of monoglycerides.

As a complement to this paper, we though it interesting to measure the coefficients of absorption of the glyceric esters of palmitic acid. While the coefficient of digestibility of a fat depends only upon the ratio between the quantity utilized by the animal and the ingested quantity, and while its determination necessitates an experimental period of several days, the coefficient of absorption is measured several hours after administration by tube of a definite quantity of fat. It is thus a function of the rapidity of the emptying of the stomach. Its individual variations can be considerable, according to the observations of Cordier (8). This is indeed what we noted for two long experimental series where the rats received monostearine or tristearine. The results obtained were too dispersed to be useful. For tripalmitine and monopalmitine, on the other hand, the differences observed were very significant from the start.

Each rat receives, by stomach tube, 60 mg of marked glyceride dissolved in 540 mg of olive oil. After 5 hours, the animal is killed. The esophogus, stomach, and intestine are removed with their contents; they are saponified in their entirety and the fatty acids are proportioned.

The quantities of palmitic acid absorbed after 5 hours are small (Table 3), but the difference this time is in favor of the tripalmitine. While being more completely absorbed than the latter, the monopalmitine is nonetheless more slowly digested. This difference in speed is perhaps caused by a difference in melting points of the two glycerides.

Table 3. Coefficients of absorption of D-monopalmitine and D-tripalmitine at 10% in olive oil.

Fat	Melting point	Rat no.	Coefficient of absorption (5)
Monopalmitine	60°	MP 13	26.5
		MP 14	22.6
		MP 15	21.8
		MP 16	21.8
Tripalmitine	53°	TP 7	36.5
		TP 8	38.5
		TP 9-11	35*
		TP 12-14	36*

*Average of 3 rats.

We can conclude these tests by the following remarks. The best form of penetration of superior saturated fatty acids is certainly constituted by the monoglycerides, and this in spite of their high melting point. If palmitine and stearine have mediocre digestibility, it is because the saturated fatty acids, too completely liberated, are badly absorbed. After administration of triglycerides, the fecal lipids contain, in fact, a large proportion of fatty acids or of soaps (1). The favorable degree of utilization of monoglycerides does not, however, allow one to conclude that fats containing a large proportion of monoglycerides may be administered indiscriminately. These substances may in fact lead to a noticeable slowing down of digestion.

These tests also confirm that the digestibility of a fatty acid depends not only on the manner in which it is joined with a molecule, but also on the composition of the mixture of fats administered.

This paper was made possible by a subsidy of Funds for the encouragement of scientific research, in Bern.

Summary

D-palmitic acid is administered to rats in free form, in the form of monoglyceride or triglyceride. The coefficients of digestibility and of absorption

are determined. Monopalmitine is digested more completely than free acid or than tripalmitine. On the other hand, monopalmitine is digested slower than the corresponding triglyceride.

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J. Lipid Res. 11(2): 167-169, 1970

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Gel permeation chromatography of neutral hydroxy lipids on Sephadex LH-20

M. Calderon and W. J. Baumann

University of Minnesota, The Hormel Institute, Austin, Minnesota 55912

SUMMARY Gel-permeation chromatography on Sephadex LH-20, using ethanol as eluent, permits the resolution of neutral hydroxy lipids according to molecular size. The influence of molecular shape, functional groups, chain lengths, and degree of unsaturation, as well as the effect of the eluent on the elution pattern are discussed. The usefulness of the method for the separation of classes of hydroxy lipids which cannot be resolved by other chromatographic procedures, is demonstrated. Examples include the separations of 1,2- and 1,3-diglycerides from long-chain alcohols, and of alkyl ethanediol monoethers from cholesterol.

SUPPLEMENTARY KEY WORDS gel filtration · lipophilic Sephadex · diglycerides · alcohols · cholesterol · dialkyl glycerol ethers · alkyl ethanediol ethers · diol lipids

In gel-permeation chromatography molecular size is a decisive parameter in determining the chromatographic behavior of the compounds to be separated. The sequence of elution of neutral lipids from lipophilic dextran gels (1, 2) is generally in the order of decreasing molecular weights, although other criteria, such as polarity of the lipid or eluent, may cause different patterns of fractionation (3–5). Nonpolar lipids which migrate in adsorption chromatography ahead of triglycerides can be readily fractionated according to molecular size on columns of Sephadex LH-20 with chloroform as eluent (1, 5). However, lipids containing hydroxy or carboxyl groups can cause severe solute–gel interaction unless more polar solvents are used. Despite the fact that such effects may be used advantageously, as was reported for the separation of isomeric diglycerides (5) or for the segregation of steroids (6), "pure molecular sieving" is usually desirable for the resolution of complex lipid mixtures.

The present investigation was undertaken to study the influence of functional groups, chain lengths, and degree and type of unsaturation on the behavior of some hydroxy lipids in gel-permeation chromatography on Sephadex LH-20 using ethanol as eluent. Model compounds were also selected in view of the possible application of the procedure to the separation of closely related hydroxy lipids which cannot be resolved by conventional chromatographic methods.

Special care was taken in preparing the dextran slurry and in packing the column. Sephadex LH-20 (Pharmacia Fine Chemicals, Inc., New Market, N.J.), 110 g, was suspended in approximately 600 ml of ethanol which had been redistilled over Drierite. Some of the solvent was removed under reduced pressure on a rotary evaporator. More ethanol was added and again evaporated under reduced pressure in order to remove air from the slurry. The gel was allowed to equilibrate for 4–5 hr at room temperature and then was poured carefully into a solvent-resistant Sephadex column, SR-25/100 (Pharmacia Fine Chemicals, Inc.), while a continuous flow of solvent was maintained through the column. The column was conditioned for 48 hr. Lipid samples, up to 100 mg and dissolved in 1 ml of ethanol, were applied to the top of the column and were eluted at room temperature at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected, and the amount of lipid material in each fraction was determined gravimetrically. The fractions were also analyzed by adsorption chromatography on layers of Silica Gel G (7) and (or) by gas chromatography after acetylation (8).

The effect of chain length and unsaturation on the elution pattern of hydroxy lipids was studied by subjecting a mixture of equal amounts of alkyl ethanediol monoethers, having chain lengths from C_{14} to C_{20} and up to two double bonds (9), to chromatography on Sephadex LH-20. As depicted in Fig. 1, the total weight curve does not indicate any fractionation. However, when the collected fractions were acetylated and then analyzed by gas chromatography, some subfractionation according to chain lengths as well as to number and configuration of double bonds became evident. The individual elution curves *A–F* (Fig. 1) demonstrate that eicosyl ethanediol ether (*A*) was eluted ahead of the octadecyl ether (*B*) which was followed by the hexadecyl (*D*) and tetradecyl ethers (*F*). Broadening of curve *C*, which represents both *cis*- and *trans*-9-octadecenyl ethanediol ethers, is due to the fact that the acetates of the two compounds were not resolved by gas chromatography under the conditions employed. Analysis of the fractions, collected from the Sephadex column, by argentation chromatography (developing solvent hexane–diethyl ether 85:15, v/v) demonstrated clearly that in gel-permeation chromatography the *trans* isomer was eluted well ahead of the *cis* isomer, most likely because of the bulkier structure of the *trans* compound. These findings are compatible with results reported for the separation of different types of geometrical isomers, namely *cis*- and *trans*-dimethylcyclohexanes on a polystyrene gel (10), which was attributed to differences in molar volumes of the isomers. As shown in Fig. 1, *cis, cis*-9,12-octadecadienyl ethanediol ether (*E*) was eluted from the Sephadex column together with the tetradecyl ether (*F*). This elution pattern is similar to that reported for long-chain methyl esters, when a Styragel column was eluted with diethyl ether (11). Thus, in gel chromatography the retardation observed for one

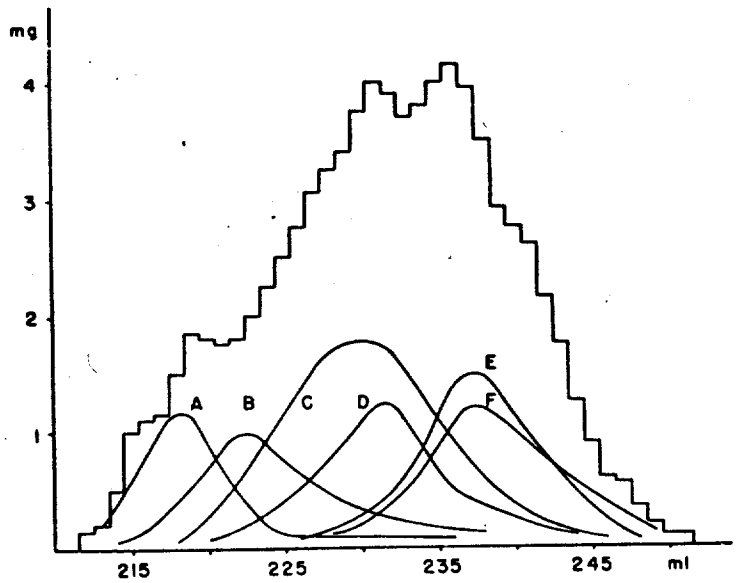

FIG. 1. Gel-permeation chromatography of a mixture of alkyl ethanediol ethers on Sephadex LH-20L. The column, 79 cm × 2.5 cm I.D., was eluted with ethanol at a flow rate of 0.5 ml/min. The composition of the fractions as determined by gas chromatography of the acetates are: *A*, eicosyl ethanediol ether; *B*, octadecyl ethanediol ether; *C*, *trans*-9′-octadecenyl followed by *cis*-9′-octadecenyl ethanediol ether; *D*, hexadecyl ethanediol ether; *E*, *cis,cis*-9′,12′-octadecadienyl ethanediol ether; *F*, tetradecyl ethane diol ether.

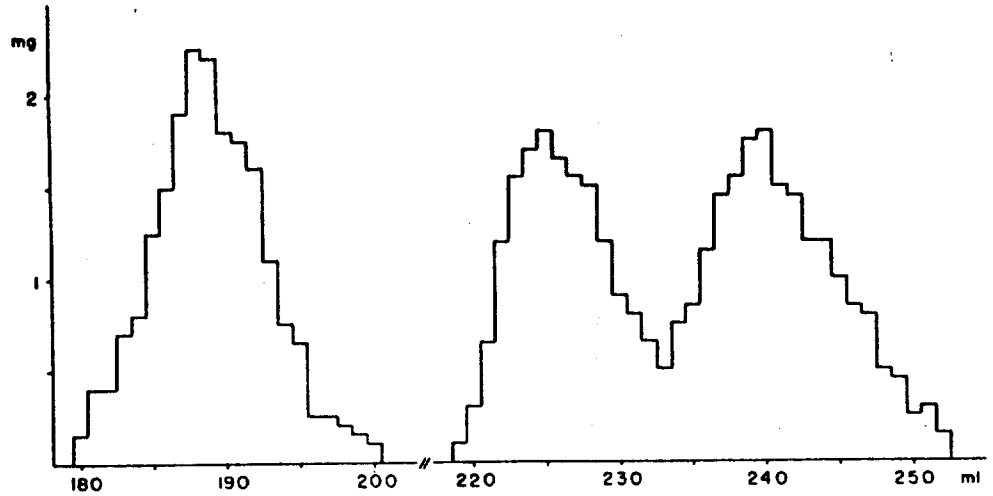

FIG. 2. Gel-permeation chromatography of a mixture of 1,2-di-*cis*-9′-octadecenyl glycerol ether (fractions 180-200), *cis*-9′-octadecenyl ethanediol ether (fractions 219-235), and cholesterol (fractions 231 252) on Sephadex LH-20. For experimental conditions, see Fig. 1.

additional *cis*-double bond is equal to that caused by a decrease in chain length by two methylene groups. The effect of one *trans*-double bond corresponds approximately to that of one methylene group.

Separations of classes of lipids according to molecular dimensions appear feasible inasmuch as subfractionation within classes, according to chain length or unsaturation, occurs only to a minor extent. Several types of lipids containing one hydroxy group show similar or identical rates of migration in adsorption chromatography. For example, cholesterol cochromatographs with ethanediol monoethers and long-chain alcohols migrate as uniform fractions together with 1,2-diglycerides.

Fig. 2 shows that in gel-permeation chromatography with ethanol as eluent, dioctadecenyl glycerol ether (12) (mol wt 593.0; fractions 180–200) is eluted ahead of and is distinctly separated from octadecenyl ethanediol ether (mol wt 312.5; fractions 219-235). A pattern of fractionation very similar to that shown in Fig. 2 was obtained when a mixture of equal amounts of 1,2-di-*cis*-9′-octadecenoyl glycerol, 1,3-di-*cis*-9′-octadecenoyl glycerol, and *cis*-9-octadecenol (The Hormel Institute

Lipids Preparation Laboratory, Austin, Minn.) was subjected to gel-permeation chromatography. Separation was not observed between the two isomeric dioleins (fractions 184–210), but both were eluted well ahead of oleyl alcohol (fractions 220–245). These hydroxy lipids are eluted quite normally in the order of decreasing molecular weights. Evidently, under the conditions employed, gel–solute interaction and differences in polarity of the lipids become negligible.

Exceptions to this normal pattern of separation were observed for compounds having vastly different shapes. Such an example is shown in Fig. 2. Although cholesterol (mol wt 386.7) has a molecular weight higher than that of octadecenyl ethanediol ether (mol wt 312.5), the ethanediol monoether (fractions 219–235) is eluted ahead of the sterol (fractions 231–252). As differences in the polarities of the two compounds are minor and of little significance with ethanol as eluent, the results can be interpreted as being due to the differences in shape or molecular volume. A similar effect was observed previously (13) and used for the resolution of paraffins from cycloparaffins.

We conclude that gel-permeation chromatography on Sephadex LH-20 with ethanol as eluent permits molecular sieving of hydroxy-substituted lipids. Chromatography on a lipophilic gel is particularly useful as a method complementary to adsorption chromatography. Mixtures of natural lipids which are not separated by adsorption chromatography can be completely resolved by gel-permeation chromatography according to differences in molecular weights or molecular shapes. For example, diglycerides of long-chain fatty acids can be separated from fatty alcohols, and long-chain monoethers of ethanediol, or the corresponding esters, can be resolved from cholesterol and other hydroxy substituted alicyclic compounds.

This investigation was supported by U.S. Public Health Service Research Grant No. AM 11255 and Program Project Grant HE 08214, as well as by The Hormel Foundation.

Manuscript received 29 August 1969; accepted 7 November 1969.

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J. Neurosurg.
21: 278-283, 1964

Oral Glycerol for the Reduction of Intracranial Pressure*

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LITERATURE of recent years reports several works on the clinical use of hypertonic solutions administered by slow intravenous infusion for reduction of intracranial pressure.[3,7,9,15,19] Among the numerous osmotic agents tested to reduce experimentally induced cerebral edema in the animal,[1] glycerol has proved particularly effective both by intravenous and oral route.[8] Considering such experimental results, glycerol has been applied on neurological,[2] ophthalmological[6,14] and, chiefly, neurosurgical[4] patients.

Glycerol (1,2,3-propanetriol) is a trivalent alcohol. It is oxidized by the organism through intermediate stages, analogous to those of the carbohydrate-oxidation cycle. As an integral part of fats (glycerides) and phosphatides, glycerol usually is present in the animal tissues at a rate of approximately 1 per cent of body weight. Oxidized to carbon dioxide and water, it produces 4.32 kg. calories per gm.[5] Such value is slightly greater than that produced by glucose. When larger amounts of glycerol are given, however, the drug is not metabolized completely by the organism since part of it is excreted in the urine.[11,12]

Glycerol increases plasmatic concentration.[13] Its mechanism of action, therefore, would be to draw liquids from the tissues, particularly from those more hydrated.

At convenient doses, glycerol promotes diuresis;[2,20] yet diuresis itself does not condition the action of the drug since reduction of intracranial hypertension in the nephrectomized animal did not differ from that obtained in the normal animal.[2]

In 1929, Ferber and Rabinowitsch[5] described the action of oral glycerol administered as food to 60 patients. No toxic effects or gastrointestinal disorders were noted. In 1933, Johnson *et al.*[10] studied the replacement of carbohydrate by glycerol in the diet of man and animal and pointed out the absolute tolerance of the drug taken orally. In order to examine the eventual toxicity of higher doses of glycerol, these authors administered daily 9 gm. of glycerol per kg. of body weight to the dog over a period of 1 year. The animals thus treated showed no toxic signs both *in vivo* and at postmortem examination. Subsequently, a group of students was submitted to a daily treatment of 1.3 gm. of glycerol per kg. of body weight over a period of 50 days. Never were toxic effects noted. Johnson *et al.*[10] therefore, concluded: "... within extensive limits glycerol compares favorably with carbohydrate as a source of energy."

We are not aware of any work, from a laboratory or clinic, dealing with therapeutic application of glycerol for the reduction of intracranial hypertension, other than those already mentioned,[2,15] which appeared in 1961.

During this study, oral administration has been applied chiefly, since oral glycerol displays the following qualities (Fig. 1):

1) Promptness and intensity of action in reducing intracranial pressure.

2) Possibility of repeating administration several times over a long period, thus avoiding "rebound overshoot," common to osmotic substances.

3) Lack of toxicity.

Method

Different kinds of glycerol are supplied. We have always used pure products.* Single oral dos-

Received for publication August 6, 1963

* Presented at meeting of Second European Congress of Neurological Surgery, Rome, Italy, April 18-20, 1963.

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* "Glycerinum RP" furnished by courtesy of Carlo Erba, Inc., Milan, Italy. Actually, Tuba-Lux Laboratories, Naples, Italy, are supplying 50 per cent glycerol solutions, suitably prepared for oral administration, under the trade name of Luxoral.

ages ranged between a minimum of 0.5 gm. and a maximum of 2 gm. per kg. of body weight. In many instances, daily dosages of over 5 gm. per kg. (1.5 gm. per kg. as initial dose, and 0.5–0.7 gm. per kg. every 3 hrs.) have been attained. Such dosages could be repeated even for several days without complaints of troublesome effects.

To attenuate the sweetish taste of glycerol, 50 per cent solutions in 0.9 per cent saline have been prepared. Lemon juice was also added. Cooling of the mixture and concurrent ingestion of small amounts of food facilitated the uptake of glycerol. Parenteral injection of an anti-emetic substance, 15–30 min. prior to ingestion of glycerol, was made in patients predisposed to vomiting. Patients, unable to drink spontaneously because of their critical condition, were given glycerol by means of gastric or duodenal tube (Fig. 2). No additional protein or electrolyte was needed during prolonged treatments with glycerol, ranging from several days to months, when patients were feeding on normal diet. Whereas in torpid or comatose patients, water-saline balance was maintained under strict control and suitable amounts of protein and electrolytes were administered.

In diuresis following administration of glycerol, excretion of electrolytes was not significant. (Data dwelling on this subject, obtained from neurosurgical and cardiopathic patients, will be discussed in a subsequent paper.

In stuporous and anesthetized patients, an indwelling catheter always was applied to prevent excessive vesical loading.

Glycerol was used both in hyperazotemic and markedly hyperglycemic patients with extremely satisfying results. In the latter case, concurrent administration of hypoglycemic agents was applied.

For intravenous infusion, sterile, 30 per cent glycerol solutions in 10 per cent inverted sugar or in 6 per cent depolymerized dextran diluted in 0.9 per cent saline have been prepared. The solution was injected at a rate of 60 drops per min. Glycerol, 0.8–1 gm. per kg. of body weight, always produced a marked anti-edematous effect, even in the most serious cases. Intravenous infusion, however, has been used only in a limited number of cases because of the frequent occurrence of transient hemoglobinuria. Further studies are being carried out presently in order to prevent hemolysis following intravenous administration.

Results

Glycerol, 1–2 gm. per kg. of body weight, was given to 258 patients, chiefly neurosurgical, from April 1961–April 1963. Oral administration was used in most cases. In a limited number of cases, intravenous infusion was applied. Glycerol was administered before, during and after surgery. In a few instances, the drug was given to patients with nonsurgical lesions of the nervous system.

Sixty-two patients suffering from space-occupying intracranial processes were treated with glycerol *before surgical intervention*. The doses administered varied according to the patient's condition. In stuporous and torpid patients, oral glycerol, 0.5 gm. per kg. every 3–4 hrs., reduced intracranial hypertension to an extent that within 30–60 min. from the start of the first administration, the patients' conditions highly improved and complete responsiveness returned. Solids and liquids were administered at the same time with glycerol, so that a convenient water, salt and protein balance could be maintained.

The most dramatic results with glycerol have been obtained in patients admitted to the hospital in comatose conditions. Administration of glycerol, 1.5 gm. per kg., was

Fig. 1. Chart of cerebrospinal fluid pressure in a patient with pneumonia. Cerebrospinal fluid pressure was measured directly from the right lateral ventricle by means of a polyethylene tube. At 3 G., oral glycerol, 1.3 gm./kg. of body weight, was first administered, subsequently, 0.6 gm./kg. every 3 hrs. was given. In spite of the marked initial hypertension, repeated administrations of glycerol were able to maintain low levels of cerebrospinal fluid pressure, thus allowing the patient to remain fairly well without complaining of cephalalgic crisis.

FIG. 2. G.P., a 45-year-old woman, weighing 66 kg., (Clinic No. 35140) was suffering from glioma of basal ganglia. Brain before (*above*) and 40 min. after administration (*below*), by means of gastric tube, of 132 gm. of glycerol in a 50-50 mixture of glycerol and 0.9 per cent saline.

begun immediately by gastric tube. Two hrs. subsequently, if the patient was still lethargic, glycerol, 1 gm. per kg., was given. Once responsiveness and cardiocirculatory conditions returned to normal, a maintaining therapy of 0.5–0.7 gm. of glycerol per kg. every 3–4 hrs. was applied until all diagnostic procedures and, eventually, surgical intervention were carried out.

Oral glycerol has been used in 75 patients in the operating room *during intracranial surgery*. The purpose was to reduce cephalic bulk, to facilitate exposure during operative procedures without sacrificing or removing cortical areas.

Glycerol was used chiefly during surgical extraction of tumors of the base of the skull (meningiomas, adenomas, craniopharyngiomas, etc.) and of every expanding intracranial process accompanied by marked cerebral edema (brain abscess, metastatic tumors, etc.). In a few cases, the drug has been administered during neurosurgical intervention on patients suffering from saccular and arteriovenous aneurysms and trigeminal neuralgias.

Usually after having introduced a gastric or duodenal tube, glycerol, 1.5–2 gm. per kg., was administered 30 min. prior to opening the dura mater. About 30 min. later, upon incision of the dura mater, evidence of significant reduction of brain volume was found. Within the subsequent 40–60 min., the brain gradually retracted, thus facilitating operative procedures tremendously.

Upon opening the skull, even during prolonged intracranial surgery, no "rebound overshoot" has ever been noted (Fig. 3). Once the desired reduction was attained, liquids accumulated in the stomach were removed to prevent vomiting.

Oral glycerol was given *postoperatively* to 84 patients who, following intracranial surgery, had signs of cerebral edema or of increased cerebrospinal-fluid pressure. Its use has been extremely valuable in resolving the postoperative course more satisfactorily.

Oral glycerol has proven very helpful, too, during roentgen-ray therapy in patients already operated upon for intracranial neoplasms to combat postradiation cerebral edema.

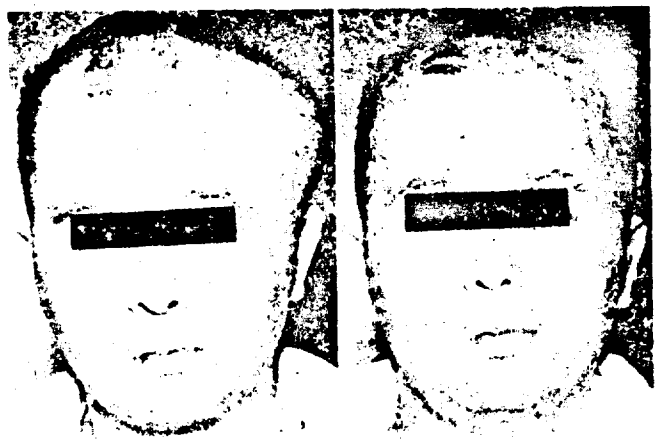

Fig. 3. L.M., a 28-year-old man (Clinic No. 3159), was operated upon 10 mos. previously for left frontotemporal glioblastoma and submitted to roentgen ray therapy. On readmission bulging of brain was remarkable (*left*). Oral glycerol, 2 gm./kg., reducing cephalic mass, caused disappearance of the tumefaction (*right*). Following the first administration of glycerol, once the desired effect was attained, the patient was treated with subsequent doses of 0.5 gm. of glycerol/kg. every 4 hrs., so that complete roentgen-ray therapy could be carried out. No further bulging of the brain was observed.

Daily doses of oral glycerol were administered for several days in the treatment of cerebral edema (25 cases), following head injuries, once diagnostic examination had excluded the presence of an intracranial hematoma.

In a limited number of patients suffering from "pseudotumor cerebri," glycerol, 0.5 gm./kg. was given for a period of 8 weeks. The drug was found to be fairly well tolerated and results were satisfying. Glycerol also was used successfully in nontumoral cephalea and headache. The number of such patients examined, however, is still too small to draw any useful indication.

Effect of Oral Glycerol on Intraocular Pressure. During previous researches carried out at the Ophthalmological Department of The Rome University,[6, 7] the action of oral glycerol on intraocular hypertension has been studied both in man and animal. Forty-six cases have been analyzed on the whole. Of these patients, 26 had suffered from various types of glaucoma and 20 had completely normal intraocular pressure. The most dramatic effect with glycerol has been obtained in cases of acute angle-closure glaucoma. Oral glycerol, 1.5 gm./kg. was shown to lower progressively intraocular pressure which, within 60–90 min., was brought to about normal levels. Such levels of pressure were maintained in some cases for several days (Fig. 4).

The effect of oral glycerol (1–1.5 gm./kg.) on other nonacute forms of glaucoma was a marked reduction of intraocular pressure, lasting 4–6 hrs. Tension, then, rose again to pretreatment levels. Tension-lowering effect induced by oral glycerol (1 gm./kg.) has

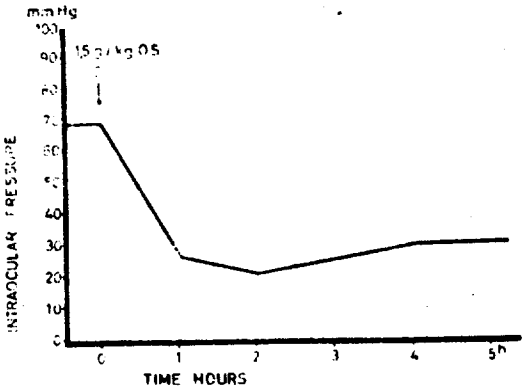

Fig. 4. Effect of oral glycerol on intraocular pressure in 6 patients during attack of acute angle-closure glaucoma. (Average values measured by Schiötz' tonometer.) At "0," oral glycerol, 1.5 gm./kg. of body weight, was administered. In all glycerol-treated patients, about 1 hr. after administration, intraocular pressure was brought to about normal levels.

proven particularly useful in patients with normal ocular pressure when intraocular hypotony was needed to proceed with orbital surgery.

From the data obtained in ophthalmology, oral glycerol is found to be highly effective in lowering intraocular hypertension.

Conclusions

Oral glycerol has proven to be a very helpful agent to lower intracranial pressure and decrease the mass of the brain in the following specific situations:

a) In patients suffering from intracranial decompensating increased pressure and herniation of the brain, especially just prior to or during diagnostic studies. Glycerol is found to improve the general conditions to an extent that diagnostic studies, and, subsequently, surgical procedures could be carried out.

b) In patients with any type of hydrocephalus with decompensation.

c) In the treatment of head injuries when symptomatology could be attributed to edema resulting from cerebral contusions.

d) In patients suffering from space-occupying processes of the base of the skull. Glycerol facilitated surgical procedures, permitting easy access to the structures beneath the brain without injuring or amputating the sound parts of the encephalon.

e) To prevent damage to the brain when intracranial tension is high and the dura mater is about to be opened.

f) In patients whose general conditions on postoperative days, following intracranial surgery, suddenly deteriorated and increased unresponsiveness developed because of "brain swelling."

g) During roentgen-ray therapy following operative intracranial procedures.

The effectiveness of glycerol, as well as of urea and mannitol, proves that the osmotic method is the most valuable for the reduction of cerebral edema and intracranial and intraocular pressure.

As compared with the other osmotic agents commonly used for this purpose, glycerol exerts its prompt and intense effect by oral route. Since oral glycerol might be considered a "food-substance," it is possible to administer this drug safely over a long period of time and to repeat its doses every 3 hrs. without dangerous "rebound overshoot," which often follows the administration of the best known osmotic agents.

Oral glycerol, furthermore, lacks those toxic side-effects which are, for the most part, bound to intravenous administration of osmotic methods.

Summary

Referring to preceding experimental and clinical studies, the authors established that oral glycerol is a highly effective agent for the reduction of cerebral edema and cerebrospinal-fluid pressure.

Value, indications and lack of toxicity of oral doses employed are described briefly. The satisfying results obtained in neurosurgical cases before, during, and after surgery are reported.

Glycerol has also been used successfully for the management of problems of increased intracranial and intraocular pressure.

It appears that oral glycerol represents a true step forward in neurosurgery since it affords the following advantages:

1) Promptness and intensity of action in reducing intracranial hypertension.

2) Possibility of repeating administrations, without secondary "rebound overshoot."

3) Lack of toxicity.

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December 11, 1972

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251 E. Chicago Avenue
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Our organization, as a contractor to the Federal Government, is engaged in the preparation of monographs on selected food additives for the Food and Drug Administration. The purpose of this research task is to review safety aspects of these substances. We anticipate that this work will be completed in mid-1973.

In this connection we are asking your permission to make fifteen (15) copies of a few selected articles from your journal, Journal of Neurosurgery. These copies will be attached to our monographs as substantiating data and presented to a review committee.

We would be happy to supply you with a complete list of articles selected if you so desire.

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British Medical Journal Vol 2.

Preliminary Communications

Oral Glycerol in Glaucoma

Since the beginning of this century various hypertonic drugs have been used in an endeavour to withdraw fluid from within the eye and lower the pressure in acute glaucoma. There was little attendant success until seven years ago, when Javid and Settlage (1956) recorded a marked fall in intraocular pressure after the intravenous administration of urea. The number of patients who required emergency surgery for acute glaucoma could thus have been drastically reduced, but the need for an intravenous drip was a disadvantage, and there was the additional but remote risk of tissue necrosis. Mannitol, which similarly lowers the osmotic and intraocular pressures, has since been introduced, but is generally less effective, and has the same disadvantages as urea.

A recent report from a group of workers in Rome (Virno *et al.*, 1963) claims equally successful results from glycerol when administered orally in 26 cases of various types of glaucoma. This preliminary communication from the ophthalmic department of Westminster Hospital confirms their satisfactory findings.

The dosage used was 1.5 g./kg. body weight (a lower dose was markedly less effective), and this was found to be most palatable when mixed in fruit juice and soda. Most of the patients experienced some degree of headache, and one was nauseated. No significant diuresis was noted.

Five patients with *acute (closed-angle) glaucoma* were treated. The response was striking in all (Fig. 1), and the following two case-notes are illustrative.

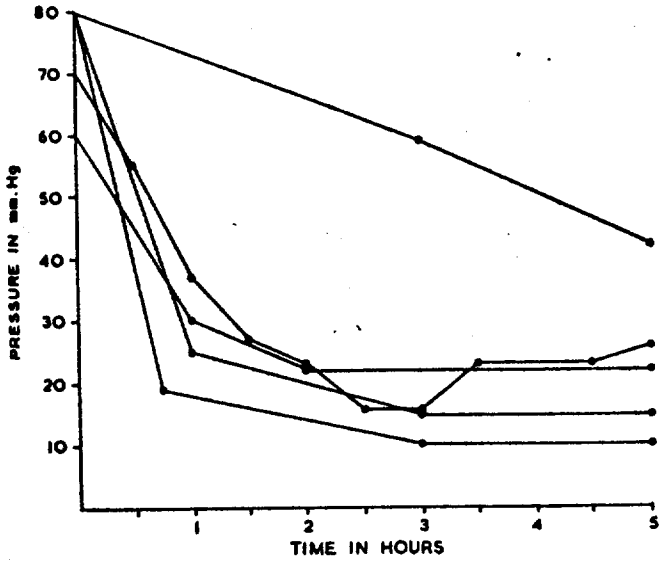

Fig. 1.—Response to glycerol in five cases of acute glaucoma.

The first patient had an acute attack for one week (treated as a conjunctivitis!). The pressure in the affected eye was over 80 mm. Hg: 45 minutes after swallowing the glycerol his intraocular pressure was 18 mm. Hg. Within the ensuing three hours there was a further fall to 10 mm. Hg.

A second patient had an acute glaucoma for three weeks, and again the pressure was over 80 mm. Hg. There was still no change in pressure three hours after the administration of 500 mg. of acetazolamide ("diamox") and the instillation of miotic drops every five minutes for half an hour. Three hours after taking glycerol the pressure was 59 mm. Hg, and after a further two hours it was 42 mm. Hg. Thereafter the pressure was further reduced to 17 mm. Hg with miotics and acetazolamide.

In a study of 16 patients with *chronic simple (open-angle) glaucoma* the relatively short-acting effects of glycerol were noted (Fig. 2). The greatest fall in intraocular pressure occurred within the first hour, but after the second hour the pressure began to rise again, and had reached its initial level in six to seven hours.

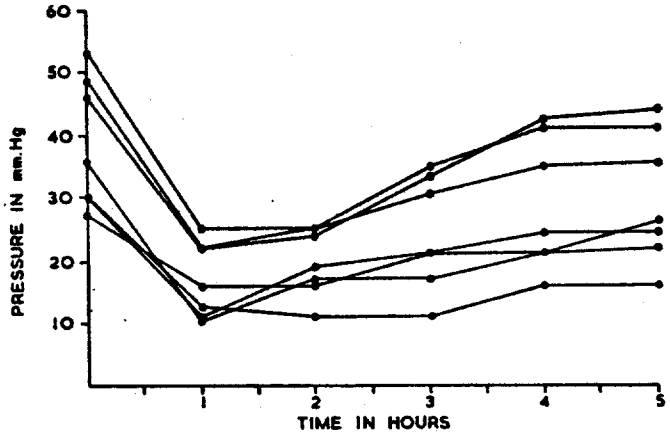

Fig. 2.—Response to glycerol in seven cases of chronic simple glaucoma.

There was an average fall in pressure of half of its initial level—for example, 45 to 22 mm. Hg. This effect was not so marked in the lower pressures, and in normal subjects the fall was typically 19 to 12 mm. Hg. One patient with simple glaucoma had a fall in pressure from 36 to 11 mm. Hg. The same patient was given acetazolamide 500 mg. orally, and the response was a fall from 30 to 22 mm. Hg.

One relatively unsatisfactory result was noted in a patient with Buerger's disease, who had thrombotic glaucoma with a tension of 80 mm. Hg. After two hours his pressure was 50 mm. Hg, but after five hours it had returned to its initial level. The same unsatisfactory response has been noted with urea, and has been thought to be due to damage to the blood-aqueous barrier.

With a dosage of 0.75 g./kg. body weight three times daily, three patients with simple glaucoma were phased for one week. Complete control of pressure was not obtained (more frequent administration is necessary), but none complained of side-effects; and it is possible that glycerol combined with miotics may have some place in treatment when acetazolamide is inadequate or cannot be tolerated.

The mechanism of action is not clear. Serum osmolarities were performed, and although they showed a rise coincidental with the fall in intraocular pressure they were not conclusive, and further biochemical studies are being performed.

In summary, oral glycerol has been used on 22 patients with various types of glaucoma, and there was a fall in tension in every case; in five cases of acute glaucoma the fall in tension was dramatic.

It is suggested that this is indeed a very useful drug, not only for the oculist in dealing with his obdurate acute glaucomas (quite apart from the many other occasions where an osmotic hypotension may be sought such as before cataract surgery in high myopia, in benign intracranial hypertension, and in the field of neurosurgery), but also for general practitioners, who can administer this safe and accessible drug as soon as the diagnosis of acute

glaucoma is made, and thus relieve the pain and avoid the serious and even tragic damage that can follow the usual delay before the patient is treated in hospital.

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J. Biol. Chem,
84(2): 553-559, 1929

THE FORMATION OF GLYCOGEN IN THE LIVER OF THE YOUNG WHITE RAT AFTER THE ORAL ADMINISTRATION OF GLYCEROL.

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(Received for publication, August 8, 1929.)

Several substances possessing 3 carbon atoms are generally thought to play a part in the intermediary metabolism of carbohydrates. The rôle of glycerol has been investigated by several authors. Since the work of Cremer (1) and more recently of Chambers and Deuel (2) on phlorhizinized animals, and that of Lüthje (3) on depancreatized dogs, it has been generally agreed that ingested glycerol is excreted as glucose in the urine by the diabetic organism. Deposition of liver glycogen after administration of glycerol, however, has not been satisfactorily demonstrated. Pflüger (4) in 1905 reviewed critically the work of earlier investigators who had reported the formation of significant amounts of glycogen after the administration of glycerol and concluded that glycogen formation in the liver after administration of glycerol was insignificant. On perfusion of the liver of a phlorhizinized dog with a 20 per cent glycerol solution, Barrenscheen (5) noted a slight loss in liver glycogen and an insignificant rise only in the sugar content of the perfusion fluid. Doyon and Morel (6) found no change in the glycogen content of dog liver after injection of glycerol into an intestinal vein over a period of 50 minutes.

Because this evidence concerning the rôle of glycerol in carbohydrate metabolism is inconsistent, and in view of the suggestive similarity in structure between glycerol and such known products of glucose catabolism as glyceric aldehyde and dihydroxyacetone, a further inquiry into the possibility of glycogen formation from glycerol was thought advisable. In the work presented here the

problem of glycerol utilization has been approached by a study of the glycogen formation in the livers of young white rats after the oral administration of glycerol. We obtained definite and prompt deposition of liver glycogen, increasing in amount up to 6 hours after feeding. The results indicate that under our experimental conditions glycerol is readily utilized in the formation of liver glycogen.

EXPERIMENTAL.

The experimental procedure was similar to that employed by Cori (7) in his studies of the absorption and metabolism of hexoses. Young white rats, weighing for the most part between 100 and 150 gm., were placed upon a standard diet of leaf lettuce, whole wheat bread, and milk. Prior to the experimental feeding period, the rats were placed in individual cages and fasted for 24 hours, an adequate supply of water being kept available at all times. The control rats were fed approximately 2 cc. of water by stomach tube, with the exception of Rats 4, 5, and 6 which were not handled. The experimental animals received 2 cc. of a 50 per cent glycerol solution by stomach tube, an amount of glycerol which caused no symptoms of gastrointestinal disturbance. With this amount, some glycerol was always observed to remain in the stomach at the end of the experimental period. In order to obtain evidence concerning the relative rates of glycogen formation from glycerol and glucose, nine rats were fed 2.5 cc. of a 50 per cent glucose solution. All of the animals were killed with chloroform, the control rats shortly after the administration of water, the others after periods of absorption as indicated in Tables II and III. The livers were quickly removed and their glycogen content determined by Pflüger's method. Glucose resulting from the hydrolysis of the glycogen was determined by the method of Hagedorn and Jensen (8).

DISCUSSION.

Our results are summarized in Tables I to III. Most of the rats used were males, but since sex did not appear to influence the results, this factor was disregarded in the tables. The livers of the rats receiving water only showed an average glycogen content of 0.09 per cent, an amount equivalent to 3.40 mg. per

100 gm. of body weight. When glycerol was fed, a significant increase in liver glycogen was noted (Table II), the average percentage rising progressively from 0.29 after 1 hour's absorption to 3.24 after 6 hour absorption periods; expressed as mg. per 100 gm. of body weight, glycogen values rose from 12.93 to 142.55. The increase of liver glycogen to 3 times that of the controls within an hour and the progressively higher figures for longer periods of

TABLE I.

Glycogen Content of Livers of Control White Rats after Fasting Periods of 24 Hours.

Rat No.	Weight after fast.	Loss in weight.	Weight of liver.	Glycogen of liver.		
	gm.	*gm.*	*gm.*	*mg.*	*per cent*	*mg. per 100 gm. body weight*
1	131	17	6.12	3.64	0.06	2.77
2	135	14	5.88	3.09	0.05	2.29
3	178	17	6.81	3.24	0.05	1.82
4	98	11	3.66	5.69	0.15	5.80
5	164	14	5.99	14.09	0.23	8.59
6	101	12	3.74	5.16	0.14	5.11
7	169	9	5.30	3.78	0.07	2.24
8	116	10	4.56	2.59	0.05	2.23
9	167	20	6.93	8.00	0.11	4.79
14	169	10	5.92	3.90	0.06	2.31
17	185	15	5.54	2.73	0.05	1.48
20	130	12	4.71	3.15	0.07	2.42
24	109	15	4.52	3.24	0.07	2.97
32	111	19	5.04	3.61	0.07	3.25
40	115	13	4.41	3.75	0.08	3.26
44	108	11	4.23	3.35	0.08	3.10
Average				4.56	0.09	3.40

absorption indicate a definite and prompt utilization of ingested glycerol.

It may be seen that with a single exception (Rat 9), less than 4 mg. of glycogen was found in the livers of control rats receiving the same treatment as experimental animals (*i.e.* administration of 2 cc. of water in place of the glycerol), and that somewhat higher values were obtained for Rats 4, 5, and 6, which received no water. The results obtained for the glycogen content of the

TABLE II.

Glycogen Content of Livers of White Rats after Oral Administration of Glycerol.

Rat No.	Weight after fast.	Loss in weight.	Period of absorption.	Weight of liver.	Glycogen of liver.		
	gm.	*gm.*	*hrs.*	*gm.*	*mg.*	*per cent*	*mg. per 100 gm. body weight*
10	127	8	1	5.01	20.55	0.41	16.18
11	131	11	1	4.72	3.43	0.07	2.62
23	124	18	1	6.06	19.35	0.32	15.60
26	136	12	1	6.20	3.75	0.06	2.76
37	113	19	1	5.65	26.88	0.47	23.78
41	105	12	1	4.31	17.45	0.40	16.62
Average					15.23	0.29	12.93
15	140	19	2	6.23	44.22	0.71	31.58
25	114	12	2	4.59	44.96	0.98	39.44
27	131	13	2	5.71	97.33	1.70	74.29
29	111	16	2	5.19	94.55	1.82	85.18
42	125	14	2	4.85	55.80	1.15	44.64
46	111	13	2	5.12	45.21	0.88	40.73
Average					63.68	1.21	52.64
12	124	12	3	5.08	60.21	1.18	48.56
13	137	14	3	5.87	82.97	1.41	60.56
16	127	14	3	5.34	92.70	1.74	72.99
18	112	14	3	4.55	113.79	2.50	101.59
22	117	13	3	5.48	120.51	2.19	103.00
33	107	16	3	5.44	122.83	2.26	114.79
Average					98.83	1.88	83.58
28	106	10	4	4.56	146.23	3.21	137.95
31	107	10	4	4.96	37.05	0.75	34.62
34	108	13	4	4.46	25.77	0.58	23.86
36	109	16	4	5.30	190.96	3.60	175.19
39	117	15	4	5.23	174.28	3.33	148.96
43	99	12	4	4.83	136.73	2.83	138.11
Average					118.50	2.38	109.78
19	167	17	6	5.50	90.61	1.65	54.25
21	140	12	6	5.45	109.38	2.00	78.13
30	130	16	6	6.04	316.11	5.23	243.16
35	108	16	6	5.06	190.96	3.77	176.81
38	110	12	6	4.49	107.53	2.39	97.75
45	117	14	6	5.42	240.09	4.43	205.20
Average					175.78	3.24	142.55

livers of the control rats after 24 hours of fasting in our series are somewhat lower than those obtained by other investigators. Although it is not definitely stated, it is probable that the control animals in these studies (9, 10) were not handled as was the case in our own experiments in connection with the use of the stomach tube.[1] The feeding apparently caused little discomfort, but it is possible that a part of the liver glycogen was mobilized or deposition slightly decreased as a result of handling the rats.

The rather wide differences in the amounts of glycogen deposited in the livers of rats during comparable absorption periods

TABLE III.

Glycogen Content of Livers of White Rats after Oral Administration of Glucose.

Rat No.	Weight after fast.	Loss in weight.	Period of absorption.	Weight of liver.	Glycogen of liver.		
	gm.	*gm.*	*hrs.*	*gm.*	*mg.*	*per cent*	*mg. per 100 gm. body weight*
48	99	14	2	4.13	66.28	1.60	66.95
50	113	16	2	4.70	60.72	1.29	53.73
52	98	13	2	3.88	46.35	1.19	47.29
54	99	14	2	4.55	45.42	0.99	45.88
Average					54.69	1.27	53.46
47	104	12	3	4.39	100.12	2.28	96.27
49	109	9	3	4.55	80.42	1.77	73.78
51	102	13	3	4.02	69.52	1.73	68.16
53	93	12	3	3.88	82.27	2.12	88.46
55	109	12	3	4.50	80.42	1.78	73.78
Average					82.55	1.94	80.09

are to be expected, since neither rate of absorption nor metabolism by other routes, such as oxidation or glycogen formation in the rest of the body, was followed. Rats 11 and 26 of the 1 hour absorption period series showed amounts of liver glycogen similar

[1] The possibility suggested itself that the low glycogen values observed might be due to increased glycogenolysis incidental to use of chloroform in killing the rats. However, the results of the experiments of Wilson (11), in which the rats were killed by a blow on the head or by chloroform, do not indicate that the use of chloroform caused appreciable changes in the glycogen content of the liver.

to those of the controls; here it seems probable that the glycerol was poorly absorbed.

The rats fed glucose, as shown in Table III, deposited amounts of liver glycogen quite comparable to those formed by the animals absorbing glycerol over similar periods. Thus the 2 hour groups showed an average of 1.27 per cent of liver glycogen after glucose feeding as compared with 1.21 per cent when glycerol was fed, and the rats killed 3 hours after feeding had average values of 1.94 per cent after glucose ingestion and 1.88 per cent after glycerol. Glycogen values in mg. per 100 gm. of body weight are also similar. It would seem that under the conditions of our experiments, for the 2 and 3 hour absorption periods at least, glucose and glycerol are almost equally good sources of liver glycogen.

Noble and Macleod (12) have reported that 10 cc. of glycerol injected into a rabbit as an antidote for insulin convulsions did not increase blood sugar or favorably influence the convulsions. Voegtlin, Dunn, and Thompson (13, 14), on the other hand, demonstrated both a curative and a preventive action of glycerol in insulin hypoglycemia when the alcohol was administered to white rats in 20 per cent solution either intraperitoneally or by slow intravenous injection. The same authors (15) have noted a hyperglycemia in rabbits after administration of glycerol both orally and intraperitoneally, and have also shown in insulinized rabbits a beneficial action of glycerol when injected intraperitoneally (13) or fed (15) in 20 per cent solution. They recognize that to achieve any curative action in insulin coma or convulsions the therapeutic agent must be promptly available, and state that if glycerol is transformed to glucose, the conversion must be very rapid. In view of the similarity in the rate of deposition of liver glycogen after the administration of glucose and glycerol in our own studies, we believe that our results tend to support the view that glycerol may be an efficient antidote for insulin intoxication.

SUMMARY.

Young white rats after fasting periods of 24 hours were fed glycerol by stomach tube. The content of glycogen in the livers at periods from 1 to 6 hours after the ingestion of glycerol increased progressively to an average maximum value of 3.24 per cent, or 142.55 mg. per 100 gm. of body weight. The livers of

control rats, which received water only, contained an average of 0.09 per cent of glycogen. The amounts of liver glycogen after 2 and 3 hour periods of absorption of glycerol were similar to those observed after absorption of glucose over the same periods. Under our experimental conditions glycerol appears to be readily metabolized and deposited as glycogen in the livers of young white rats.

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J. Biol. Chem.
65: 21-29, 1925

ANIMAL CALORIMETRY.

THIRTIETH PAPER.

THE METABOLISM OF GLYCEROL IN PHLORHIZIN DIABETES.

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(Received for publication, June 24, 1925.)

Glycerol is ordinarily listed with the intermediary products of carbohydrate metabolism, which can be completely converted into glucose in the diabetic organism, although no published data showing its total recovery as urinary sugar are to be found. Hirschfeld (1), Satta (2), and Lang (3) have reported a decrease in acetonuria after giving glycerol to diabetic and to normal fasting individuals. Voegtlin, Dunn, and Thompson (4) have shown that glycerol will replace glucose in relieving the hypoglycemia and toxic effects of insulin injections. Cremer (5) found that about 40 per cent of the glycerol ingested by a phlorhizinized dog was excreted in the urine as "extra glucose," and Lüthje (6) obtained similar results with depancreatized dogs. Recently McCann, Hannon, Perlzweig, and Tompkins (7) and Thomas (8) have used this conversion figure of 40 per cent as a basis for calculating the antiketogenic value of glycerol in the diets of diabetic patients. To determine more exactly the quantitative conversion of glycerol into glucose, experiments were made on phlorhizinized dogs in which the urine was collected at hourly intervals.

EXPERIMENTAL DATA.

The technique of phlorhizinizing, collecting the urine, and calculating the results is published in a previous paper (9). Glycerol, U.S.P., was given by stomach tube in aqueous solution or injected subcutaneously. The specific gravity of the glycerol, determined

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by pycnometer, was 1.2497 which, according to the table in "Watts' Dictionary of chemistry" (10), equals by interpolation 94.76 per cent. It should be noted in calculating the recovery of glycerol as glucose that 2 molecules of glycerol yield 1 molecule of glucose and 4 atoms of hydrogen

$$2C_3H_8O_3 \rightarrow C_6H_{12}O_6 + 4H$$

or 100 gm. of glycerol yield 97.8 gm. of glucose and 2.2 gm. of hydrogen.

The complete data of five experiments made on the three female dogs are given in the protocols. In the first experiment (Dog 56, January 20, Protocol 1) 15.16 gm. of glycerol were ingested and only 55.9 per cent were recovered in the urine as extra glucose.

TABLE I.

Summary.

Dog No.	Glycerol ingested.	Glucose equivalent.	Glucose recovered.	
	gm.	*gm.*	*gm.*	*per cent*
56	15.16	14.85	8.30	55.9
56	8.53*	8.33	5.83	70.0
56	8.53	8.33	4.46	53.8
57	8.53	8.33	8.20	98.4
51	8.53	8.33	8.07	96.9

* Subcutaneous injection.

The following day into the same dog a smaller amount, 8.53 gm., was injected subcutaneously to eliminate the factor of absorption. The yield of extra glucose was 70 per cent. When the same amount, 8.53 gm., was ingested the next day the return was 53.8 per cent.

The other two experiments with Dogs 57 and 51 are given in Protocols 2 and 3. From 8.53 gm. of ingested glycerol 8.20 and 8.07 gm. of extra glucose were obtained in the urine, a yield of 98.4 and 96.9 per cent, respectively. These results relating to the amount of glycerol converted into glucose are summarized in Table I.

In two of the dogs (Dogs 57 and 51) there was an almost complete recovery of the glycerol in the form of glucose, while the third dog (Dog 56) yielded about 60 per cent. The metabolism

of the last dog was therefore determined in the respiration calorimeter to see if there was an oxidation of the unrecovered portion of glycerol.

On the day following the experiments tabulated in Protocol 1 the basal metabolism of Dog 56 was determined for 2 consecutive hours, 8.53 gm. of glycerol were then ingested and the metabolism was again observed for the succeeding 2nd, 3rd, and 4th hours. The data are given in Table II.

The respiratory quotients average 0.703 for the basal metabolism and 0.678 for the 3 hours after the glycerol ingestion. There is a slight decrease in the heat production of questionable significance, as the averages are 19.91 calories before and 18.80 calories after taking the glycerol.

TABLE II.

Metabolism of Dog 56 before and after Ingesting Glycerol in Phlorhizin Glycosuria.

Hourly period ending.	Experiment.	CO_2	O_2	R.Q.	N	Non-protein R.Q.	Total calories.
p.m.		*gm.*	*gm.*		*gm.*		
3.41	Basal metabolism.	5.84	6.25	0.680	0.304	0.691	19.47
4.41		6.10	6.10	0.727	0.304	0.754	20.34
Averages.		5.97	6.18	0.703	0.304	0.723	19.91
7.14	After ingesting 8.53 gm. glycerol at 4.59 p.m.	5.28	5.79	0.663	0.293	0.672	18.42
8.14		5.22	5.41	0.702	0.312	0.724	18.20
9.14		5.69	6.17	0.671	0.312	0.680	19.77
Averages.		5.40	5.79	0.678	0.306	0.692	18.80

DISCUSSION.

Our experiments on three dogs indicate that a complete conversion of glycerol into glucose is found in some dogs and that the lower figures of earlier workers might be explained on the basis of the difference in individual animals. A 70 per cent recovery on subcutaneous injection and one of 55 per cent after ingestion in Dog 56 suggest that an incomplete absorption may account for a part of the unrecovered portion of the glycerol. The calorimeter

experiment excludes the oxidation of the glycerol as an explanation for the unrecovered balance.

The decrease in the respiratory quotient from a basal of 0.703 to 0.678 on the ingestion of glycerol (Table II) is a significant one. As shown in the equation above, the conversion of 2 molecules of glycerol to 1 of glucose yields in addition 4 atoms of hydrogen. The recovery of extra glucose was not determined in the calorimeter experiment, but on the previous day (January 22, Protocol 1) 3.53 gm. of extra glucose were excreted during the 2nd, 3rd, and 4th hours after giving *per os* the same amount of glycerol, 8.53 gm. If oxidized, the hydrogen from the amount of glycerol converted into glucose requires 0.628 gm. or 0.440 liter of additional oxygen. If this be subtracted from the oxygen used in the oxidation of the non-protein fraction of the total metabolism, one may calculate the respiratory quotient of the rest of the material oxidized. The formula is as follows:

$$\text{R.Q.} = \frac{6.37 \text{ liters of } CO_2}{9.23 - 0.44 \text{ liters of } O_2} = 0.725$$

This agrees almost exactly with the non-protein quotient of the preceding period (0.723) in which no glycerol was given. Since the respiratory quotient of glycerol is 0.857, it follows that glycerol itself could not have been oxidized.

One must consider that when 3.53 gm. of glucose (13.24 calories, Rubner) are produced from 3.61 gm. of glycerol (15.57 calories, Stohmann), 2.33 calories are liberated in the reaction. One also may estimate that, if the hydrogen liberated in this reaction united directly with oxygen, 2.72 calories would have been produced. Therefore approximately one-third of 2.33 calories, or 0.80 calorie, has been added to the computed hourly heat production in this part of the experiment. The results of these metabolism experiments, therefore, indicate that in the phlorhizinized dog there may be a complete conversion of glycerol to glucose and no detectable oxidation of the glycerol or the glucose which is formed from it, except as to the hydrogen liberated in the reaction.

McCann and Hannon (11) have reported a decrease in the respiratory quotient after the ingestion of glycerol by diabetic patients. However, they have found a similar effect in the same patients when glucose was taken.

The hourly rate of excretion of ingested glycerol is practically the same as that of the monosaccharides. Thus, the same dog (Dog 51) which was used in both kinds of experiments gave almost identical figures for the percentage of extra glucose eliminated during the first 3 hours after the administration of both glycerol (Protocol 3) and galactose.[1] From the figures of hourly nitrogen excretion given in the protocols it is seen that glycerol produces a sparing of the protein metabolism in the same manner as do the monosaccharides.

No gastrointestinal disturbances were apparent following the administration of the glycerol in the small amounts used in these experiments.

SUMMARY.

A practically complete conversion of glycerol to glucose was exhibited by two phlorhizinized dogs. After the ingestion of 8.53 gm. of glycerol, 96.9 and 98.4 per cent, respectively, of extra glucose were recovered in the urine.

A third dog yielded 55 per cent conversion after ingestion and 70 per cent after subcutaneous injection of glycerol. A decrease in the respiratory quotient from 0.703 to 0.678 after giving glycerol indicates that there was no general oxidation of the ingested glycerol, which has a respiratory quotient of 0.86, nor of the glucose into which it was converted. The reduction in the respiratory quotient from 0.703 to 0.678 may be exactly accounted as due to the oxidation of the 2 atoms of hydrogen liberated in the reaction glycerol → 1/2 glucose.

In the rate of excretion and the sparing of protein metabolism the reaction of glycerol was similar to that of the monosaccharides.

To Professor Graham Lusk the authors are indebted for his kindly suggestions and criticism.

[1] Deuel and Chambers (9), Protocol 3, p. 18.

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PROTOCOL 1.

Glucose and Nitrogen Elimination during Glycerol Experiments.

Dog 56. Weight, 9.8 kilos.

Date.	Time.	Glucose.	Nitrogen. For period.	Nitrogen. Per hr.	D:N ratio.	Extra glucose.		Remarks.
1925		*gm.*	*gm.*	*gm.*		*gm.*	*per cent*	
Jan. 18	7.45 p.m.							
" 19	9.17 a.m.	17.67	4.754	0.351	3.72			
	10.17 "	1.44	0.364	0.364	3.94			
	11.17 "	1.60	0.415	0.415	3.87			
	12.17 p.m.	1.45	0.372	0.372	3.92			
	1.17 "	1.44	0.375	0.375	3.85			
	2.17 "	1.48	0.371	0.371	3.98			
	6.26 "	5.40	1.448	0.348	3.73			
" 20	9.25 a.m.	18.66	5.495	0.360	3.30 3.55*			
	10.25 "	1.09	0.340	0.340	3.21			
	11.25 "	1.15	0.346	0.346	3.31			15.16 gm. glycerol ingested at 11.33 a.m.
	12.33 p.m.	2.17	0.438	0.388	5.04	0.64	7.71	
	1.33 "	2.60	0.301	0.301	8.64	1.55	18.66	
	2.33 "	2.85	0.260	0.260	10.96	1.94	23.38	
	3.33 "	2.39	0.215	0.215	11.11	1.44	17.35	
	4.33 "	2.07	0.254	0.254	8.15	1.18	14.22	
	5.33 "	1.54	0.257	0.257	5.99	0.64	7.71	
	6.33 "	1.19	0.238	0.238	5.00	0.36	4.34	
	7.33 "	1.01	0.240	0.240	4.21	0.17	2.05	
	8.33 "	1.00	0.236	0.236	4.23	0.17	2.05	
	9.33 "	1.05	0.276	0.276	3.80	0.04	0.48	
	10.33 "	1.17	0.287	0.287	4.07	0.17	2.05	
						8.30		
" 21	9.33 a.m.	10.93	2.894	0.263	3.78 3.68*			
	11.49 "	2.53	0.609	0.269	4.15			8.53 gm. glycerol injected subcutaneously at 11.56 a.m.
	12.56 p.m.	2.18	0.379	0.340	5.75	0.72	12.36	
	1.56 "	2.53	0.295	0.295	8.58	1.40	24.01	
	2.56 "	2.85	0.293	0.293	9.73	1.72	29.50	
	3.56 "	2.63	0.324	0.324	8.12	1.39	23.84	
	4.56 "	1.69	0.351	0.351	4.81	0.34	5.83	
	5.56 "	1.37	0.308	0.308	4.45	0.19	3.26	
	6.56 "	1.20	0.294	0.294	4.08	0.07	1.20	
						5.83		
	7.56 "	1.13	0.306	0.306	3.69			
	11.01 "	3.14	0.845	0.274	3.72			

PROTOCOL 1—*Concluded.*

Date.	Time.	Glucose.	Nitrogen. Per period.	Nitrogen. Per hr.	D:N ratio.	Extra glucose.		Remarks.
1925		*gm.*	*gm.*	*gm.*		*gm.*	*per cent*	
Jan. 22	9.51 a.m.	12.99	3.322	0.307	3.92 3.85*			8.53 gm. glycerol ingested at 9.55 a.m.
	11.55 "	4.10	0.605	0.294	6.77	1.77	39.68	
	1.55 p.m.	4.60	0.623	0.312	7.38	2.20	49.33	
	3.55 "	2.49	0.517	0.259	4.82	0.49	10.99	
						4.46		
	5.55 "	2.04	0.572	0.286	3.57			
" 23	11.53 a.m.	19.48	5.192	0.288	3.75			

* The last portion of the night sample was collected by catheter and the D:N ratio determined separately.

PROTOCOL 2.

Hourly Elimination of Glucose after the Ingestion of Glycerol.

Dog 57. Weight, 7.8 kilos.

Date.	Time.	Glucose.	Nitrogen. Per period.	Nitrogen. Per hr.	D:N ratio.	Extra glucose.		Remarks.
1925		*gm.*	*gm.*	*gm.*		*gm.*	*per cent*	
Feb. 2	2.29 p.m.							
" 3	9.13 a.m.	19.62	4.992	0.266	3.93 3.97*			8.53 gm. glycerol ingested at 9.17 a.m.
	10.17 "	2.37	0.312	0.288	7.60	1.13	13.78	
	11.17 "	3.02	0.238	0.238	12.69	2.08	25.37	
	12.17 p.m.	2.51	0.213	0.213	11.78	1.66	20.24	
	1.17 "	1.54	0.174	0.174	8.85	0.87	10.61	
	2.17 "	1.37	0.215	0.215	6.37	0.52	6.34	
	3.17 "	1.25	0.208	0.208	6.01	0.42	5.12	
	4.17 "	1.12	0.200	0.200	5.60	0.33	4.02	
	5.17 "	1.16	0.220	0.220	5.27	0.29	3.54	
	6.17 "	1.04	0.208	0.208	5.00	0.21	2.56	
	7.17 "	0.92	0.214	0.214	4.30	0.07	0.85	
	8.17 "	0.97	0.222	0.222	4.37	0.09	1.10	
" 4	9.01 a.m.	11.83	2.864	0.225	4.13 3.62*	0.53	6.47	
						8.20		

* The last portion of the night sample was collected by catheter and the D:N ratio determined separately.

PROTOCOL 3.

Hourly Elimination of Glucose after the Ingestion of Glycerol.

Dog 51. Weight, 8.42 kilos.

Date.	Time.	Glucose.	Nitrogen. Per period.	Nitrogen. Per hr.	D:N ratio.	Extra glucose.		Remarks.
1925		*gm.*	*gm.*	*gm.*		*gm.*	*per cent*	
Jan. 27	11.19 a.m.							
	5.19 p.m.	7.12	1.545	0.258	4.61			
" 28	9.08 a.m.	14.77	3.718	0.235	4.20 3.88*			8.53 gm. glycerol ingested at 9.33 a.m.
	10.33 "	2.23	0.364	0.258	8.64	0.82	10.16	
	11.33 "	2.80	0.276	0.276	10.14	1.73	21.44	
	12.33 p.m.	2.70	0.228	0.228	11.84	1.82	22.55	
	1.33 "	1.74	0.194	0.194	8.97	0.99	12.27	
	2.33 "	1.46	0.222	0.222	6.58	0.60	7.43	
	3.33 "	1.18	0.203	0.203	5.81	0.39	4.83	
	4.33 "	1.15	0.228	0.228	5.04	0.27	3.35	
	5.33 "	1.24	0.237	0.237	5.23	0.32	3.97	
" 29	9.58 a.m.	15.37	3.684	0.224	4.21 3.67*	1.13 8.07	14.00	

* The last portion of the night sample was collected by catheter and the D:N ratio determined separately.

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Arch. Ophthal.
75: 201-203, 1966

Adverse Effects After Glycerol Orally and Mannitol Parenterally

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No serious side effects have been reported following the use of glycerol orally for the production of ocular hypotony. An elderly, senile patient received glycerol orally for acute angle-closure glaucoma, and experienced signs which were initially interpreted as due to cardiac arrest. Mannitol then was given for persistent ocular hypertension before cataract surgery, and it compounded her problem.

Report of Cases

CASE 1 (SFGH 112542).—An 82-year-old Caucasian woman had been followed for primary angle-closure glaucoma in the right eye. She refused surgery and was treated with 4% pilocarpine and 125 mg acetazolamide (Diamox) three times a day. The left eye was blind from glaucoma.

She experienced pain in the right eye on Aug 24, 1964. Visual acuity was 20/200, and the ocular tension was 60 mm Hg (Schiotz). She was given 200 cc* of 50% glycerol by mouth at 3 PM. Carbachol 1.5% and neostigmine (Prostigmin) 5% were given every 15 minutes. A 4 PM the intraocular pressure was 17 mm Hg.

While riding home on a bus, the woman developed a headache in the occipital region, shaking of the right arm, quivering of the eyes, and nausea. Soon after this she was taken to an emergency hospital. A diagnosis of convulsive seizure with cardiac arrest was made. There was an associated hypertension and possible cerebral vascular accident. Her blood pressure was 258/88 mm Hg. External cardiac massage and oxygen were given.

She was transferred to the San Francisco General Hospital at 6:15 PM. The blood pressure was 220/90 mm Hg, the pulse was 80 beats per minute, and a few rales were present in the base of the right lung. Cardiac examination revealed cardiomegaly. An electrocardiogram showed left ventricular hypertrophy and frequent ventricular premature contractions often with ventricular bigeminy. The diagnosis was that of "possible cerebrovascular accident." Another electrocardiogram taken one hour after the first showed disappearance of the premature ventricular contractions. Quinidine was given orally to suppress the ventricular premature contractions.

Hospital Course.—Physical examination revealed a pale, elderly woman in no acute distress. She denied chest pain or dyspnea. Blood pressure one hour following admission was 180/90 mm Hg. The right pupil was 2 mm and the left pupil 6 mm in diameter. The right pupil reacted sluggishly to light.

On the following day, visual acuity was counting fingers at 2 inches in the right eye and light perception in the left eye. The right intraocular pressure was 60 mm Hg, the cornea was clear, and a cataract was present. A diagnosis of angle-closure glaucoma and cataract was made in the right eye. Repeated instillations of pilocarpine 3% and eserine ¼% failed to lower the intraocular pressure.

In view of the persistently high intraocular pressure it was felt that cataract surgery should be done, preceded by mannitol administration. During the next hour 200 cc of 20% mannitol were administered intravenously. At the end of this period the patient was in the operating room, and the intraocular pressure of the right eye was 30 mm Hg. Prior to entrance of the anterior chamber, the intraocular pressure was 15 mm Hg. The mannitol infusion was discontinued after 300 cc had been given. With local anesthesia and α-chymotrypsin (Zolyse) an uncomplicated intracapsular cataract extraction was done. During the entire surgical procedure an anesthesiologist was present, monitoring the cardiac status. The anesthesiologist noted premature ventricular contractions early in the course of surgery, but these later disappeared.

Postoperative Hospital Course.—On return to the ward, the patient became restless and agitated and required restraint.

The following morning she still was disoriented, and medical consultation was obtained. The impression was: (1) dehydration secondary to osmotic diuresis, and (2) acute brain syndrome. Blood-serum studies showed the following: CO_2, 32 mEq/liter; chloride, 112 mEq/liter; potassium, 3.6 mEq/liter; sodium, 155 mEq/liter; blood urea nitrogen (BUN), 23 mg/100 cc; and creatinine, 1.2 mg/100 cc. It was felt the elevated serum sodium level was secondary to a water deficit and that the low potassium and elevated CO_2 levels reflected a metabolic alkalosis secondary to potassium loss during the osmotic diuresis. Calculations showed the water deficit to be 2.93 liters. Intravenous fluid therapy of 5% dextrose with potassium chloride was initiated and continued for three days.

On the second postoperative day, blood-serum chemistries showed the following: CO_2, 31 mEq/liter; chloride, 105 mEq/liter; sodium, 143 mEq/liter; potassium, 4.1 mEq/liter; FBS, 175 mg/100

Submitted for publication July 12, 1965.

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* The patient's weight was later found to be 54.6 kg (120.12 lb).

cc; BUN, 12 mg/100 cc; and creatinine, 0.8 mg/100 cc. Though the electrolyte balance was improved, the patient still was disoriented. This state persisted until the 15th day after surgery. During her hospitalization the operated eye showed no complications. She was discharged 10 days following surgery. At this time the intraocular pressure was 9 mm Hg in the right eye, and 40 mm Hg in the left eye.

Follow-Up—The patient was seen on April 23, 1965, at which time her visual acuity in the right eye was 20/100 with an aphakic correction. The poor vision was attributed to macular degeneration. Intraocular pressure in the right eye was 18 mm Hg. Gonioscopy showed a wide-open angle circumferentially with a few peripheral anterior synechias superiorly. The patient was receiving 1% pilocarpine three times a day.

Comment: The initial symptoms of occipital headache, arm tremors, and nausea experienced by patient 1 presented, are felt to be due to the oral glycerol given for acute angle-closure glaucoma. These symptoms occurred about 1 to 1½ hours after glycerol ingestion and coincided with the approximate time of maximal effect of the drug.[1,2,3] Drance[1] has shown that in normal eyes an increased blood osmolarity corresponded to the decrease in intraocular pressure observed, the maximal effect occurring about one hour after the ingestion of glycerol. One can speculate that the glycerol caused the symptoms experienced in this arteriosclerotic patient by altering circulatory dynamics sufficiently to produce a cerebrovascular ischemic state: the increased blood osmolarity due to glycerol leading to an increased blood volume, adding extra work to an arteriosclerotic cardiovascular system. The initial high systolic blood pressure of 258/88 may have been the result of the increased blood volume. Glycerol may also dehydrate the peripheral nerves[4] and this may have played a part in the patient's symptoms.

Reported side effects following the use of glycerol orally (0.7 to 1.5 mg/kg) have included mild to marked headache,[1,3,5,6] transient nausea,[1,5,6] dizziness,[2,6] lightheadedness,[1] and erythema of the face.[2] In the present case, the symptoms were severe enough to warrant treatment in an emergency hospital.

Though it is difficult to know all the factors responsible for the patient's symptoms, at least three should be considered: (1) dose of glycerol, (2) her fasting or nonfasting state, and (3) her mental state. The most commonly used dosage of oral glycerol varies between 1 to 1.5 gm/kg body weight.[3,5,6] This patient received 2.3 gm/kg of glycerol,* which is more than is usually prescribed. Johnson et al[7] administered 1.3 to 2.2 gm/kg of glycerol orally for 50 days to 14 subjects without observing ill effects. Their subjects, however, were graduate students. Had they used fourteen 82-year-old ladies, they may have seen a few side effects. Miner and Dalton[4] cite studies in which oral doses of glycerol up to 3 gm/kg of body weight were given to humans without harmful effects.

The second factor which may have played a role in the patient's symptoms would be her fasting or nonfasting state. This factor is seldom a problem when intravenous therapy (urea and mannitol) is used. With oral glycerol, this can be a major consideration, especially in debilitated patients. In the present case, the glycerol was given about three hours after a lunch consisting of a cup of tea and an egg. Johnson et al[7] state that it is "easy to conceive of ingestions of large quantities of glycerol causing serious if not fatal changes through osmotic effects alone especially if the stomach is empty."

How can we explain the patient's prolonged disorientation during her hospitalization? Though disorientation and agitation have been observed following urea,[8] these signs are not felt to occur following administration of mannitol.[9] Hill[8] has reported disorientation following intravenous urea in two elderly female patients. Both patients were oriented within 24 hours. In the present case, if dehydration secondary to mannitol were responsible for her signs, one would expect regression of symptoms within a few days. It was noted by one of us (W.F.) that the patient was mentally senile for the period of time prior to glycerol administration. Therefore, the patient's prolonged disorientation in the hospital was in no small part due to her senile mentality; her "normal" status. When the patient was examined on April 23, 1965, she had no recollection of her hospitalization or the events immediately preceding it.

Another patient was observed to exhibit side effects attributed to glycerol.

Case 2 (SFGH 115363).—A 68-year-old obese diabetic woman was seen in the Eye Clinic complaining of severe pain in the right eye, of one week's duration. An uncomplicated cataract extrac-

*The density of glycerol is 1.26; therefore, 4 ml is equal to about 5 gm.

tion was done in the right eye seven months previously. Her diabetes was controlled with 25 units of isophane insulin suspension daily. Intraocular pressure in the right eye was 62 mm Hg (Schiotz). A diagnosis of open-angle glaucoma secondary to rubeosis irides was made, and she was given 60 cc of 50% glycerol by mouth and admitted to the hospital. The next day intraocular tension remained elevated in spite of two doses of 60 cc of 50% glycerol and 250 mg of acetazolamide four times a day. On the third hospital day she was given 100 cc 50% glycerol by mouth, but 1¼ hours later the intraocular tension remained elevated at 54 mm Hg.

Two days later the patient became confused and semicomatose, and a cerebrovascular accident was suspected. Intraocular tension at this time was 17 mm Hg in the right eye. The following day the patient's condition remained critical. Medical consultation was obtained, and a diagnosis of "electrolyte imbalance" and hypovolemia secondary to diuresis (she had lost 13 lb [6 kg] since admission) was made. Fasting blood sugar was 455 mg/100 cc and serum CO was 15 mEq/liter. Diabetic acidosis was now considered, and intravenous therapy supplemented with insulin was instituted. Three days later the patient was completely recovered.

Comment: The adverse effects of glycerol were considered when the patient was first seen in the Eye Clinic, and a small, and perhaps homeopathic dose (60 cc) of glycerol was given. Additional glycerol was given the following two days. One would have to speculate that the glycerol had cummulative effects in this diabetic patient to account for her symptoms several days following admission. Whether this occurred can only be surmised. Wishnofsky et al [10] have shown that following oral administration of glycerol, the blood sugar in nondiabetic and diabetic patients is increased, the increase being greater in the latter group. Recently, Bietti [11] has seen coma occur in two diabetic patients following administration of glycerol orally. He has suggested that insulin be given simultaneously with glycerol when treating diabetic patients.

Cases of two patients who had adverse effects after oral administration of glycerol are presented.

Summary

The first patient, an 82-year-old hypertensive, mentally senile woman, received glycerol orally for primary angle-closure glaucoma and experienced signs which were misdiagnosed as due to cardiac arrest. Following hospitalization, cataract extraction was done. Mannitol, given preoperatively to lower intraocular pressure, may have been responsible for the patient's prolonged confused state.

The second patient, a 68-year-old diabetic woman, was admitted for treatment of glaucoma secondary to rubeosis irides. Orally administered glycerol was felt to be responsible for the ensuing severe diabetic acidosis.

Though complete studies were not done at the time, the evidence presented indicate that oral glycerol may produce serious side effects. These effects may be most marked in the elderly patients especially if they are already dehydrated and the glycerol is given on an empty stomach. Glycerol therapy is not innocuous and should be used cautiously especially in the elderly, senile patient.

Thus, in addition to exercising caution when administering glycerol to elderly senile patients, special care must be given to the elderly diabetic patient.

Gary Kardos, MD, and Marvin Smoller, MD, managed medical and electrolyte problems for patient 1.

Generic and Trade Names of Drug

Chymotrypsin—*Alpha Chymar, Avazyme, Chymar, Chymar Aqueous, Chymar Buccal, Chymolase, Enzeon, Quimotrase, Zolyse.*
Acetazolamide—*Diamox.*
Neostigmine—*Prostigmin.*

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Glycerol

—*Effects upon Rabbits and Rats*—

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BECAUSE of the wide use of glycerol as a solvent for compounds administered experimentally or therapeutically to animals and men, and in view of the discrepancies in the published reports on the toxicity of glycerol[1 to 9] certain observations have been made and are recorded in the following paragraphs.

The Toxicity of Glycerol

GLYCEROL (Merck's reagent) was administered subcutaneously, intraperitoneally, orally, cutaneously, and by intracardiac injection, to albino rabbits and rats.

Repeated applications of glycerol and of 50% aqueous solution of glycerol upon the abdominal skin of rabbits, in individual doses equivalent to 4.5 cc. glycerol per kilogram of body weight, did not result fatally in any instance. The animals were kept under restraint for two hours after each application. The undiluted glycerol, applied daily for 12 days, excepting Sunday, caused no reduction in the rate of weight increase, and no blood changes or other evidences of intoxication. The aqueous glycerol solution, applied daily for 25 days, exceptnig Sunday, was similarly without obvious toxic effects, except that the animals remained stationary in weight during this period, resuming normal growth at its conclusion.

TABLE 1
SUMMARY OF LETHAL EFFECTS OF GLYCEROL ON WHITE RATS

Number of Rats	Dose cc/kg	% Dead	Time Till Death
Oral Administration			
5	13	0	
5	15	0	
5	17	20	3 hr.
5	19	20	10 hr.
5	21	40	2 hr.
5	23	60	2–10 hr.
Subcutaneous Administration			
5	7	20	3 days
5	9	0	
10	11	20	3 hr., 4 days
10	13	30	1 to 3 days
10	15	60	3 hr. to 3 days
10	16	70	10 hr. to 4 days
10	17	90	1 to 9 hr.
Subcutaneous Administration to Rats Starved for 3 Days			
5	13	80	1 to 3 hr.
5	15	80	3 to 4 hr.
5	16	100	2 to 3 hr.
5	17	80	1 to 3 hr.
Intraperitoneal Administration			
5	1		
10	3	10	3 days
10	4	20	25 min. to 2 days
10	5	40	50 min. to 3 days
10	6	80	30 to 100 min.
5	7	100	20 to 50 min.
5	9	100	30 to 60 min.
5	13	100	15 to 30 min.

Regardless of the mode of administration, lethal doses of glycerol produced restlessness, mild cyanosis, diminished arterial blood pressure, increased rate and magnitude of respiration, followed by decreased respiration, debility, diuresis, tremor, collapse, clonic convulsions, coma, and death in respiratory and circulatory failure.

Sublethal doses produced similar but less severe effects except that the debility, diuresis, loss of appetite and the consequent loss of weight were more pronounced. Several rats remained inactive for four days, but both rabbits and rats recovered after having suffered moderately severe convulsions. Rats showed no changes in alimentary activity, while rabbits, particularly those treated orally, occasionally showed a mild diarrhea.

Tables 1 and 2 summarize the facts with respect to the lethal and sublethal concentrations of glycerol.

Effect of Glycerol on the Blood Picture of Rabbits

A SINGLE lethal subcutaneous injection in the dorsal region caused a pronounced and prompt (in three hours) drop in the number of erythrocytes (from 5.6 to 1.6 millions per cu. mm.) and leucocytes (from 9.2 to 4.1 thousands per cu. mm.) and in the concentration of hemoglobin (from 11 to 7 gm. per 100 cc. blood). Weekly blood examinations on a group of five rabbits, each of which was given 16 subcutaneous injections of 2 to 6 cc. of glycerol per kilogram of weight over a period of seven weeks, revealed wide fluctuations in the leucocytes, diminution of erythrocytes from an average of six millions to four millions per cu. mm., and reduction in hemoglobin concentration from 13 gm. to 8 gm. per 100 cc. of blood, with prompt recovery upon discontinuance of the injections.

TABLE 2
SUMMARY OF EFFECTS OF GLYCEROL ON ALBINO RABBITS

Number of Rabbits Employed	Dose cc/kg	Fate
Single Oral Administration		
4	6 to 12	inactivity, tremor, recovered
3	14 to 18	convulsions, death in 100 to 120 min.
Single Subcutaneous Administration		
6	4 to 8	slight diarrhea, recovered
5	10 to 18	inactivity, tremor, death in from 2 hours to 3 days
Single Intraperitoneal Administration		
4	4 to 6.5	inactivity, convulsions, recovered
3	7 to 9	convulsions, death in 60 to 110 min.

Each of five rabbits was given two injections either of 2 or 2.6 cc. per kg. of glycerol into the heart, at intervals of 24 hours. Each injection was followed by a decrease in erythrocytes (from six to four millions per cu. mm.) and the hemoglobin concentration (from 13 to 10 gm. per 100 cc. blood), and by a slight increase in leucocytes.

Sublethal intraperitoneal injections, given to six rabbits, induced no significant changes in the num-

ber of erythrocytes or in the hemoglobin concentration during the first four to six hours after the injection, but the leucocyte counts were increased by seven to 25 thousands per cu. mm. of blood. There was a reduction in the hemoglobin concentration at the end of 24 hours, the concentration had dropped from an average of 12 to 9.5 gm. per 100 cc. of blood.

The oral administration of lethal and repeated sublethal doses of glycerol produced no reduction in erythrocytes, leucocytes, or hemoglobin, in excess of the observed variations in control animals.

The Production of Hemoglobinuria

HEMOGLOBINURIA, observed in both rabbits and rats after subcutaneous injections of glycerol, was also seen in a few rabbits after intravenous and intraperitoneal injection, but never after oral or cutaneous administrations.

Gross pathology

THE gross pathological changes observed in the tissues of these animals agree with those reported by Plosz[10], Simon[6], and Kobert[2].

Summary

THE lowest lethal concentrations of glycerol for albino rabbits were approximately 7 cc. per kg. when given intraperitoneally, 10 cc. per kg. subcutaneously, and 14 cc. per kg. orally.

Doses of 5 to 6 cc. per kg., given intraperitoneally, killed from 40 to 80% of the rats; 15 or 16 cc. per kg. given subcutaneously killed from 60 to 70%; and 21 to 23 cc. per kg. administered orally killed from 40 to 60% of these animals.

Repeated and extensive applications of glycerol, alone or in 50% aqueous solutions, upon the skin of rabbits and rats did not induce definite or fatal intoxication.

Lethal subcutaneous doses of glycerol caused great reduction in the number of erythrocytes and leucocytes and in the hemoglobin content of the blood of rabbits, while repeated sublethal doses produced corresponding but less severe effects upon the erythrocytes and hemoglobin, and a wide fluctuation in the numbers of leucocytes. Glycerol injecetd into the heart produced a decrease in the number of erythrocytes and in the hemoglobin content of the blood, together with a slight increase in the number of leucocytes. Intraperitoneal injections resulted in considerable increases in leucocytes, but no other significant changes in the formed elements of the blood.

Hemoglobinuria occurred regularly after subcutaneous injection, occasionally after intravenous and intraperitoneal injection, but never after cutaneous or oral administration.

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The separation of polyols by thin-layer chromatography*

Many procedures have recently been proposed for the separation of polyols by thin-layer chromatography on silica gel, alumina or Kieselguhr G plates using various developing solvents[1]. In some cases, adsorbents modified with complexing agents such as boric acid[2] or ammonium borate[3] have also been tried.

In previous work[4-6] it has been shown that in an alkaline medium complex formation takes place between lead(II) and polyols (P) containing at least two adjacent hydroxyl groups, according to the following equilibrium:

$$Pb(OH)_3^- + P \leftrightharpoons PbP^- + nH_2O.$$

Because the complex formation is affected by the number and position of the hydroxyl groups and the equilibrium constants are quite different, lead(II) has been used here as a complexing agent in the adsorbent layer to separate polyols by thin-layer chromatography. The influence of complex formation with lead(II) and of eluent composition on the mobility of polyols has been studied.

Experimental

Thin-layer plates were prepared according to the following procedure: 30 g of Silica Gel H (Merck) were mixed with 70 ml 0.1 *M* $Pb(NO_3)_2$ (Erba RP, Milan) or 70 ml water (untreated plates) by shaking in a stoppered conical flask, and were then transferred to the spreader (Chemetron, Milan). The thickness of the layer was set at 0.25 mm. All the plates were activated by heating at 110° for 30 min. Samples were applied to the starting line of the chromatograms as dilute solutions in alcohol or water–alcohol, in chromatography tanks. Alcohol–ammonia or alcohol–ammonia–water mixtures were used as developing solvents, and the polyols were detected by spraying with potassium permanganate containing sodium carbonate solution. Experiments carried out with Silica Gel G plates have not given satisfactory results. This seems to be due to the reaction of lead(II) with calcium sulfate present in the Silica Gel G.

TABLE I

FORMATION CONSTANTS WITH LEAD (II) OF POLYOLS IN ALKALINE MEDIUM

Polyol	*log K*
1,3-Propanediol	−0.20
Ethylene glycol	0.30
1,2-Propylene glycol	0.30
1,2,5-Trihydroxypentane	0.40
1,2,4-Butanetriol	0.45
Glycerol	1.15
meso-Erythritol	1.93
d-Mannitol	2.78
Dulcitol	2.90
d-Sorbitol	3.42

* Work carried out with the aid of the "Consiglio Nazionale delle Ricerche".

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The polyols listed in Table I were examined, their formation constants, K, with lead(II) in alkaline medium (0.1 M OH^-) having been previously determined.

Typical chromatograms of some polyols on untreated Silica Gel H and lead(II)-impregnated Silica Gel H for three developing solvent systems are reproduced in Fig. 1. The influence of lead(II) is evident: the mobility of the polyols decreases with an increase of the number of adjacent hydroxyl groups, as the formation constants between lead(II) and polyols become larger.

In Table II the R_F values measured for the polyols investigated are collected. The R_F values for glycols are slightly affected by the presence of lead(II) in the adsorbent layer, while the mobility of other polyols is considerably decreased by

TABLE II

R_F VALUES × 100 OF THE INVESTIGATED POLYOLS ON SILICA GEL H (A) AND LEAD(II)-IMPREGNATED SILICA GEL H (B)

Developing solvents: (I) ethanol saturated with gaseous ammonia; (II–VII) ethanol–conc. ammonia (32%)–water, respectively: II (21:2:0), III (23.5:2.2:1), IV (23:2.2:2), V (21:2:2.4), VI (21:2:3.5) and VII (20:2:4).

Polyols	*I*		*II*		*III*		*IV*		*V*		*VI*		*VII*	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
1,3-Propanediol	76	68	82	75	80	78	86	79					82	82
Ethylene glycol	62	59	67	60	64	60	66	62	66	62	74	68	71	66
1,2-Propylene glycol	69	65	73	67	72	67	73	69	72	70	80	76	77	74
1,2,5-Trihydroxypentane	65	61	69	64	68	64		66	70	65	78	73	76	74
1,2,4-Butanetriol	60	53	62	60	64	60	66	62	65	62	73	68	71	67
Glycerol	48	22	53	42	50	46	56	48	57	52	62	54	63	55
meso-Erythritol	33	6	39	22	38	30	45	34	49	39	51	43	56	45
d-Mannitol	7	0	14	3	15	6	22	9	30	14	27	17	37	22
Dulcitol	4	0	12	0	14	3	21	3	29	8	24	10	35	18
d-Sorbitol	4	0	11	0	12	2	17	5	26	7	22	9	34	14

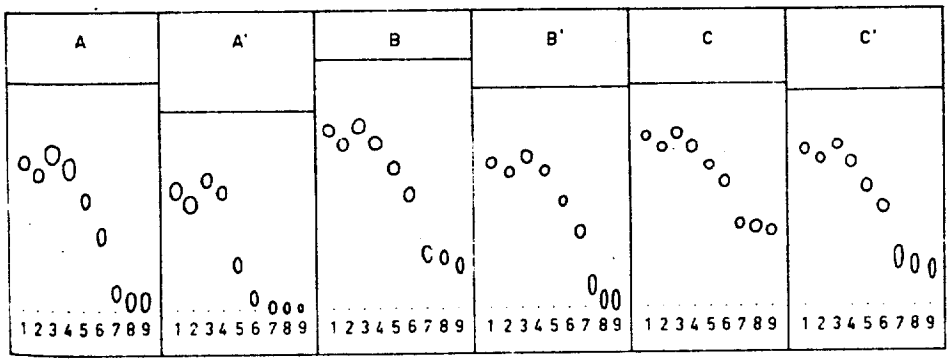

Fig. 1. Thin-layer chromatograms on Silica Gel H (A, B, C) and lead(II)-impregnated Silica Gel H (A', B', C') of the following polyols: (1) 1,2,5-trihydroxypentane; (2) 1,2,4-butanetriol; (3) 1,2-propylene glycol; (4) ethylene glycol; (5) glycerol; (6) *meso*-erythritol; (7) *d*-mannitol; (8) dulcitol; (9) *d*-sorbitol. Developing solvents: (A,A') ethanol saturated with gaseous ammonia; (B,B') ethanol–conc. ammonia (32%)–water (23:2.2:2); (C,C') ethanol–conc. ammonia (32%)–water (20:2:4).

the presence of the metal. A marked influence of the complexing ability of the added metal can be observed by running compounds that do not bind lead(II) on the two types of plates. Amines such as benzylamine yield identical R_F values when 95 % ethanol–25 % ammonia (4:1) is used as eluent.

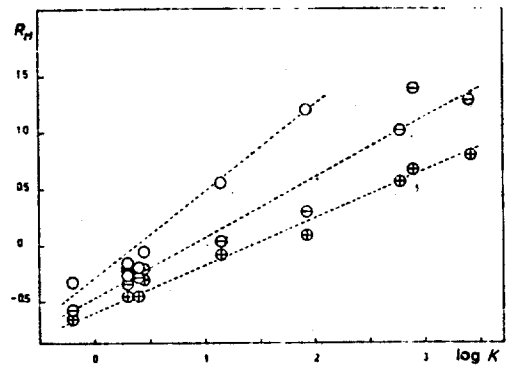

Fig. 2. R_M values plotted *versus* logarithm of the formation constants with lead(II). Developing solvents: (○) ethanol saturated with gaseous ammonia; (⊖) ethanol–conc. ammonia (32 %) water (23:2.2:2); (⊕) ethanol–conc. ammonia–water (20:2:4).

The mobility of the polyols changes noticeably with the eluent composition. Since the polyol–lead(II) complexes are polar, their migration is affected by the polarity of the elution medium consisting of ethanol and ammonia, with and without water. The migration of glycols is slightly affected by the eluent composition. Glycerol and erythritol exhibit a large difference in the R_F values when they are chromatographed on untreated and lead(II)-treated silica gel plates when water is present in the eluent. Higher polyols show a larger difference in the R_F values on the two types of plates when the eluent does not contain water.

More favourable conditions for the separation of triols and higher polyols from diols are obtained when anhydrous ethanol saturated with ammonia is used. In this medium the higher polyols have a smaller mobility because their complexes with

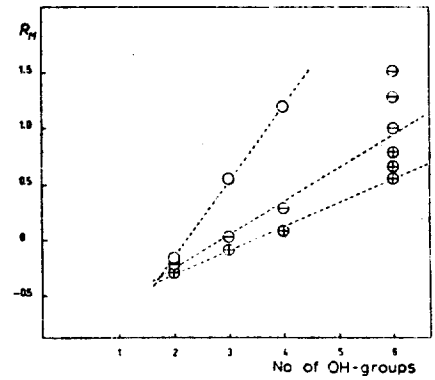

Fig. 3. R_M values plotted *versus* number of hydroxyl groups of various polyols. Developing solvents: (○) ethanol saturated with gaseous ammonia; (⊖) ethanol–conc. ammonia (32 %)–water (23:2.2:2); (⊕) ethanol–conc. ammonia–water (20:2:4).

lead(II) are stronger and very slightly soluble. It is possible to separate glycerol from erythritol and the latter from hexahydric alcohols by increasing the eluent polarity by adding water.

The dependence of the chromatographic behaviour on the formation constants of lead(II)-polyol complexes is readily observed in Fig. 2, where the R_M values obtained for three different eluents have been plotted *versus* the logarithm of the formation constants reported above. The plot is only of a qualitative nature because the formation constants were obtained under different experimental conditions (0.1 *M* OH^- and 1 *M* Na^+). It clearly shows, however, that the R_M values increase gradually as log *K* increases. From this plot the effect of the eluent is quite clear because the slopes of the hypothetical curves drawn through the experimental points are different.

Since R_M is a linear function of the number of equal groups responsible for the chromatographic behaviour, the R_M values obtained on lead(II)-treated plates have been plotted *versus* the number of hydroxyl groups of the polyols investigated. There is a linear relationship for diols, triols and tetrols, whereas deviations are observed for the hexahydric alcohols; this is probably due to interactions among –OH groups which influence each other.

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First received July 10th, 1968; revised manuscript received July 29th, 1968

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Emulsifiers. III. Periodic Acid Analysis for 1-Monoglycerides

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Two collaborative studies were conducted to compare the official AOCS method, Cd 11–57, and a revised Miner procedure. The second study used known materials to obtain accuracy information. Studies showed that the revised Miner procedure is more accurate and more precise at low monoglyceride levels such as those encountered in commercial shortenings. It is recommended that the Miner procedure be adopted as official first action. It is further recommended that the joint AOAC-AOCS methods study of these two procedures be continued so that a single common method can be made official by both societies.

In an earlier report (1), data were presented which indicated that a revised Miner procedure for the analysis of 1-monoglycerides is more precise and probably more accurate than the official American Oil Chemists' Society (AOCS) method, **Cd 11–57**. The multimethods study of the AOCS in 1957, which led to the adoption of that procedure (2), also showed that at low levels the original Miner procedure was more precise. Regulatory interest in methods for measurement of monoglycerides is greatest for low levels such as those encountered in commercial shortenings. It seemed wise, therefore, to subject the revised Miner and the **Cd 11–57** procedures to collaborative studies. For this purpose, a joint AOAC-AOCS Committee was formed and two collaborative studies were conducted.

Collaborative Study I

Six samples of commercial products, four shortenings, one "commercial" concentrate, and one distilled concentrate, with monoglyceride levels ranging from about 2 to about 90%, were submitted to twelve participating laboratories. The analytical methods, worksheets for results with space for comments, and the composition of a control sample were provided. Seven collaborators also used the column chromatographic procedure, which analyzes for di- and triglycerides as well as monoglycerides (3, 4). These individuals were supplied with the silica gel necessary for this determination.

A summary of the monoglyceride values obtained by the three procedures is given in Table 1. Table 2 presents the data in detail. Eight of the twelve laboratories used the AOCS and Miner procedures in duplicate.

The data were statistically examined further by the calculation of Neuman Keuls least significant differences. With a least significant difference of 0.467 and assuming an alpha risk

Table 1. Summary of results for monoglycerides: Study I

Method	Statistic	Sample 1	2	3	4	5	6
AOCS	Range, %	10.07-10.75	8.09-10.34	3.40-4.92	2.20-3.73	34.22-43.52	83.18-94.61
	Av., %	10.47	8.69	3.74	2.52	41.71	90.39
	Median, %	10.48	8.60	3.68	2.37	42.08	90.31
	Std dev., %	0.19	0.42	0.33	0.37	1.89	3.13
Miner	Range, %	10.05-11.30	7.36-9.26	2.59-3.54	1.34-1.86	37.58-44.82	81.41-92.36
	Av., %	10.45	8.26	3.06	1.64	41.13	88.54
	Median, %	10.45	8.21	3.03	1.65	41.26	89.44
	Std dev., %	0.26	0.48	0.20	0.12	1.79	2.96
Column	Range, %	10.4-12.1	8.2-9.5	1.0-4.0	1.9-3.0	37.3-44.0	86.7-92.1
	Av., %	11.0	8.5	3.1	2.2	41.0	90.3
	Median, %	10.7	8.4	3.4	2.0	41.0	91.1
	Std dev., %	0.71	0.41	0.91	0.45	1.92	1.82

of 5%, the two methods were found to be significantly different, with the Miner method being the more precise. Also of practical significance was the observation that the AOCS method has a high bias at the lower levels. This observation was based on formulation knowledge of a number of the products examined.

Confirming the earlier results, the column chromatographic procedure for monoglycerides is less precise. A comparison of the column procedure results with the AOCS method, the former measures both 1- and 2-monoglycerides, substantiates the observation that the AOCS procedure has a high bias.

Collaborative Study II

In view of the above, a second collaborative study was conducted to include known materials. Monostearin and monoolein were synthesized from carefully purified fatty acids. The resulting monoglycerides were recrystallized and characterized by melting point, per cent monoglyceride, periodic acid analysis, hydroxyl values, and thin layer chromatography. They were judged greater than 99 and 98% pure, respectively. To serve as a base to which these materials could be added, a nonemulsifier commercial shortening was made free of monoglycerides by molecular distillation. As judged principally by thin layer chromatography, the product was for all practical purposes devoid of any monoglyceride (<0.02%). Using the monostearin and the monoglyceride-free base, three samples of simulated shortenings were formulated: Samples 1, 2, and 3. The monostearin and 1:1 blend of monostearin-monoolein in preweighed quantities were submitted as simulated distilled concentrates (Samples 7 and 4, respectively). The last two samples, 5 and 6, were a commercial shortening and a "commercial" concentrate. The purpose of these two samples was to correlate more completely the two studies. Single analyses were run by the Miner and AOCS procedures by eight participating laboratories. Four laboratories also used the column chromatographic procedure. Worksheets, a control sample, and reagents were again supplied.

The results are presented as before in Tables 3 and 4. The data on the "knowns" were again analyzed by the Neuman Keuls approach, assuming a 5% alpha risk. The shortening- and concentrate-type samples were treated separately.

It was concluded that the accuracy of the AOCS method is slightly better on concentrates. For the shortenings, however, the accuracy of the Miner procedure was substantially greater with no distinguishable difference in precision.

Gas Chromatography

Gas chromatography as a technique for monoglyceride analysis has been studied in a number of laboratories. It, of course, offers the distinct advantage of speed over the periodic acid oxidation approach. A study was, therefore, made of several available methods. It appeared that the precision of present methods was insufficient to warrant any methods

Table 2. Individual results for monoglycerides (%): Study I

Coll.	Sample 1	2	3	4	5	6
AOCS Method: Monoglycerides						
1	10.25	8.60	3.88	2.60	40.95	88.79
2	10.07	8.74	4.01	2.26	40.87	83.18
3	10.43	8.83	3.77	2.59	43.52	94.32
4	10.64	8.64	3.66	2.28	42.08	91.84
	10.41	8.60	3.42	2.49	42.11	86.24
5	10.27	8.35	3.57	2.55	34.22	83.51
6	10.67	8.87	3.70	2.26	43.22	94.20
	10.63	8.97	3.68	2.30	42.91	94.61
7	10.48	8.09	4.04	2.97	42.55	92.74
	10.16	8.57	3.59	2.79	42.20	89.99
8	10.60	8.50	3.53	2.32	41.49	90.31
	10.42	8.42	3.49	2.21	41.57	90.18
	10.75	8.61	3.58	2.33	41.29	91.93
9	10.56	8.49	3.70	2 36	41.14	91.88
	10.66	8.64	3.60	2.43	42.35	92.21
10	10.74	8.76	3.76	2.51	42.94	93.42
	10.53	8.55	3.76	2.37	41.59	89.78
11	10.40	8.60	3.40	2.20	41.40	89.60
	10.30	8.50	3.50	2.20	41.60	90.30
12	10.39	10.34	4.92	3.73	43.31	88.59
	10.51	8.80	3.99	3.12	42.62	90.57
Miner Method: Monoglycerides						
1	10.55	8.10	3.15	1.66	42.01	90.90
2	11.30	8.74	3.22	1.86	41.53	89.63
3	10.74	7.88	3.05	1.69	41.23	89.12
4	10.53	9.26	3.07	1.70	39.50	84.20
	10.64	8.52	3.15	1.75	40.95	92.07
5	10.23	8.21	3.05	1.65	41.40	12.0[b, c]
6	10.40	8.45	3.54	1.73	43.35	92.36
	10.37	8.56	3.46	1.76	43.38	91.33
7	10.05	7.81	2.85	1.34	37.94	81.98
	10.45	7.36	2.59	1.37	37.58	81.41
8	10.45	8.12	3.02	1.63	41.22	89.63
	10.49	8.14	3.00	1.58	41.26	89.57
	8.13[b]	8.22	2.99	1.59	41.28	89.01
9[a]	10.31	9.21	2.99	1.70	44.82	90.18
	10.26	8.84	2.96	1.71	43.36	86.98
10	10.41	8.29	3.03	1.65	41.74	89.44
	10.45	8.23	3.08	1.59	41.51	89.13
11	10.10	7.70	2.90	1.60	38.50	87.40
	10.20	7.70	3.00	1.60	40.30	90.00
12	10.47	8.01	2.97	1.58	40.38	87.86
	10.52	8.09	3.17	1.60	40.57	88.64
Mono-, Di-, Triglyceride Method: Monoglycerides						
1	10.9	8.3	3.4	1.9	40.2	91.5
2	10.7	8.4	3.1	2.0	41.0	44.1[b]
3	12.1	9.5	4.0	2.8	44.0	92.1
4	11.9	8.5	3.7	2.1	42.0	91.1
5	—	8.6	1.0	3.0	37.3	86.7
6	10.6	8.3	3.3	1.9	40.9	90.1
	10.4	8.4	3.4	1.9	40.6	89.5
7	10.4	8.2	3.1	1.9	42.1	91.3

[a] Recalculated values.
[b] Not included in calculations.
[c] Wrong weight.

Table 3. Summary of results for monoglycerides (%): Study II

Method	Statistic	Sample 1	2	3	4	5	6	7
AOCS	Range, %	1.80–3.50	3.30–4.05	7.60–10.12	96.5–102.5	2.10–4.12	35.0–39.2	92.5–103.0
	Av., %	2.59	3.67	8.97	99.3	2.67	37.1	98.2
	Median, %	2.58	3.72	9.16	99.4	2.58	37.1	98.9
	Std dev., %	0.59	0.29	0.91	1.86	0.62	1.30	3.06
Miner	Range, %	1.59–2.00	3.00–3.55	7.10–8.61	92.7–100.7	1.80–1.99	31.30–45.13	89.6–101.3
	Av., %	1.68	3.22	8.27	97.8	1.88	36.76	98.0
	Median, %	1.65	3.15	8.45	98.3	1.89	36.40	98.7
	Std dev., %	0.15	0.16	0.49	2.51	0.06	3.83	3.43
Calculated values		1.6	3.0	8.0	98.5			99.0
Column chromatography	Range, %	1.8–3.9	2.2–3.7	8.2–9.3		2.0–2.4		
	Av., %	2.5	3.1	8.8		2.2		
	Median, %	2.4	3.3	8.8		2.2		
	Std dev., %	0.99	0.64	0.45		0.17		

study at this time. However, because of expressed interest by five collaborators, one of these methods, its reagents, and additional quantities of the test materials were submitted for analysis; *see* Table 5.

The variance of the three methods was analyzed by an F test comparison. It was concluded: (*1*) at low levels the Miner and gas chromatographic methods have significantly more precision than AOCS; (*2*) at higher levels, the AOCS method is significantly more precise; and (*3*) on the simulated shortenings, gas chromatography is the most accurate, followed by the Miner procedure, then AOCS method. In view of the surprisingly good results on the shortenings, it appears that additional investigation is in order.

Collaborators' Comments

The main comment by the collaborators was the general preference for the AOCS procedure, probably because of greater familiarity with that procedure. None of those familiar with the Miner procedure preferred the AOCS; the reverse was not true. Also, in general, the participants found the two procedures about equivalent in analysis and analyst times.

It should be recalled, in conjunction with both of the above points, that the Miner procedure also gives free glycerine contents. Such data are of little consequence with commercial shortenings but are frequently of value with concentrates. It is a matter of individual laboratory preference as to whether or not such information should be obtained.

Recommendation

In view of the observed superiority in accuracy of the Miner procedure on commercial shortenings, it is recommended that it be adopted as official first action. Since industry uses mainly the AOCS procedure and has for many years, the joint committee is continuing its study before recommending a single procedure for both societies.

Acknowledgments

The authors wish to thank the following collaborators for their efforts in performing the analytical work and for their comments and suggestions.

Table 4. Individual results for monoglycerides (%): Study II

Coll.	Sample 1	2	3	4	5	6	7
AOCS Method: Monoglycerides							
1	2.39	4.05	9.16	99.4	2.47	36.9	98.9
2	2.80	3.40	9.30	99.3	2.62	37.2	100.3
3	2.58	3.72	10.12	100.1	2.58	38.4	99.1
4	2.09	3.43	8.79	97.1	2.34	36.0	96.5
5	2.26	3.59	7.75	99.1	2.69	37.1	92.5
6	3.30	3.90	9.10	100.1	2.40	36.9	97.9
7	3.50	3.99	9.90	102.5	4.12	39.2	103.0
8	1.80	3.30	7.60	96.5	2.10	35.0	97.0
Miner Method: Monoglycerides							
1	1.61	3.15	8.39	97.8	1.87	35.3	99.5
2	1.65	3.12	8.51	98.3	1.89	45.1	98.9
3	1.75	3.29	8.50	98.8	1.93	36.4	98.6
4	2.00	3.55	8.59	—	1.99	38.9	101.3
5	1.64	3.13	8.40	84.2[a]	1.88	36.0	98.7
6	0.33[a]	3.28	8.45	99.1	1.89	37.0	98.1
7	1.60	3.10	7.90	97.2	1.90	33.6	96.6
8	1.72	3.33	8.61	100.7	1.81	37.2	100.6
9	1.50	3.00	7.10	92.7	1.80	31.3	89.6
Column Chromatography Method: Monoglycerides							
1	3.9	3.7	8.2		2.1		
2	2.4	2.2	9.3		2.4		
3	1.8	3.2	8.7		2.0		
4	1.8	3.3	8.8		2.2		

[a] Not included in calculations.

Study I

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The authors also want to express their appreciation to W. D. Pohle of Swift & Company, the AOCS representative, for his participation in this program and to Marjorie Edwards of Procter & Gamble's Mathematics Service Department for the statistical treatments of the data.

Lastly, the authors thank Paul A. Larson for submission of his gas chromatographic method for evaluation.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee C and was adopted by the Association. See *This Journal* 52, 331–332 (1969).

This report of the Associate Referee, F. J. Baur, was presented at the 82nd Annual Meeting of the Association of Official Analytical Chemists, Oct. 14–17, 1968, at Washington, D.C.

Table 5. GLC results for analyses of monoglycerides (%): Study II

Coll.	Sample 1	2	3	5	6
1	2.2[a]	3.8[a]	9.5[a]	2.2[a]	30.5[a]
2	1.5[a]	3.1[a]	8.9[b]	1.9[c]	30.6[d]
3	1.4[b]	2.7[b]	8.4[b]	1.1[c]	23.6[c]
4	1.6	3.0	8.4	1.8	—
5	1.6[b]	3.0[b]	8.5[b]	1.8[c]	38.3[a]
	1.7	2.9	8.6	1.8	38.7
Range, %	1.4-2.2	2.7-3.8	8.4-9.5	1.1-2.2	23.6-38.7
Av., %	1.7	3.1	8.7	1.8	32.3
Median, %	1.6	3.0	8.6	1.8	30.6
Std dev., %	0.28	0.38	0.43	0.36	6.30
Calculated values	1.6	3.0	8.0		

[a] Includes monopalmitin-monoolein and monostearin.
[b] Includes monoolein and monostearin.
[c] Includes monoolein and monopalmitin.
[d] Includes monopalmitin and monostearin.

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Arch. Ophthalmol. 72(4): 491-493, 1964

Effect of Oral Glycerol on Intraocular Pressure in Normal and Glaucomatous Eyes

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The osmotic agents have recently reappeared in the management of acute rises of intraocular pressure and as an adjunct in producing preoperative hypotension required prior to ocular surgery. Urea and mannitol have the obvious drawback of intravenous administration and have been reported to produce pulmonary edema and anginal pain due to the rather sudden increase in blood volume. The appearance of reports[1-3] that 50% glycerol by mouth can produce comparable osmotic changes with a reduction in intraocular pressure was therefore significant. The literature also suggests that tromethamine (THAM,[4] tris-(hydroxymethyle)aminomethane) may be an even better oral osmotic agent but it has yet to be tried in a large number of patients.

Submitted for publication April 14, 1964.

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Supported in part by Federal Provincial grant No. 609-7-58.

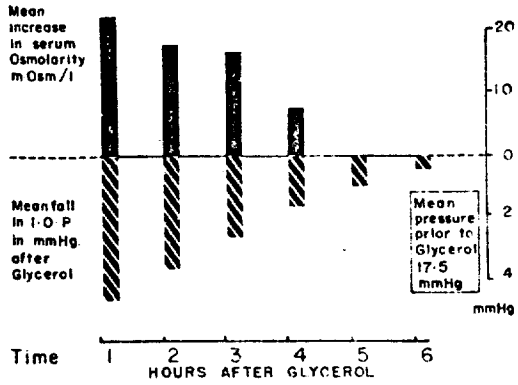

Fig 1.—Mean fall of intraocular tension following glycerol in ten normal eyes recorded below 0 line; mean serum osmolarity changes are recorded above the 0 line.

The present report deals with the effect on intraocular pressure and serum osmolarity of 50% glycerol by mouth in 10 nonglaucomatous and 14 glaucomatous eyes.

Method

All patients had their intraocular pressures recorded with a certified Schiotz tonometer using the 1955 calibration scale. Three readings were obtained within five minutes of one another to establish the steady state level of intraocular pressure. Glycerol, 50%, was then administered in dosage of 1.5 gm/kg weight with a flavoring agent. Fluid intake was restricted. The intraocular pressures were rechecked at hourly intervals, and in some of the nonglaucomatous patients blood samples were taken and serum osmolarity was measured by means of a freezing point technique.

Results

Normal Eyes.—The changes in intraocular pressure produced by the glycerol in the ten normal eyes are illustrated in Table 1. Fig 1 shows the mean changes of intraocular pressure in the ten eyes and the changes in serum osmolarity. In all eyes, the intraocular pressure fell within the first hour and tended to go back to normal by the end of the fourth or fifth hour. It can also be seen that the increased blood osmolarity corresponded to the pressure reduction.

Glaucomatous Eyes.—All glaucomatous eyes showed a reduction in intraocular pressure within the first hour, as shown in Table 2. In this series the maximal reduction of pressure occurred two hours after the ingestion of glycerol as shown in Fig 2 which illustrates the mean fall of the intraocular pressure. Some eyes showed a fairly marked reduction in intraocular pressure even six hours after the administration of glycerol. A typical pressure record is shown in Fig 3.

TABLE 1.—*Change of Intraocular Pressure (Schiøtz) in 10 Normal Eyes Following Oral 50% Glycerol*

Case	Steady State IOP Prior to Glycerol, Mm Hg	Change in IOP, Mm Hg (Schiøtz)					
		1 Hr	2 Hr	3 Hr	4 Hr	5 Hr	6 Hr
1	20	−4	−7	−2	−2	0	
2	18	−4	−4	−2	−1	−4	0
3	20	−4	−4	−6	−6	−3	−3
4	17	−5	−5	−5	−4	0	0
5	17	−4	−3	−3	0	0	
6	14	−6	−2	−2	0	0	
7	17	−4	−3	0	0	0	
8	17	−4	−2	−2	0	0	
9	16	−3	−4	−4	−4	−3	0
10	20	−5	−3	0	0	0	0
Mean IOP, mm Hg	17.5	Mean change of IOP −4.3	−3.7	−2.6	−1.7	−1	

Side Effects

Some patients experienced nausea but there was no vomiting. No difficulty in swallowing the glycerol was experienced. Almost all patients had a headache which amounted to marked throbbing in some but in the majority was fairly mild in nature. The headache was accompanied by some light-headedness. Diuresis was not prominent and was not as urgent a problem as with mannitol or urea. There were no cases of anginal pain even in the preoperative cases which were not included in this series.

Conclusions

The present studies confirm the effectiveness of oral glycerol as an ocular hypotensive agent with milder side effects, easier administration, and almost as profound ocular hypotensive action as the intravenous osmotic agents used for this purpose. The ocular hypotensive changes are accompanied by a rise in serum osmolarity.

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Generic and Trade Names of Drugs

Mannitol—Mannitol.
Tromethamine—Talatrol.

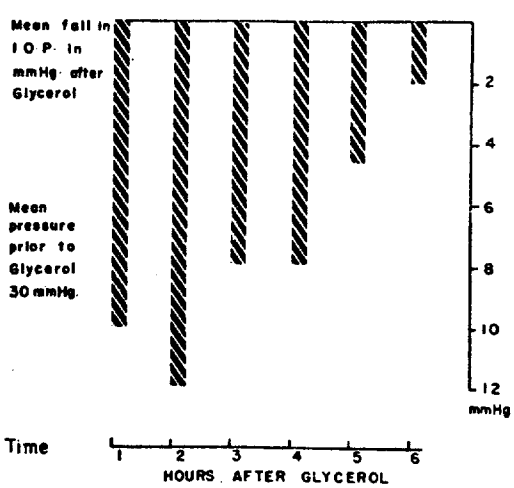

Fig 2.—Mean fall of intraocular tension following glycerol in 14 glaucomatous eyes.

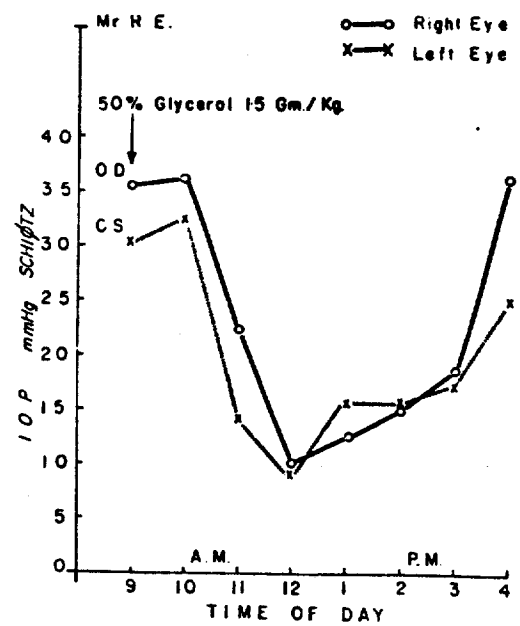

Fig 3.—Characteristic ocular hypotension in both eyes of a glaucomatous patient following oral glycerol.

TABLE 2.—*Changes of Intraocular Pressure (Schiøtz) Following 50% Glycerol by Mouth in 14 Glaucomatous Eyes*

Case	Steady State IOP Prior to Glycerol, Mm Hg		Change in IOP, Mm Hg (Schiøtz)					
			1 Hr	2 Hr	3 Hr	4 Hr	5 Hr	6 Hr
1	35		—13	—25	—22	—20	—16	0
2	33		—19	—24	—17	—17	—16	—7
3	36		—10	—8	—5	—10	—5	—5
4	26		—4	0	—4	—4	—4	0
5	28		—22	—21	—12	—12	0	0
6	26		—20	—18	—7	—15	0	0
7	25		—8	—5	—3	—3	0	0
8	36		—6	—10	—9	—9	—6	0
9	23		—9	—9	—8	—9	—9	—5
10	18 *		—8	—8	—4	—8	—7	—6
11	36		—8	—16	—14	—14	—12	—10
12	21 *		—5	—7	—5	—6	—6	—3
13	31		—17	—18	—13	—4	0	0
14	42		—24	—28	—20	—15	0	0
Mean IOP, mm Hg	30	Mean change in IOP	—12	—14	—10	—10	—65	—2

* Applanation readings in these eyes were always elevated.

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Inhibition by sucrose was directly proportional to the concentration in the range 0·04 to 0·5 *M*. In the case of lactose a similar relationship was observed to hold from 0·1 to 0·5 *M*: with the lower concentrations such proportionality did not exist, owing perhaps to the relatively greater influence in the low concentrations of the reducing group of the sugar. It is proposed in further studies to compare the effects of low concentrations of various sugars, reducing and non-reducing, in order to determine more exactly the relation between the reducing powers of the sugars and their inhibitory properties. The degree of inhibition by glycerol was, within experimental error, directly proportional to the concentration between the limits 2 and 12 %.

It may be suggested that the inhibition by glycerol and the sugars is closely related to the well known fact that these compounds become hydrated in solution, possibly through coordinate linkages of the atoms of the hydroxyl groups with water molecules in the manner indicated by Jordan-Lloyd and Phillips [1933] in the case of proteins. The degree of hydration of sucrose has been investigated by several workers. McBain and Kistler [1929], who review the earlier literature, obtained evidence from direct measurements by ultrafiltration methods that sucrose in dilute solution is hydrated with not less than 4 molecules of water per molecule of sugar. Scatchard [1921] concluded from an analysis of his own and other data that the formation of a penta- or hexa-hydrate best explained the experimental results, though the concentration affected in some measure the degree of hydration. Total removal of water by hydration increases, of course, with the concentration of the sugar. One may assume also that caseinogen in solution becomes hydrated, possibly by coordinate linkage of water molecules with the atoms of the hydroxyl, carboxyl, amide, amino- and imino-groups, but in the case of proteins accurate assessment of the degree of hydration of the various groups is much more difficult. In any case it is probable that the sugars and glycerol, when added to enzyme-protein solutions, reduce in greater or less degree the effective water concentration by competition with both enzyme and its substrate for water.

Polysaccharides. Dextrin and soluble starch as inhibitors were less effective than the sugars: gum acacia was without significant effect (Table II).

Amino-acids. The effects of several amino-acids and of asparagine on the activity of the enzyme were examined at p_H 8·7 according to Procedure 1, the enzyme preparation being a 1 % Na_2CO_3 extract of pancreas powder. The results are shown in Tables V and VI.

Table V. *The effects of amino-acids on proteinase activity.*

The figures represent initial velocity as % of that in control digests.

Substance	Concentration *M*															
	0·0020	0·0024	0·0026	0·0028	0·0030	0·013	0·020	0·024	0·026	0·028	0·030	0·050	0·060	0·065	0·070	0·075
Histidine	94	—	—	—	—	—	92	—	—	—	—	97	—	—	—	—
Phenylalanine	—	97	—	—	—	—	—	94	—	—	—	—	97	—	—	—
Cysteine	—	—	82 85	—	—	100	—	—	110 109		—	—	—	97	—	—
Leucine	—	—	—	—	99	—	—	—	—	—	99	—	—	—	—	98
Aspartic acid	—	—	—	—	106	—	—	—	—	—	117	—	—	—	—	121
Glutamic acid	—	—	—	96	—	—	—	—	—	105	—	—	—	118	—	—
Asparagine	—	—	—	—	98	—	—	—	—	—	—	—	—	—	—	125

Of the amino-acids examined histidine, phenylalanine and leucine had no very significant effects in the concentrations used. Cysteine (0·026 *M*) exercised a slight accelerating effect in two experiments; with aspartic and glutamic acids

Table VI. *Effect of amino-acid mixtures on proteinase activity.*

The figures represent initial velocity as % of that in control digests.

Mixture	Concentration %			
	0·06	0·30	0·60	1·50
Monoamino-acid mixture prepared from caseinogen	111	115	119	—
Arlington aminoids	97	—	105	107

and asparagine the activation was somewhat more marked. Activation by the amino-acid mixture (Table VI) was similarly quite definite but the identity of the activator is obscure.

Triglycerides. Emulsions (20% by volume) of triacetin, tributyrin and triolein in 3% gum acacia were prepared. The first two emulsions were reasonably stable but triolein in gum acacia solution gave a very unstable emulsion which separated, immediately after shaking, into two layers. A very stable emulsion of triolein was obtained with the aid of 0·2% sodium oleate solution. Portions of the emulsions were transferred to the digestion mixtures to give the concentrations listed in Table VII. Strictly speaking, these cannot be regarded as truly molecular concentrations since the substances, at least in the higher concentrations, were not in true solution.

Table VII. *Effect of triglycerides on proteinase activity.*

The figures represent initial hydrolysis as % of that in control digests.

Substance	Concentration *M*									
	0·0081	0·020	0·041	0·066	0·081	0·092	0·132	0·184	0·264	0·368
Triacetin	—	—	—	—	—	56	—	41	—	29
Tributyrin	—	—	—	80	—	—	63	—	40	—
Triolein (gum acacia)	95	—	96	—	102	—	—	—	—	—
Triolein (sodium oleate)	104	91	74	—	—	—	—	—	—	—

The three triglycerides, when properly emulsified, had marked inhibitory effects. In the case of triolein emulsified in gum acacia solution the degree of inhibition was relatively very slight but triolein in sodium oleate was definitely inhibitory in a concentration corresponding to 0·04 *M*. Control experiments indicated that part of this effect was due to the sodium oleate. Inhibition by the well-emulsified triglycerides was probably due to the formation of protective films around the micelles of the reacting substances. The opposite effect of emulsions was observed by Wasteneys and Borsook [1928] in experiments on protein synthesis. In this case the emulsion apparently provided means for the extension of the surface area of enzyme or substrate or both.

Bile and bile salts. Sodium taurocholate and sodium glycocholate (Eastman) and fresh ox bile were used. The enzyme was a 33% glycerol extract of pancreas powder. The results are summarised in Tables VIII and IX. They confirm the observations of Ringer [1921; 1922], of Vonk *et al.* [1933] and of others, that in alkaline solution bile and bile salts diminish the rate of breakdown of protein by trypsin. The results with sodium glycocholate are, however, contrary to those of Willstätter and Persiel [1925] who observed that this salt, in 0·002 *M* concentration, had no effect on tryptic activity determined by the increase in acid titratable in alcoholic solution.

Table VIII. *Effect of ox bile on proteinase activity.*

% concentration	% inhibition
0·002	3
1·0	0
10·0	33
20·0	77
40·0	88

Table IX. *Effect of bile salts on proteinase activity.*

The figures represent % inhibition.

	Concentration *M*		
	0·001	0·01	0·04
Sodium glycocholate	11	22	62
Sodium taurocholate	32	51	77

Indicators and dyes. In carrying out these experiments the solution of the indicator was first mixed with freshly prepared aqueous enzyme extract and the mixture was allowed to stand before addition to the reaction mixture. 2 ml. portions of enzyme solution were pipetted into several small test-tubes; varying amounts of *M* 5 phosphate-borax buffer solution (p_H 8·7) and of the indicator solution were then added to give the desired concentrations of indicator in a total volume of 3 ml. Control tubes contained 2 ml. enzyme solution and 1 ml. buffer solution. After standing for 20 min. at 25°, 1 ml. indicator-enzyme solution was added to a solution containing 1·5 ml. 3 % caseinogen and 2·5 ml. buffer solution; the reaction was followed as in Procedure 1. In experiments in which Procedure 2 was used the reaction mixture contained twice the above amounts of all consti-

Table X. *Effect of indicators on proteinase activity.*

The figures represent initial velocity as % of that in control digests.

Substance	Concentration $M \times 10^{-4}$														
	0·42	0·59	0·65	0·72	0·84	1·2	1·3	1·4	4·8	8·4	9·6	12	13	17	96
Phenol red	—	—	108	—	—	—	94	—	—	—	—	—	89	—	—
Bromophenol blue	—	104	—	—	—	106	—	—	—	—	—	96	—	—	—
Thymol blue	—	—	—	107	—	—	—	100	—	—	—	—	—	—	—
Bromothymol blue	97	—	—	—	95	—	—	—	—	94	—	—	—	—	—
Phenolphthalein*	—	—	—	—	—	—	—	—	95	—	85†	—	—	—	74‡
Methyl orange	—	—	—	—	95	—	—	—	—	—	—	—	—	94	—

* This was dissolved in 30 % aqueous alcohol solution. The alcohol introduced into the reaction mixture with the indicator was shown by a control experiment to have no effect on the rate of hydrolysis.

† A small precipitate was formed.

‡ A heavy precipitate developed.

Table XI. *Effect of indicators and dye-stuffs on proteinase activity.*

The figures represent initial velocity as % of that in control digests.

Substance	Concentration $M \times 10^{-4}$							
	0·084	0·17	0·34	0·84	1·7	3·4	17·0	34·0
Congo red	107	100	—		88	—		
Safranine	—	—	—	97	113		100	—
Trypan red	—	—	—	110	110		106	—
Methylene blue	—	94	93			92		—
Rosolic acid	—	91	99			101		—
Bismarck brown	—	—	—	—	101	100	—	110

tuents. The caseinogen concentration in the digests was therefore 0·9%; the concentrations of the indicators shown in Tables X and XI refer to those in the indicator-enzyme solutions before addition to the substrate. The concentrations in the reaction mixtures were, therefore, one-fifth of these values.

Quastel [1931; 1932] has shown that certain indicators and dyestuffs have marked inhibitory effects on the oxidation of glucose, lactate, succinate and formate by *Bact. coli* and on the action of fumarase and urease. Both basic and acidic dyes were toxic toward fumarase, whereas only basic dyes inhibited oxidations by *Bact. coli*. Acidic dyes were entirely inert toward urease although most basic dyes were toxic. These and other observations led Quastel to the conclusion that the groupings responsible for the attachment of urease to its substrate consist of the primary amino-group and the secondary imino-group. Investigations of this kind would appear to offer a very promising approach to the study of the nature of the active groups of enzymes in general. Our own efforts in this direction were concerned with a survey of the effects of representative dyestuffs on proteinase activity with the hope that a subsequent study of the possible protective effects of known chemical compounds in the case of toxic dyes might lead to some understanding of the nature of the chemical groups responsible for the union of the proteinase with its substrate. It has been found however that, even though used in concentrations considerably higher than those which Quastel found to be decidedly toxic in many cases towards the enzymes enumerated above, the dyestuffs examined were quite inert toward the proteinase. Furthermore, the acidic or basic nature of the dye had little or no relation to its effect on the enzyme. The results which are recorded have, therefore, little positive interest. They do suggest that the active group of the proteinase differs from those of the enzymes studied by Quastel. But the question of the degree of purity of the enzyme is one which introduces difficulties of interpretation. It is quite possible that impurities present in the enzyme preparations protected the enzyme from the toxic influence of added dyestuff, though enzyme and dyestuff were in preliminary association for 20 min. Quastel's urease preparation was a partially purified aqueous extract; the fumarase solutions were cell-free preparations made from bacteria, erythrocytes and brain; for the oxidation experiments suspensions of bacteria and of minced muscle were used. Some of these preparations, like the enzyme solutions used in the present work, must have contained large amounts of non-enzymic material. Further work on the influence of dyestuffs on the activity of purified proteinase preparations is now in progress.

Salts. In Tables XII and XIII the effects of various salts are recorded. In all experiments the enzyme-salt mixtures were allowed to stand for 20 min. at 25° before addition to the substrate-buffer solution. An aqueous solution of the enzyme was used and the method of preparing the digests was similar to that employed in the case of the indicators. The enzyme solutions containing calcium chloride and lead acetate developed precipitates on standing, the amounts varying with the concentration of the salt. Sodium nitroprusside caused the development of a purple colour in the enzyme solution: the other salts caused no visible effect. The concentrations of the salts given in the tables are those in the enzyme-salt solutions; in the digests the salt concentrations were one-fifth of the recorded values.

Unlike many enzymes the proteinase was apparently unaffected by the presence of salts of the heavy metals in concentrations as high as 3×10^{-3} *M*.

Each of the compounds calcium chloride, sodium cyanide, potassium ferrocyanide and potassium ferricyanide exercised an activating effect on the pro-

Table XII. *Effect of salts of heavy metals on proteinase activity.*

The figures represent initial velocity as % of that in control digests.

Substance	Concentration $M \times 10^{-4}$ 0·67	3·3	9·9	33
Mercuric chloride	106	102	101	—
Silver nitrate	101	98	101	—
Cupric chloride	106	95	101	112
Lead acetate	—	108	—	99

Table XIII. *Effect of various salts on the activity of pancreatic proteinase.*

The figures represent initial velocity as % of that in control digests.

Substance	Concentration $M \times 10^{-4}$ 0·67	1·65	3·3	6·7	9·9	16·5	33
Magnesium sulphate	—	100	—	98	—	—	97
Sodium fluoride	—	108	—	—	104	—	98
Sodium nitroprusside	100	—	97	—	—	96	—
Calcium chloride	121	—	141 135	—	110	—	106
Sodium cyanide	119	—	140 134 136	172	107	—	99
Potassium ferrocyanide	100	—	129	—	—	102	—
Potassium ferricyanide	102	—	127	—	—	118	—

teinase (Table XIII). Some years ago it was generally accepted that calcium salts activate trypsin, but experiments of Waldschmidt-Leitz [1924] caused a revision of this opinion. In the present experiments the activity of the proteinase was definitely stimulated by calcium. It is possible that this activation was due to the removal of concomitant inhibitors present in the rather impure enzyme preparation employed. It is proposed to test this possibility by using more highly purified preparations.

The activation by cyanide was quite definite. Willstätter *et al.* [1926] obtained results which led them to conclude that cyanide inhibits the activity of the protease of the pumpkin. However, Ambros and Harteneck [1929], on examining the problem, showed that the effect was due to the inhibitory influence of cyanide on the peptidases present in the enzyme preparation. The result was a marked reduction in the total acid titratable in alcoholic solution. When the effect on the peptidases was taken into account, the proteinase was shown to be activated by cyanide, a finding with which the present results with pancreatic proteinase are in agreement. Activation by cyanide has been well established in the case of other proteolytic enzymes such as papain and bromelin.

The mechanism of the acceleration of enzyme action by cyanide has, in recent years, been a subject of considerable interest. Krebs [1930] suggested that cyanide activation is due to the removal of toxic heavy metals by the formation of cyanide-metal complexes. In the present work, however, salts of heavy metals in moderately high concentration had no apparent inhibitory or toxic effects even after preliminary association with the enzyme for 20 min. Activation of the enzyme by cyanide cannot, therefore, be explained on the basis of such a detoxication process. Cyanide appears to exercise a positive activating influence: this is true, also, of potassium ferrocyanide and potassium ferricyanide. The explanation in each case must await further experiments.

Summary.

1. The influence of various chemical compounds on the initial rate of hydrolysis of caseinogen at p_H 8·7 by pancreatic proteinase was investigated with the aid of micro-methods for the determination of residual proteins. The substances investigated included carbohydrates, glycerol, amino-acids, asparagine, triglycerides, bile and bile salts, indicators and dyes, salts of heavy metals and other salts.

2. Mono- and di-saccharides in moderately high concentrations (*e.g.* 0·5 *M*) exercised marked inhibitory effects upon the activity of the proteinase. These effects are believed to be largely independent of the configurations of the sugars and to be related to a disturbance of the effective water concentration of the digestion mixture.

3. Dextrin and soluble starch were less effective inhibitors than the sugars. Gum acacia was without significant effect in moderately high concentration.

4. The degree of inhibition by glycerol was in direct proportion to the concentration. The effect observed is believed to be related to a disturbance of the effective water concentration of the enzyme digest.

5. Of the amino-acids investigated, aspartic and glutamic acids exercised definite accelerating effects: acceleration by cysteine was less marked; the others were relatively ineffective.

6. Asparagine caused definite activation.

7. Triacetin, tributyrin and triolein, when properly emulsified, were inhibitory. Unstable emulsions of triolein in gum acacia had no appreciable effect.

8. Fresh ox bile and bile salts were inhibitory.

9. Indicators and dyes, used in concentrations which Quastel found to be inhibitory towards fumarase and urease, had no significant effect on proteinase activity.

10. Salts of heavy metals in concentrations as high as 3×10^{-3} *M* were without effect.

11. Calcium chloride, sodium cyanide, potassium ferrocyanide and potassium ferricyanide stimulated the activity of the enzyme.

12. Activation of the proteinase by cyanide was apparently not due to removal of toxic heavy metals.

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Chemical Structure and Pharmacological Effects of Glycols, Particularly 1,3-Butylene Glycol

Article by L. Fischer, R. Kopf, A. Loeser and G. Meyer of the Pharmacological Institute, Munster University, in Zeitschrift fur die Gesamte Experimentelle Medizin 115:22-39 (1949).

The divalent alcohols ethylene glycol, propylene glycol, butylene glycol, etc. grouped together under the summary designation of glycols, have in common with the univalent alcohols their narcotic effect, and with the most important representative of the trivalent alcohols, glycerine, the sweet taste and solubility in water. They differ from glycerine in that their effect on the central nervous system is generally stronger. While certain glycols cause partial or permanent damage to the body even in small doses, this does not appear to be valid for others, or possibly under special conditions only.

As regards the true glycols, toxic effects have been reported to date for ethylene glycol (2,3,6,9,10) and, as reported recently by the authors, for 1,4-buthylene glycol (5,7). Comparison of average lethal doses (LD50) of the best known glycols shows that they differ strongly with respect to magnitude and that they cannot be correlated either with the length of the carbon chain or the position of the hydroxyl groups with respect to each other. Rather, they exhibit all transitions from a practical lack of toxicity (1,2-propylene glycol, 1,3-butylene glycol) to the highest effectiveness of very small doses (1,4-butylene glycol, Table 1). With the exception of values given for 1,3-propylene glycol and 2,3-butylene glycol (10), all of the data originate in the authors' own toxicity determinations. The average lethal dose (LD50) determined by the method developed by Karber (1) indicates the amount of the substance per kg of body weight which kills 50% of the experimental animals within 24 hours. The deviations found from numerous literary data (3, 10) should be attributed in part to the higher degree of purity of the glycols used. In part, they are certainly due to the fact that the authors' investigations were conducted on a very large animal material.

In this connection, mention should be made of a fact, which, although generally known, has not been considered sufficiently, according to the authors' knowledge, in the pharmacological and toxicological evaluation of glycols. As is known, univalent alcohols are classified as primary, secondary and tertiary. Accordingly, glycols also differ according to the type of the alcoholic hydroxyl group. If one therefore considers not only the number but also the quality of the hydroxyl groups, three subgroups may be established within the common class of glycols. Group 1 includes glycols which contain the CH2OH group twice (diprimary glycols, e.g. ethylene glycol), Group 2 those which contain the CH2OH group of primary alcohols and the CH.OH group of secondary alcohols (primary-secondary glycols, e.g. 1,2-propylene glycol) and, finally, Group 3, in which the CH.OH group of the secondary alcohols appears twice (disecondary glycols, e.g. 2,3-butylene glycol). Let us mention for the sake of completeness that primary-tertiary, secondary-tertiary and ditertiary glycols are also possible.

TABLE 1. Chemical Structure and Toxicity of Glycols Arranged by the Number of Carbon Atoms

1 Substanz	2 Konstitution	3 LD 50 per os cm³/kg Maus (24 Stunden)
4 Äthylenglykol	$CH_2(OH)-CH_2(OH)$	13,79
1,2-Propylenglykol	$CH_3-CH(OH)-CH_2(OH)$	30,76
1,3-Propylenglykol	$CH_2(OH)-CH_2-CH_2(OH)$	6,0
1,3-Butylenglykol	$CH_3-CH(OH)-CH_2-CH_2(OH)$	23,31
1,4-Butylenglykol	$CH_2(OH)-CH_2-CH_2-CH_2(OH)$	2,14
2,3-Butylenglykol	$CH_3-CH(OH)-CH(OH)-CH_3$	9,0

Key: 1. Substance, 2. Structure, 3. LD50 per os, cm3/kg mouse (24 hours), 4. Ethylene glycol.

The arrangement of glycols listed in Table 1 by these aspects yields two remarkable facts (Table 2). First, it is seen from Table 2 that the toxicity of the individual compounds within the same group appears to be dependent on the length of the carbon chain. This observation is especially obvious in the group of diprimary glycols. If the extension of the investigation to other glycols should result in a repetition of this observation, it would perhaps be possible to establish a rule similar to that found with primary alcohols, where i is known that their toxicity also increases with the number of carbon atoms (12, 13). It should be noted in this context, however, that with divalent alcohols other effects, such as the position of OH groups with respect to each other and other factors can create exceptions from such a schematic interpretation. The second fact derived from Table 2 is the observation that glycols which contain both the CH2OH group of the primary alcohols and the CHOH group of the secondary alcohols, display an astonishingly low toxicity in comparison with all of the other groups. Thus, it is known that 1,2-propylene glycol greatly ressembles in its toxicological behavior the most prominent representative of trivalent alcohols, glycerine (Table 3). Without trying to establish a connection, attention is called to the fact that glycerine also contains a primary and a secondary alcoholic hydroxyl group; it should be noted, however, that the CH2OH group of primary alcohols is represented twice in the compound.

TABLE 2. Chemical Structure and Toxicity of Glycols Arranged by the Type of Hydroxyl Groups

1 Substanz	2 Konstitution	3 LD 50 per os cm³/kg Maus (24 Stunden)
4 I. Zweiwertige diprimäre Alkohole.		
5 Äthylenglykol	$CH_2(OH)-CH_2(OH)$	13,79
1,3-Propylenglykol	$CH_2(OH)-CH_2-CH_2(OH)$	6,0
1,4-Butylenglykol	$CH_2(OH)-CH_2-CH_2-CH_2(OH)$	2,14
6 II. Zweiwertige primär-sekundäre Alkohole.		
1,2-Propylenglykol	$CH_3-CH(OH)-CH_2(OH)$	30,76
1,3-Butylenglykol	$CH_3-CH(OH)-CH_2-CH_2(OH)$	23,31
7 III. Zweiwertige disekundäre Alkohole.		
2,3-Butylenglykol	$CH_3-CH(OH)-CH(OH)-CH_3$	9,0

Key: 1. Substance, 2. Structure, 3. LD50 per os, cm3/kg of mouse (24 hours), 4. Divalent diprimary alcohols, 5. Ethylene glycol, 6. Divalent primary-secondary alcohols, 7. Divalent disecondary alcohols.

TABLE 3. Chemical Structure and Toxicity of Di- and Trivalent Primary-Secondary Alcohols

1 Substanz	2 Konstitution	3 LD 50 per os cm³/kg Maus (24 Stunden)
4 I. Zweiwertige primär-sekundäre Alkohole.		
1,2-Propylenglykol	$CH_3-CH(OH)-CH_2(OH)$	30,76
1,3-Butylenglykol	$CH_3-CH(OH)-CH_2-CH_2(OH)$	23,31
5 II. Dreiwertige primär-sekundäre Alkohole.		
1,2,3-Propantriol (Glycerin)[1]	$CH_2(OH)-CH(OH)-CH_2(OH)$	20,71

6 [1] wasserfreies Glycerin.

Key: 1. Substance, 2. Structure, 3. LD50 per os, cm3/kg of mouse (24 hours), 4. Divalent primary-secondary alcohols, 5. Trivalent primary-secondary alcohols, 6. Anhydrous glycerine.

The exceptional behavior of 1,2-propylene glycol has been known for a long time and has been the subject of numerous investigations (3, 10). It is explained by the fact that the molecule is loosened by the adjacent position of the OH groups and made susceptible to decomposition in the body (10).

A similar behavior is exhibited by 1,3-butylene glycol, which should also be interpreted as a primary-secondary alcohol (Table 2). The authors have investigated the effects of the latter.

1,3-Butylene glycol is a colorless, viscous liquid of a bitter-sweet taste and an odor vaguely ressembling butter. It is miscible with water and alcohol in all proportions and insoluble in ether, lipoid solvents and fatty oils. The boiling point of the pure substance is given differently in the literature, e.g. 185-195o, 203-204o (760 mm) or 207-208o (769 mm). The specific gravity is given as 1.0258 at 20o.

According to Gross (3), 1,3-butylene glycol is used in technology occasionally as a lubricant. Based on its physical and chemical properties, it has been proposed as a substitute for glycerine in the cosmetic, tobacco, paper and textile industries. The material is readily available as an intermediate of synthetic rubber (Buna) production.

The effect of 1,3-butylene glycol, following the oral and subcutaneous administration of sublethal amounts to rats and mice is characterized by the fact that the animals become sluggish and tired after 10-20 minutes, lie on their bellies, lose muscle tone, exhibit accelerated respiration, later they lie on their sides and show respiratory spasms. The phenomena gradually disappear after 4-5 hours following the administration of the substance, their duration and intensity being a function of the amount given.

The same obvious changes occurring as the result of sublethal doses, also appear in cases of lethal intoxication. Here, however, the narcosis deepens rapidly, reflexes disappear and the animals perish because of respiratory paralysis. The mean lethal dose (LD50) for mice was found to be 23.31 cm3 per kg of body weight (per os). The dose depends on the purity of the product and amounts to 19.16 cm3/kg using the commercial grade with oral administration. LD50 for a purified grade of 1,3-butylene glycol is 22.52 cm3/kg and, finally, for the high-purity grade 23.31 cm3/kg (Table 4). The variation of LD50 is the same for rats. The degree of purity also affected the dosage following subcutaneous injection (Table 4).

A comparison of the mean lethal dose of 1,3-butylene glycol with that of glycerine, which generally is considered nontoxic, shows that the LD50 for 1,3-butylene glycol given to mice per os is approximately the same as the LD50 of anhydrous glycerine. With subcutaneous administration, 1,3-butylene glycol was even somewhat less toxic in mice and rats than hydrous glycerine (DAB VI).

TABLE 4. Comparison of the Toxicity of 1,3-Butylene Glycol of Different Grades of Purity in Mice and Rats (cm3/kg, 24 hours)

Reinheitsstufe	Maus		Ratte	
	per os	subcutan	per os	subcutan
1,3-Butylenglykol, technisch[1] . . .	19,16	15,80	28,38	19,42
1,3-Butylenglykol, gereinigt	22,52	–	—	—
1,3-Butylenglykol, Reinprodukt . .	23,31	16,51	29,42	20,06

[1] Nach den Angaben der Herstellerfirma Chemische Werke Hüls sind die Konstanten:

Key: 1. Degree of purity, 2. Mice, 3. Rats, 4. Commercial grade, 5. Purified, 6. High-purity product, 7. According to data provided by the manufacturer, the Huls Chemical Works, the constants are the following:

Technisches Produkt: $d_4^{20} = 1{,}0035$, $n_D^{20°} = 1{,}4399$, Kp = 202,5°—206,2° (768 mm);

1,3-Butylenglykol: $d_4^{20} = 1{,}0046$, $n_D^{20°} = 1{,}4398$, Kp = 203,0°—206° (760 mm).
(Reinprodukt)

It is remarkable that with rats the intervals of mean lethal doses in case of the oral and subcutaneous administration of glycerine (DAB VI), are far apart (Table 5).

TABLE 5. Comparison of the Toxicity of 1,3-Butylene Glycol and Glycerine in Mice and Rats (cm3/kg, 24 hours)

Substanz	Maus		Ratte	
	per os	subcutan	per os	subcutan
1,3-Butylenglykol (Reinprodukt). .	23,31	16,51	29,42	20,06
1,3-Butylenglykol (Reinprodukt, als 90%ige wäßrige Lösung) . .	24,44	—	—	—
Glycerin, wasserfrei	20,71	—	—	—
Glycerin, DAB VI[1].	30,21	10,78	46,72	16,69

[1] Glycerin DAB VI (83%—87% reines Glycerin).

Key: 1. Substance, 2. Mice, 3. Rats, 4. Pure product, 5. Pure product as a 90% aqueous solution, 6. Anhydrous glycerine, 7. Glycerine DAB VI (83%-87% pure glycerine).

The blood pressure of dogs and rabbits is not changed by the intravenous injection of 0.01-0.2 cm3 1,3-butylene glycol in a physiological table sale solution as 1%, 5%, 10% and 50% solutions.

Only administrations of 0.5 cm3 (50% solution) or 1.0 or 2.0 cm3 pure, undiluted 1,3-butylene glycol depress the blood pressure of dogs by a maximum of 12 mm Hg and that of a rabbits by 58 mm Hg. The degree of depression is a function of the magnitude of the dose given. After 45 and 100 seconds the blood pressure always returns to normal. If glycerine is given in place of 1,3-butylene glycol intravenously in the same concentrations, again only doses of 0.5-2.0 cm3 cause temporary blood pressure depressions in 45-165 seconds, which in the authors experiments amounted to a maximum of 50 mm Hg in dogs and 36 mm Hg in rabbits. A similar behavior was observed by Schubel (11) in cats, following the intravenous injection of glycerine.

The respiration of dogs and rabbits is temporarily accelerated only by doses which also depress the blood pressure. In mice and rats, high doses of both substances also increase the frequency of respiration, which, with a rising severity of intoxication, may develop into convulsive breathing.

Investigation of the sugar content of the blood of sober rabbits by the Hagedorn-Jensen method showed, after a single administration of 3 cm3 of a 50% solution of 1,3-butylene glycol in water per kg of body weight (per os, pharingeal probe), the same fluctuations during a 24-hour observation period as that produced by the ingestation of glycerine (3 cm3/kg, 50% glycerine solution in water, Fig. 1).

The effect of 1,3-butylene glycol on organs with smooth muscles is different. The isolated small intestine of rats is only slightly affected by a 1:400 1,3-butylene glycol solution. Higher concentrations of 1:100 to 1:40, however, result in a strong decline in tone and a cessation of contractions. This inhibiting effect cannot be eliminated by subsequent doses of Prostigmin. As found in agreement with Schubel (11), the isolated small intestine is also calmed by the effect of corresponding glycerine concentrations. However, in this case the intestine tends to recover spontaneously and remains responsive to Prostigmin.

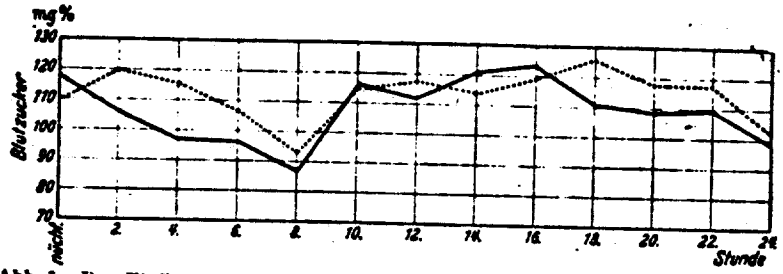

Abb. 1. Der Einfluß von 1,3-Butylenglykol und Glycerin auf den Zuckergehalt des Blutes von Kaninchen. Abszisse: Zeitpunkt der Blutentnahmen in Stunden. Ordinate: Zuckergehalt des Blutes in mg-%. Ausgezogene Linie: 1,3-Butylenglykol (3 cm³/kg, 50%ige Lösung in Wasser). Gestrichelte Linie: Glycerin (3 cm³/kg, 50%ige Lösung in Wasser). Jeder Punkt der Kurven entspricht den Mittelwerten von je 5 nüchternen Kaninchen, denen nach Bestimmung der Ausgangswerte die angegebenen Mengen 1,3-Butylenglykol bzw. Glycerin per os (Schlundsonde) zugeführt worden waren.

FIGURE 1. The effect of 1,3-Butylene glycol and Glycerine on the Sugar Content of Rabbit Blood. Abscissa: Time of blood sampling, hours. Ordinate: sugar content of the blood, mg-%. Solid line: 1,3-butylene glycol (3 cm3/kg, 50% aqueous solution). Broken line: glycerine (3 cm3/kg, 50% aqueous solution), Each point on the curves corresponds to the mean values of 5 sober rabbits, which had been given, following the determination of initial values, the amounts of 1,3-butylene glycol or glycerine per os (pharingeal probe)

Key: 1. Blood sugar, 2. Hour

Natural movements and movements reinforced by a pituitary extract ("Physormon" Promonta) of the uterus of guinea pigs were also inhibited by concentrations of 1,3-butylene glycol sufficient to eliminate the mortility of intestines. Lower concentrations, e.g. 1:400, produce more numerous and more productive contractions. The effect of 1,3-butylene glycol on the tone of the uterus depends on its initial position; it declines when the uterus is initially contracted, but remain unaffected if moderately or completely relaxed.

The special position of 1,3-butylene glycol within the common class of glycols is derived not only from the above investigations, but also from local effects on the skin and mucous membranes and from the behavior of the body in case of continued administration of the substance.

Body Behavior in the Case of Localized Action of 1,3-Butylene Glycol

1. Animal Experiments:

Application of 1,3-butylene glycol for 2 hours to the shorn belly of rats in urethane sleep produces no visible irritation. The condition of the skin, whether intact or artifically injured, has no effect. The same result was obtained with guinea pigs. The experimental procedure consisted of applying once daily 19.16 cm3 of undiluted 1,3-butylene glycol (commercial) to the shorn, whole (14 days) or sacrified (4 days) skin of the belly of guinea (per kg of body weight), i.e. an amount corresponding to the mean

lethal dose of mice and sufficient, if given subcutaneously, to induce narcosis in guinea pigs. The licking of the substance was prevented by immobilizing the animals. After a 2-hour exposure, the glycol was washed off with warm water, the animals dried under an electric dryer and placed in a thermostat. On the 5th or 15th experimental day the animals were killed and their kidneys, livers and bladder histologically examined.

No harmful effect of 1,3-butylene glycol was found during the entire treatment period. All of the guinea pigs behaved normally. Narcotic phenomena were specifically absent. As seen from daily weight measurements, body weights remained unaffected. No protein was found in the urine. No macroscopic or microscopic pathological changes were found in the organs.

In a similar experimental procedure, ethylene glycol (13.79 cm3 per kg of body weight, corresponding to the LD for mice) caused no pathological changes in guinea pigs. In contrast, 1,4-butylene glycol (2.14 cm3 per kg of body weight, corresponding to the LD50 for mice) produced a clear narcotic effect when applied to the sacrificed skin. The depth of narcosis was a function of the extent of the artificially inflicted injuries of the skin. About 75-90 minutes after the application of the substance the animals appeared to be dazed, lay on their bellies and exhibited initially superficial and accelerated breathing, followed by deep and slow respiration. After about 100-110 minutes they turned on their sides and breathed spasmodically. Reflexes were maintained. In the case of one animal, hyperactive reflexes and spasmodic breathing was observed clearly after 330 minutes. After about 480 minutes the narcotic phenomena in general disappeared and the animals resumed normal behavior. The narcotic effect did not occur when 1,4-butylene glycol was applied to the intact skin. 1,4-butylene glycol is thus not capable of penetrating normal skin. It penetrates, however, injured skin in amounts sufficient to cause obvious absorptive remote phenomena.

The sensitive mucous membranes again do not show harmful effects of 1,3-butylene glycol. Thus, a drop of the undiluted substance was applied to the eyes of rabbits and dogs without observing changes in the conjunctive membrane and the cornea. Similarly, visible damage was absent when 1,3-butylene glycol was applied with a brush once or several times to the mucous membrane in the mouth of dogs. 1,3-butylene glycol had no effect on the mucous membrane of the colon. Enema of 1,3-butylene glycol were ineffective in the case of rats, guinea pigs and dogs, in contrast to glycerine enema. The nerves of mucous membranes again showed no reaction to the effect of 1,3-butylene glycol in the sense of irritation -- no vomiting in dogs after the feeding of a probe of 1 cm3/kg, nor any paralysis -- and no anesthesia of the cornea in rabbits. The absence of effects on peripheral nerves in the case of local application is confirmed by the observation that 1,3-butylene glycol, used as eye drops, does not affect the pupilla reaction.

Following the intracutaneous injection of 0.15 cm3 and the subcutaneous injection of 0.8 cm3 of 1,3-butylene glycol in dogs, reactions at the locations of application are no more visible than after the subcutaneous or intramuscular administration of 0.2 cm3. In a similar manner, no changes were found at the vein in the ear of rabbits following the intravenous administration of 1 cm3 of the substance diluted 50% with water.

2. Observations on Humans:

Negative results, similar to those obtained in the animal experiments, resulted from investigations performed with members of the institute, who volunteered their services. In agreement with Gross (3), no local irritation was observed following the application of 1,3-butylene glycol to the intact skin of the upper of forearms. Similarly, no local irritation resulted when a cotton wad saturated with approx. 5 g undiluted 1,3-butylene glycol was applied for 16 days for 24 hours every other day to the skin (3 x 3 cm), covered with Billroth fabric, to expose the skin to the action of 1,3-butylene glycol. The skin also tolerates a repeated exposure to 1,3-butylene glycol after a pause of 12 days, without irritation. None of the experimental persons experienced interference with general well being, the occurrence of protein or blood pigment in the urine, or pathological components in urine sediments. Application to the mucous membrane of the mouth caused no disagreeable sensations or local anesthesia, with the exception of a slight, rapidly passing sensation of heat (heat of solution at the location of the application which is common, more or less, to all glycols). Similarly, no irritation of the mucous membranes of the mouth was observed following the continuous use (4 weeks) of 1,3-butylene glycol-containing toothpaste (menthol 1, 0, Sapo medicat. 10.0, calc. carb. 35.0, magn. carb. 10.0, saccharin 0.05, ol. menth. gtt. XXX, ol. caryoph. gtt. V, 1,3-butylene glycol ad 105.0). Intracutaneous (0.2 cm3) and subcutaneous (0.1 cm3) injection of 1,3-butylene glycol sterilized at 100o causes no irritation at the location of the injection, except for a rapidly passing sensation of burning. No difference was observed in this case between the commercial product and the pure substance. The urine of all the persons was free of protein during the entire experimental period.

Summarizing the results of the above investigations, it may be stated that 1,3-butylene glycol causes no detectable changes of the skin and mucous membranes under the given conditions. This observation results both from animal experiments and observations of humans.

Behavior of Organisms During Continuous Application of 1,3-Butylene Glycol.

The necessity for conducting extended experiments is the result of the empirical fact that substantial differences may exist between the toxicity of a substance in single or multiple applications, which cannot or not always be determined by the results of a

toxicity test. The authors therefore agree with Scholz (10) that "it is not permissible to declare a substance to be harmless on the basis of a single toxicity determination".

1. Experiments on Animals:

In order to study the reaction of the body to the repeated administration of 1,3-butylene glycol, 180 young male rats weighing 75-110 g, maintained under identical conditions and fed kitchen scraps, bran and oats, were divided into three groups. The first group received a daily dose of 1 cm3 of a solution of 1,3-butylene glycol solution per 100 g of body weight, the second 1 cm3 of a solution of glycerine in water, and the third 1 cm3 of distilled water (20 rats). The experimental period was 44 days in each case. 1,3-butylene glycol and glycerine was given in the form of 1%, 5%, 10% and 20% solutions to 20 rats in each group, pharingeal probe. Following the completion of the experiments, the animals were killed and their kidneys, livers and bladders examined histologically.

Although the greater part of the rats in their treatment with 1,3-butylene glycol or glycerine in the given concentrations received approximately a single, double or triple mean lethal dose, their behavior did not differ from that of animals receiving only water. Narcotic effects or diarrhea were not observed. Daily weight controls revealed an increase in body weight in every case. (Fig. 2). This finding deserves special emphasis, because in the experiments young, growing animals were used of which it is known that interference with their normal growth is an especially sensitive indicator for chronically acting poisons. No difference was found with respect to lethality between the treated and the control animals. It amounted to approx. 15% for all groups of animals.

The compatibility of the substance is also supported by the observation that a young dog (4.3 kg) tolerated without reaction repeated doses of a total of 23 cm3 undiluted 1,3-butylene glycol within a period of 14 days. No precipitation of protein in the urine or hematuria was observed.

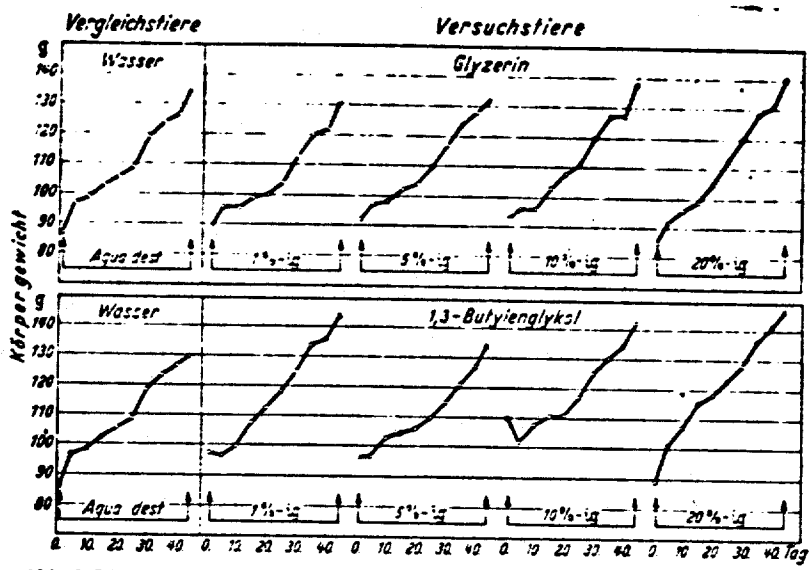

Abb. 2. Wachstumskurven männlicher Ratten bei täglicher Einwirkung von 1,3-Butylenglykol und Glycerin verschiedener Konzentration (per os, Schlundsonde). Links Vergleichstiere (1 cm³ Aqua dest. je 100 g Körpergewicht und Tag). Rechts Versuchstiere (1 cm³/100 g 1,3-Butylenglykol bzw. Glycerin als 1%ige, 5%ige, 10%ige und 20%ige Lösung in Aqua dest.). Abszisse: Behandlungszeit in Tagen. Ordinate: Körpergewicht in Gramm. Jeder Punkt der einzelnen Kurven entspricht den Mittelwerten von je 20 Ratten. Die Pfeile geben die Dauer der Behandlung an.

FIGURE 2. Growth Curves of Male Rats Under the Daily Effect of 1,3-Butylene Glycol and Glycerine of Different Concentrations (per os, pharingeal probe). Left: Control animals (1 cm3 of distilled water per 100 g of body weight and day). Right: Experimental animals (1 cm3/100 g of 1,3-butylene glycol or glycerine as 1%, 5%, 10% or 20% aqueous solutions in distilled water). Abscissa: treatment period in days. Ordinate: body weight in grams. Each point on the curves represents the average value for 20 rats. The arrows indicate the duration of the treatment.

Key: 1. Control animals, 2. Experimental animals, 3. Water, 4. Glycerine, 5. Distilled water, 6. Body weight, 7. Days.

The histological investigation of livers, kidneys and bladders showed no pathological changes.* Specifically, the kidneys lacked any indication of damage. The possibility that with other animal species or following the administration of larger amounts of 1,3-butylene glycol the organs may suffer damage cannot be excluded. Such an occurrence is known in the case of other glycols. They may also be observed when glycerine is used. It should be noted here that the liver, kidneys and bladders of animals which perished during toxicity determinations within 24 hours also lacked pathological changes.

* The authors thank Professor Dr. Giese, Pathological Institute, Municipal Hospitals, City of Bremen, for his review of the histological preparations.

In all experimental groups, in some rats worm eggs were found in the bladder and ocassionally worms in the liver.

During the investigation of the behavior of the organism following the repeated administration of 1,3-butylene glycol, the consumption of oxygen and the blood count were also tested continuously. (Fig. 3).

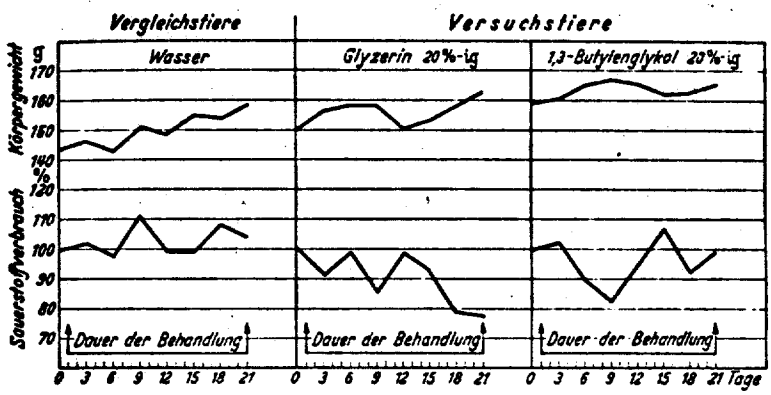

Abb. 3. Sauerstoffverbrauchswerte männlicher Ratten bei täglicher Einwirkung von 1,3-Butylenglykol und Glycerin (per os, Schlundsonde). Links Vergleichstiere (1 cm³ Wasser je 100 g Körpergewicht und Tag). Rechts Versuchstiere (1,3-Butylenglykol bzw. Glycerin; 1 cm³/100 g Körpergewicht und Tag als 20%ige Lösung in Wasser). Abszisse: Behandlungszeit in Tagen (Pfeile). Ordinate: Sauerstoffverbrauch je g/Stunde in Prozent des gleich 100 gesetzten mittleren Ausgangswertes. Obere Kurven Körpergewicht in Gramm. Jeder Punkt der Kurven der Vergleichs- und der mit 1,3-Butylenglykol behandelten Ratten entspricht den Mittelwerten von je 8 Tieren. Die Punkte der Kurven von den Ratten, welche Glycerin erhalten hatten, entsprechen am 6. Versuchstage den Mittelwerten von 7, am 9. denen von 6, am 12. denen von 5, am 15. denen von 4 und am 18. und 21. Tage denen von 3 Tieren.

FIGURE 3. VAlues of Oxygen Consumption in Male Rats Under the Daily Effect of 1,3-Butylene Glycol and Glycerine (per os, pharingeal probe). Left: Control animals (1 cm3 water per 100 g body weight and day). Right: Experimental animals (1,3-Butylene Glycol or glycerine; 1 cm3 per 100 g body weight and day as a 20% aqueous solution). Abscissa: treatment period in days (arrows). Ordinate: oxygen consumption in g/hour as percentage of the average initial value set equal to 100. Upper curves, body weight in grams. Each point on the curves of the control animals and the rats treated with 1,3-butylene glycol corresponds to the mean value of 8 animals. The points of the curves of rats receiving glycerine represent the mean values of 7 rats on the 6th experimental day, of 5 rats on the 12th day, of 4 rats on the 15th and of 3 rats on the 18th and 21st days.

Key: 1. Control animals, 2. Experimental animals, 3. Body weight, 4. Consumption of oxygen, 5. Water, 6. Duration of treatment, 7. Days.

Oxygen Consumption:

Twentyfour male rats weighing 140-160 g were divided into 3 groups of 8 animals each. The rats of Group 1 received daily per os (pharingeal probe) 1 cm3 of tap water and served as the control animals. The animals of Group 2 were given a daily dose

of 1 cm3 of a 20% aqueous solution of glycerine, and the rats of Group 3 1 cm3 of a 20% aqueous solution of 1,3-butylene glycol in water per 100 g body weight. The experimental period was identical for all groups and amounted to 21 days. The consumption of oxygen was determined by the method described by Loeser & al. (8).

The values of oxygen consumption determined show that neither the 20% glycerine nor the 20% 1,3-butylene glycol solutions have clear effects on metabolism in the amounts given. The oxygen consumption of the control animals varied around the mean initial value (set 100%). The oxygen values of rats treated with the 20% glycerine solution exhibited a slight temporary decline on the 3rd and 9th day of treatment. Since, due to the great mortality in this group, only 2 animals remained alive on the 18th day of the experiment, further evaluations had to be discontinued. Following the administration of the 20% 1,3-butylene glycol solution, the oxygen consumption of rats declined temporarily only on the 6th, 9th and 12th day by 10, 17 and 5% (Fig. 3 and Table 6).

Blood Count:

Ten rats weighing approx. 75-100 g received for 44 days a daily dose of 1 cm3 of a 10% aqueous solution of 1,3-butylene glycol per 100 g body weight per os (pharingeal probe). Simultaneously, a second group of 10 male rats of equal weight were given a daily dose of 1 cm3 of a 10% aqueous glycerine solution, and a third group of 10 male rats received daily 1 cm3 of distilled water per 100 g of body weight for the same period of time, per os. The first examination of the blood took place on the 2nd day of treatment, i.e. 7 days after the determination of initial values. The examination was repeated in 7-day intervals. The blood was taken from the tail vein of the animals and examined by conventional clinical hematological methods: hemoglobin determination by the Sahli method, erythrocite and leucocyte count with conventional pipettes in the new Thoma-Zeiss chamber. Differential blood picture: staining in accordance with Pappenheim, evaluation according to Schilling.

As is evident from Fig. 4, the hemoglobin content, the number of erythrocytes and leucocytes, and the values of the white blood cells behaved in a similar manner with respect to both experimental and control animals. Only individual fluctuations were observed. The increase in eosinophiles in animals treated with 1,3-butylene glycol and glycerine is presumably the result of a worm infection. The presence of worms was confirmed in the dissection of the animals.

To complete the above experiments, the hemolytic effect of 1,3-butylene glycol was compared with that of glycerine. It was shown that 1,3-butylene glycol -- and glycerine -- does not cause hemolysis up to a concentration of 40% in a 0.9% table salt solution. Only an 80% concentration of 1,3-butylene glycol caused an incomplete hemolysis after 1 1/2 hours, while glycerine

resulted in complete hemolysis after one hour, using the same concentration.

TABLE 6. The Effect of Glycerine and 1,3-Butylene Glycol on the Oxygen Consumption in Rats. Dosage: 1 cm3 of a 20% aqueous glycerine or 20% aqueous 1,3-butylene glycol solution per 100 g of body weight and day per os. Treatment period: 1-21 days.

Tabelle 6. *Der Einfluß von Glycerin bzw. 1,3-Butylenglykol auf den Sauerstoffverbrauch von Ratten. Dosierung: je 100 g Tier und Tag 1 cm³ einer 20%igen wäßrigen Glycerin- bzw. 20%igen wäßrigen 1,3-Butylenglykollösung per os (Schlundsonde). Behandlung vom 1.—21. Tage.*

1 Tier Nr.	2 O_2-Verbrauch in cm³ je g/Stunde vor der Behandlung (Ausgangswert)				3 Mittelwert	4 O_2-Verbrauch in cm³ je g/Stunde bzw. in Prozent des mittleren Ausgangswertes während der Behandlung													
						5 Tag der Behandlung													
						3.		6.		9.		12.		15.		18.		21.	
	I	II	III	IV		cm³	%	cm³	%	cm³	%	cm³	%	cm³	%	cm³	%	cm³	%
6 Vergleichstiere.																			
1309	1,573	1,619	1,521	1,699	1,603	1,556	97	1,213	76	1,574	98	1,325	83	1,516	95	1,969	123	1,610	100
1310	1,838	1,542	1,286	1,659	1,581	1,485	94	1,588	104	1,429	90	1,459	92	1,497	95	1,514	96	1,894	120
1311	1,466	1,375	1,443	2,106	1,598	1,644	103	1,353	85	1,535	96	1,402	88	1,551	97	1,621	101	1,678	105
1312	1,596	1,603	1,407	1,624	1,558	1,604	103	1,147	74	1,788	115	1,465	94	1,626	104	1,672	107	1,766	113
1313	1,572	1,374	1,370	1,569	1,471	1,596	109	1,525	104	1,919	131	1,576	107	1,539	105	1,575	107	1,233	84
1314	1,433	1,532	1,244	1,555	1,441	1,566	109	1,810	126	1,893	131	1,740	121	1,626	110	1,469	102	1,583	110
1315	1,550	1,486	1,441	1,790	1,592	1,745	110	1,673	105	1,646	103	1,524	96	1,360	85	1,835	115	1,187	75
1316	1,371	1,157	1,221	1,152	1,225	1,081	88	1,317	108	1,516	124	1,434	117	1,200	98	1,403	115	1,577	129
							102		98		111		99		99		108		104
Glycerin 20%ig.																			
1339	1,821	2,011	1,527	1,670	1,757	1,324	75	1,754	100	1,162	66	1,636	93	1,385	79	1,951	111	1,739	99
1342	1,937	2,332	2,514	2,514	2,253	2,088	93	1,757	78	1,640	73	1,392	62	1,608	90	1,407	63	1,487	66
1343	1,649	1,473	1,495	1,716	1,584	1,268	80	1,349	85	1,227	77	1,237	78	1,271	80	0,991	63	1,080	68
1344	1,701	1,314	1,093	1,376	1,366	1,351	99	1,648	121	1,532	112	1,781	130	1,691	124	†			
1340	1,490	1,233	1,569	1,361	1,413	1,492	106	1,749	124	1,518	107	1,857	131	†					
1338	1,458	1,820	1,572	1,478	1,582	1,115	71	1,625	103	1,306	83	†							
1341	1,609	1,481	1,684	1,647	1,605	1,340	83	1,780	85	†									
1337	1,053	1,471	1,110	1,333	1,242	1,472	119	†											
							91		99		85		99		93		79		78
1,3-Butylenglykol 20%ig.																			
1345	1,355	1,189	1,696	1,505	1,436	1,570	109	1,175	82	1,245	87	1,237	86	1,426	99	1,294	90	1,328	93
1346	1,970	2,187	2,522	2,326	2,251	1,990	89	1,482	66	1,279	57	1,566	70	1,808	80	1,313	58	1,714	76
1347	1,352	1,593	1,823	1,478	1,562	1,647	106	1,276	82	1,425	91	1,206	77	1,471	94	1,078	69	1,237	79
1348	2,186	1,191	1,574	1,524	1,619	1,582	98	1,136	71	1,630	101	1,425	88	1,532	95	1,458	90	1,450	90
1349	1,430	1,529	0,938	1,079	1,244	1,553	125	1,693	136	1,016	82	1,666	134	1,914	154	1,712	136	1,575	127
1350	1,511	1,706	1,272	2,720	1,806	1,622	89	1,459	81	1,155	64	1,591	88	1,852	103	1,570	87	1,547	86
1351	1,406	1,422	1,416	1,478	1,435	1,530	107	1,402	98	1,186	83	1,615	113	1,754	122	1,511	105	1,933	135
1352	1,299	1,276	1,273	1,283	1,283	1,237	96	1,282	100	1,229	96	1,323	103	1,406	110	1,313	102	1,367	107
							102		90		83		95		107		92		99

Key: 1. Number of animal, 2. O_2 consumption in cm3 per g/hour prior to the treatment (initial value), 3. Mean value, 4. O_2 consumption in cm3 per g/hour and % of the mean initial value during the treatment; 5. Day of treatment, 6. Control animals.

A spectroscopic examination of the blood of 4 rats each of the animal groups mentioned above, performed on the 44th day of the experiment, gave no indication of the formation of methemoglobin in

animals treated with the 10% aqueous solution of 1,3-butylene glycol or the 10% aqueous glycerine solution. No such indication was found in a cat after the administration for 3 days of 5 cm3/kg 1,3-butylene glycol per day.

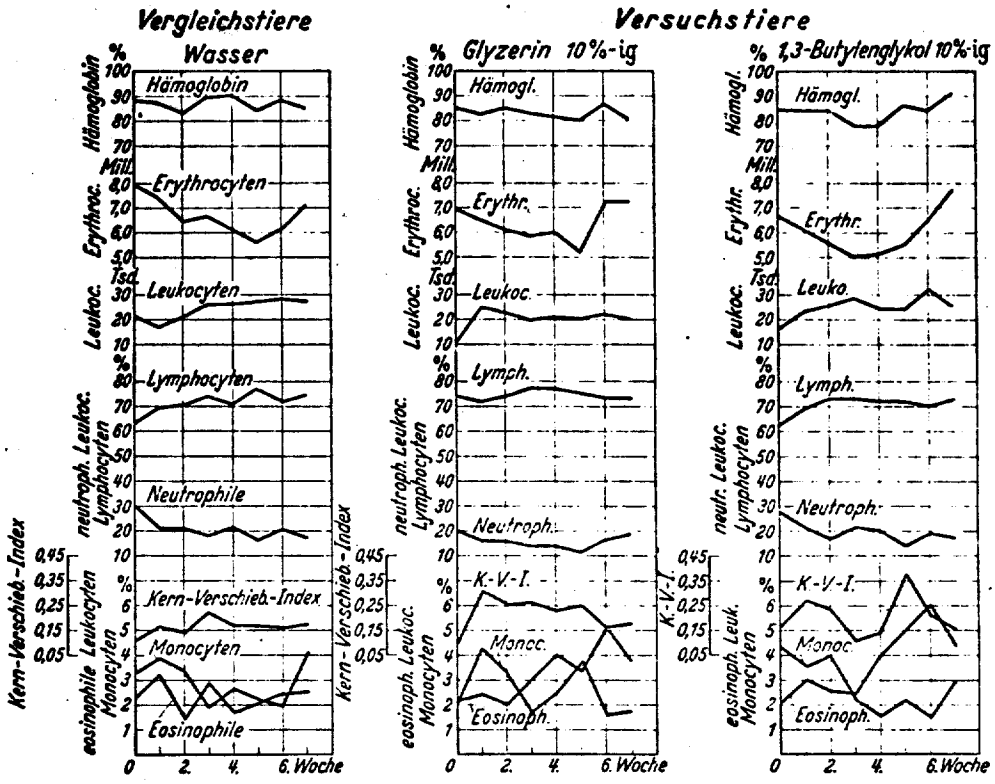

Abb. 4. Differentialblutbild von Ratten bei 44tägiger Einwirkung von täglich 1 cm³ 1,3-Butylenglykol bzw. Glycerin (10 %ige Lösung in Aqua dest.) bzw. 1 cm³ Aqua dest. je 100 g Körpergewicht per os (Schlundsonde). Blutentnahme in 7tägigen Abständen. Die Punkte der einzelnen Kurven entsprechen den Mittelwerten von je 10 Ratten. Abszisse: Zeit in Wochen. Ordinate: Hämoglobin in Prozent, Erythrocyten in Millionen, Leukocyten in Tausend, neutrophile und eosinophile Leukocyten, Lymphocyten sowie Monocyten in Prozent und Kern-Verschiebungsindex.

FIGURE 4. Differential Blood Picture of Rats During a 44-Day Administration of a Daily Dose of 1 cm3 1,3-butylene glycol or Glycerine (10% solution in distilled water) or 1 cm3 distilled water per 100 g of body weight per os (pharingeal probe). Blood sampling in 7-day intervals. The points of the individual curves correspond to the median values of 10 rats in each group. Abscissa: time in weeks. Ordinate: hemoglobin, %. Erythrocites in millions, leucocytes in thousands, lymphocytes and monocytes in % and nuclear displacement index

Key: 1. Control animals, 2. Experimental animals, 3. Water, 4. Nuclear shift index, 5. Weeks.

Observations on Humans:

Auto experiments showed that 1,3-butylene glycol, as a 50% aqueous solution taken on 3 consecutive days internally, does not cause changes in the general condition in doses of 1 cm3 or 2 cm3. Daily urine examinations indicated that the urine was free of protein or other pathological components, both during and after the experimental period.

With respect to the absorption, the destination in the body and the elimination of 1,3-butylene glycol it may be stated, based on observations during the determination of toxicity, that -- measured by the occurrence in time of the narcotic effect -- the substance is absorbed relatively rapidly by the mucous membrane of the digestive tract and the subcutaneous cell tissue. Nothing is known concerning its destination in the body and its location. It is conceivable that in the decomposition of 1,3-butylene glycol in the organism beta-oxybutyric acid and acetoacetic acid are formed as intermediate products. However, these compounds were not detected by the use of conventional clinical investigation methods in the urine.

These investigations were based on the fact that the mean lethal doses of 1,2-propylene glycol and 1,3-butylene glycol are remarkably high in comparison with other glycols, i.e. the toxicity of these bivalent alcohols is astoundingly low. Both substances contain both the CH2OH group of primary alcohols and the CHOH group of secondary alcohols. Based on these approximations, it was initially assumed that the compounds are similar in other effects as well. The assumption has been confirmed by the investigations of the authors. They show that 1,3-butylene glycol, under the given conditions and amounts, does not cause changes in humans and animals indicating any damage to the body. In this respect, the results of the authors are in agreement with the results of Hildebrandt (4), who, based on a few preliminary experiments considered the substance to be practically nontoxic, although he observed a slight precipitation of protein in the case of a dog fed undiluted 1,3-butylene glycol (0.2 g/kg) 14 times in 2 weeks.

A review of the divalent alcohols grouped together in the common pharmacological class of glycols shows that the former unique position of 1,2-propylene glycol with respect to its toxicity cannot be maintained, as shown on the example of 1,3-butylene glycol. As in other groups of substances, there are certain representatives of the glycol series, which on the basis of their effects may be considered practically nontoxic. It is therefore not correct to speak of the toxicity of glycols in general. Every one of them must be evaluated individually. Particular attention must be paid to the glycol derivatives -- esters, ethers, etc. -- which, according to the literature (3, 10), have in general proved to be especially toxic.

The authors have emphasized repeatedly that both in 1,2-propyelen glycol and in 1,3-butylene glycol the CH2OH group

of the primary alcohols and the CHOH group of the secondary alcohols are present. If further investigation should reveal that other primary-secondary alcohols also exhibit such low toxicities, it may be possible to establish a regular relationship between chemical structure and pharmacological and toxic effects. Results obtained to date are inadequate for this purpose; they can serve only as an incentive for additional study.

Summary:

Arrangement of the divalent alcohols ethylene glycol, propylene goycol, butylene glycol and their isomers by the type of hydroxyl groups, they contain indicates that 1,2-propylene glycol as a representative of the primary-secondary alcohols differs from other glycols by its remarkably low toxicity.

According to the present investigation, 1,3-butylene glycol occupies a similar position.

Its repeated application to the skin and mucous membranes of humans and animals for short periods of time did not result in harmful effects. Given per os under the conditions of the experiments, even the repeated administration of aqueous solutions of 1,3-butylene glycol (1, 5, 10 and 20%) left the general condition, growth oxygen consumption and blood count of rats unaffected. Specifically absent are pathological changes in kidneys, livers and bladders, such as caused by other glycols. In humans, the urine remains free of protein after the repeated ingestion of small amounts of 1,3-butylene glycol (50% aqueous solution, per os).

Being an alcohol, 1,3-butylene glycol has a narcotic effect when given in high doses. The mean lethal dose (LD50) was found to be 23.31 cm3/kg given per os and 16.51 cm3/kg subcutaneously for mice, 29.42 cm3/kg per os and 20.06 cm3/kg subcutaneously for rats. The values are a function of the purity of the substance. The effect of 1,3-butylene glycol on respiration, blood pressure, blood sugar and the isolated intestine and uterus were investigated.

The potential significance of the type of hydroxyl groups with respect to the effect of glycols is discussed.

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TOXICOLOGY AND APPLIED PHARMACOLOGY 1, 315-331 (1959)

Chronic Oral Toxicities of Four Stearic Acid Emulsifiers

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Received January 7, 1959

During the past decade extensive use has been made of stearic acid-type emulsifiers in foods and pharmaceutical preparations. While the quantity incorporated into individual food items is relatively small, as indicated by Pratt (1952), the ever-increasing use of these compounds is paralleled by an increasing exposure hazard.

Numerous investigations have been carried out in an effort to prove safety of the Myrj,[3] Tween[3] and Span[3] types of emulsifiers for food use. The voluminous records of "bread hearings" before the Federal Security Agency (1948-1949), indicated an extensive use for the polyoxyethylene stearates in retarding the firming of bread. In applying for the inclusion of Myrj 45 as a permissive ingredient in bread, and for which a definition and standard of identity would be necessary, certain toxicologic evaluations of safety were given in testimony on this and related types of compounds. However, such evidences were judged by the Administrator to be inadequate. Results of the then available oral toxicity studies on the polyoxyethylene stearates were reviewed in the report by the Food Protection Committee of the Food and Nutrition Board of the National Research Council (1953). This report concluded that available experimental data did not demonstrate the safety of polyoxyethylene stearates for use in food under all patterns of dietary consumption and for all segments of the human population.

Much of the data published since 1953 indicate some degree of safety of these substances to animals but do not establish clearly the no-effect

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[3] Trade names of Atlas Powder Co., Wilmington, Delaware.

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levels. According to Brush *et al.* (1957), Myrj 52 at feeding levels of 1–10% of the diet showed no histopathology in mice. Diarrhea and growth retardation data led to the conclusion that 5% of Myrj 52 is near the upper limit for feeding of this surface-active agent to hamsters. These authors concluded also that no urinary tract concretions or digestive tract irritation resulted from feeding 1% and 5% Myrj 45 to twelve hamsters for one year; however, 5% was considered to be near the upper limit of safety for hamsters. Krehl *et al.* (1955) found no deleterious effect of feeding Myrj 45 to cats at a concentration of 20% of total diet for one year. Oser and Oser (1957c) fed hamsters at 5, 10, 15, and 20% levels of Myrj 45 for 10 weeks. Feces were softer with increasing dosages but there was no diarrhea. The only pathology noted was some increase in kidney weight and enlargement of the cecum at the 20% level. Orten and Dajani (1957) found incidental renal calculi and no other consistent pathology when hamsters were fed for 22 weeks at the 15% level. Eagle and Poling (1956) found renal concretions in hamsters fed this substance for 29–39 weeks at 5, 10, and 15%. There were nephropathology and some calculi in the urinary bladder of the hamsters. Chronic diarrhea was observed in the test animals, especially at the high levels.

Poling *et al.* (1956) reported that polyoxyethylene monostearate retarded growth, increased mortality, and produced fecal occult blood in rats fed at 25% in the diet for 21 weeks. Two of these authors (Eagle and Poling, 1956) reported renal concretions in rats at this dosage; however, kidney weights were not significantly heavier than the controls. Oser and Oser (1956a, b, 1957a, b) reported satisfactory growth and food utilization by rats fed stearates of the Myrj, Tween, and Span types for two years at levels of 5, 10, and 20% in a diet composed of wheat, corn, milk solids, and meat supplemented with minerals and vitamins. Male rats fed 20% levels of the same emulsifiers, with the exception of those fed 20% of Myrj 45, showed less weight gain than controls. Progeny through three succeeding generations showed some decrease in survival at the 10% and 20% dosage levels and impairment of lactation efficiency at the 20% level. These authors found one bladder stone in thirty-two rats fed Myrj 45 for two years at the 20% level. Groups fed at the 5% and 10% level had no stones. Similarly Graham and associates (1954; Graham and Grice, 1955) found no bladder stones or other deleterious effects in rats fed 11.25% Myrj 45 in a bread diet for one year. György *et al.* (1958) evaluated Myrj 45 under stress conditions and found that the addition of 2% and 10% did not affect the course of cirrhosis of the liver or of nephrosis in rats, nor did 1% or 8% affect the course of hepatic necrosis in rats.

Experiments with dogs are very limited. Brush *et al.* (1957) fed weanling puppies a special diet prepared for growing young dogs and containing 5% and 10% Myrj 45, Myrj 52, or Tween 60 for 9 months to a year. No gross pathology was noted in any dog, and those that were sectioned showed no histologic change due to treatment.

Kruesi and Van Itallie (1956) in work with ten patients suffering from a variety of liver disorders concluded that 3–6 g per day for periods of up to 66 days produced no effect as evidenced by liver function tests and clinical progress. Oler and Craemer (1955) found no effect of 3-g dosages of a 1% concentration of Myrj 45 or Myrj 52 on the gastrointestinal motility of humans when given in test meals. Gastric analysis revealed no effect on gastric acidity but revealed a decrease in presence of gross mucus in most gastric samples.

METHOD

Rat experiment. Compounds tested were Myrj 45 (polyoxyethylene-8-stearate), Myrj 52 (polyoxyethylene-40 stearate), Span 60 (sorbitan monostearate), and Tween 60 (polyoxyethylene-20 sorbitan monostearate). Myverol 18-00[4] was fed as a comparative control.[5] The basal diet was composed of ground commercial rat biscuit, used extensively in this laboratory and shown to be complete for growth and reproduction. Diets were prepared at the 0, 2, 5, 10, and 25% levels by weight, replacing an equivalent amount of the basal diet, except that Myverol was fed only at the 25% level. All diets were prepared in quantities approximating a 2-week supply.

Span 60 and Tween 60 were fed concurrently to groups of 24 rats on each level of each compound with a single control group. Each of the other studies was compared to its own concurrent control group. Groups of 24 weanling rats, weighing between 40 and 50 g and equally divided as to sex, were selected from our colony of Osborne-Mendel strain[4] for

[4] Trade name of Distillation Products Industries, Rochester, New York, and composed of "distilled monoglycerides made from hydrogenated lard."

[5] Materials employed in these experiments were bought from Atlas Powder Co., except Myrj 45, which was purchased on the open market. These are not single chemical compounds, but mixtures of variable composition depending upon manufacturing procedures.

each test. In an expanded two-year feeding study Myrj 45 was fed to groups of 200 animals at each level except 25%, where 150 animals were used. All animals were selected for the various groups on a littermate basis. Rats were housed in individual cages and were allowed food and water ad libitum. Animal weights and food intake were recorded weekly for two years, at which time survivors were sacrificed by exsanguination and autopsied. Animals surviving 102 or more of the 104 weeks on experiment were counted as "survivors."

Complete blood counts were taken from ten animals of each of the several groups twice during the test period. General physical condition of individual rats was observed frequently and abnormalities were noted; moribund animals were sacrificed. Organ weights and gross pathology were recorded for all sacrificed animals, and organs and tissues were fixed in formalin solution for histopathologic study. Organs from animals found dead were excluded from organ weight calculations, but were saved for histopathologic study if not markedly autolyzed.

Dog Experiment. Dogs from 1 to 6 years of age of both sexes, some mongrels and some Irish terriers, were fed Myrj 45 and Span 60 in a semisynthetic diet at 5% of the total food intake. Two of four dogs fed Myrj 45 were previously given 1 g/kg per day of this chemical in capsules for 21 months while maintained on dog biscuits. These two dogs were then fed 5% Myrj 45 mixed with the diet at the time the other dogs, including the controls, were started, and all dogs were then continued for 19 or 20 months. The total intake of Myrj 45 was somewhat less than 1 g/kg per day on the latter regimen. The basic diet consisted of sucrose 50%, casein 19%, corn oil 6%, lard 5%, vegetable fat 5%, liver extract 4%, salt mixture 4%, yeast 3%, cod liver oil 2%, and beet root pulp 2%. Diets which contained 5% of test substance had casein, corn oil, and sucrose reduced 1, 1, and 3%, respectively. Animals were weighed each week and food intake was measured when the dogs were fed.

RESULTS

Rats

Average weight gain was calculated after 12 weeks on the diets to measure the effect on the early and rapid growth, and again after one year on the diet. Significant growth depression occurred in some groups at the 25%-feeding level but in none at 10% or below. Adverse effects were more pronounced in males than in females, only Span 60 producing growth depression in both sexes at the highest dietary level. Span 60 and Myrj 52 at 25% retarded growth of male rats throughout the first year of the experiments, whereas the inferior weight gain shown by male rats on Tween 60 was statistically significant only at the 12-week period. Myverol, the glycerol-containing compound, produced normal growth of both sexes at 25% throughout the experiment. In the feeding test of Myrj 45 in which 24 animals were used in each group, early growth was not affected but male rats fed 25% of the material did not attain the size of controls for that group at 1 year of age. Female rats fed 10% of Myrj 45 were significantly heavier than their controls after one year on the diet. The second feeding test of Myrj 45 in the same dietary concentrations, but using littermated animals in much larger numbers, yielded somewhat similar results. In this test of Myrj 45, early growth of males fed 25% was depressed while females fed 10% of the material in the diet showed superior weight gain both at 12 weeks and at 52 weeks. Other groups were not affected.

In evaluating the effects on growth of these emulsifiers, the food efficiency of the various diets was considered; a summary of results at 12 weeks for the highest dosage level is presented in Table 1, along with the weight gains. The animals on Myrj 52 showed the greatest difference from the controls. In all levels food intake increased and food efficiency, or grams of weight gained per gram of diet consumed, decreased as the concentration of Myrj 52 in the diet was increased. Growth of males was markedly depressed at the 25% level in spite of increased food consumption. Here food intake was 16.7% above controls for the first 12 weeks and 13.1% above controls for the first year. These figures indicate decreased food efficiency beyond the 18.75% nonnutritious polyol in the total diet since growth retardation was greater, particularly at the 12-week period, than can be accounted for by a difference in food intake. This difference is relatively small and its significance is questionable. Since Myrj 52 caused diarrhea at all except the lowest feeding level, it is probable that less of this total diet was absorbed. Values shown in Table 1 for Span 60 present a different picture. Here it is estimated that, in order to supply an equal number of calories, rats fed 25% of the emulsifier should consume 8.5% more than controls. Actual food intake of males was 2.4% below controls at 12 weeks. Females consumed almost as much of the diet for the first 12 weeks as controls, yet growth was significantly depressed. Early growth depression in males fed 25% of Tween 60 was

also accompanied by a reduced food efficiency. Myverol was well consumed and found to be nutritionally adequate in a 25% diet. Myrj 45, in the expanded test, proved to be nutritionally adequate for females in

TABLE 1

FOOD EFFICIENCY OF DIETS CONTAINING 25% STEARATE EMULSIFIERS FED TO RATS FOR 12 WEEKS

Substance	Dietary level (%)	No. animals	Sex	Average weight gain[a] (g)	Average food intake (g)	Food efficiency (g wt. gain/100 g food)
Myverol	0	12	M	333.4 ± 11.4	1988	16.8
		12	F	184.3 ± 4.3	1568	11.7
	25	12	M	311.2 ± 11.2	2021	15.4
		12	F	182.6 ± 5.8	1549	11.7
Span 60	0	12	M	313.2 ± 15.3	1752	17.8
		12	F	178.6 ± 9.8	1269	14.1
	25	12	M	192.5 ± [illegible]	170[illegible]	1[illegible]
		12	F	151.0 ± 8.1	1214	12.4
Tween 60	0	12	M	313.2 ± 15.3	1752	17.8
		12	F	178.6 ± 9.8	1269	14.1
	25	12	M	271.0 ± 6.3[b]	1589	17.1
		12	F	175.7 ± 5.1	14[illegible]	11.9
Myrj 52	0	12	M	329.7 ± 5.7	1784	18.5
		12	F	181.6 ± 9.1	1554	11.[illegible]
	25	12	M	288.0 ± 9.4[c]	2082	13.8
		12	F	176.5 ± 4.5	1730	10.2
Myrj 45	0	12	M	355.3 ± 16.6	1677	21.2
		12	F	187.4 ± 6.0	1268	14.8
	25	12	M	328.9 ± 17.4	1729	22.1
		12	F	181.8 ± 4.7	1455	12.4
Myrj 45	0	10[illegible]	M	311.6 ± 3.6	1787	17.4
		10[illegible]	F	173.2 ± 1.6	1497	11.6
	25	75	M	289.5 ± 4.1[b]	1934	15.2
		75	F	168.4 ± 2.2	1528	11.0

[a] Gain and standard error. [b] $P < 0.05$. [c] $P < 0.01$.

all levels except the highest. In the test in which 24 rats were fed in each group, superior weight gain resulted from increased food intake at the 10% level after one year on the diet although actual weight gain per gram of food consumed is less at 12 weeks. Among males in both tests inadequate caloric intake is indicated.

Rats which died or were sacrificed when moribund during the course

of the experiments were totaled for each six-month period of the tests. A review of these data on survival revealed no difference from that shown by the survival rate at two years (Table 2). Span 60 and Myrj 45 caused decreased survival at the 10% and 25% dosages. No effect on survival was noted in any other group.

TABLE 2

TWO-YEAR SURVIVAL RATES OF RATS FED TEST DIETS

Substances	No. of animals	Dietary level (%)	No. of survivors	Survival rate (%)
Myverol	24	0	13	54.2
	24	25	10	41.7
Myrj 52	24	0	14	58.3
	24	2	9	37.5
	24	5	10	41.7
	24	10	14	58.3
	24	25	13	54.2
Span 60	24	0	12	50.0
	24	2	12	50.0
	24	5	10	41.7
	24	10	6	25.0[a]
	24	25	6	25.0[a]
Tween 60	24	0	12	50.0
	24	2	15	62.5
	24	5	14	58.3
	24	10	12	50.0
	24	25	9	37.5
Myrj 45	224	0	123	54.9
	224	2	112	50.0
	224	5	111	49.6
	224	10	99	44.2[b]
	174	25	66	37.9[c]

[a] $p < 0.05$. [b] $p < 0.01$. [c] $p < 0.001$.

Hematology studies showed normal values for hemoglobin, red and white blood cells, and differential counts for all groups.

Gastrointestinal tract. Diarrhea was apparent throughout life in rats fed Tween 60 and Myrj 52, severe at 25%, moderate at 10%, and slight at 5%. Myrj 52 may be considered somewhat more damaging in this respect since diarrhea was noted occasionally in some rats fed 2% of this material in the diet, whereas the same level of Tween 60 produced no diarrhea. Oser and Oser (1957a) showed that the degree of laxation was

related to the molecular weight and the concentration of the polyol moiety released upon hydrolysis. Laxation could be induced equally well by the longer-chain polyols themselves. Also Myrj 52 and Tween 60 are characterized as the most hydrophilic of the tested substances.

Organ weight. Organ weights were calculated on the basis of grams of organ per kilogram of rat at the time of sacrifice, separate calculations being made for males and females of each group and also for the group as a whole. Most animals on the highest emulsifier dosage had livers which were enlarged and more friable than normal. No significant liver enlargement occurred in rats at lower dosages (Table 3). The increase

TABLE 3

LIVER WEIGHTS IN GRAMS PER KILOGRAM OF MALE AND FEMALE RATS FED STEARATE EMULSIFIERS FOR TWO YEARS

	Dietary levels				
Substances	0%	2%	5%	10%	25%
Myverol	23.5 ± 1.35[a]	—	—	—	27.9 ± 1.62[c]
Myrj 52	26.4 ± 1.72	25.5 ± 1.51	27.0 ± 1.07	28.1 ± 1.13	29.1 ± 1.45
Span 60	25.8 ± 0.98	27.8 ± 1.98	25.7 ± 0.91	27.1 ± 1.56	40.3 ± 2.28[d]
Tween 60	25.8 ± 0.98	27.6 ± 0.90	28.5 ± 1.32	27.8 ± 1.05	29.9 ± 1.6[b]
Myrj 45 (120 rats)	26.1 ± 0.89	25.7 ± 1.07	28.3 ± 2.28	26.8 ± 0.90	31.1 ± 1.35
Myrj 45 (950 rats)	26.8 ± 0.55	27.6 ± 0.48	30.0 ± 0.85	29.0 ± 0.71[c]	35.6 ± 0.85[d]

[a] Standard error. [b] $p < 0.05$. [c] $p < 0.01$. [d] $p < 0.001$.

in weight was highly significant for Myverol, Span 60, and Tween 60, and the large group of Myrj 45 animals. Also in this Myrj 45 experiment liver weight was significant at both 10% and 5% dietary levels. Likewise in the second test with Myrj 45 there was a slight but significant increase in the weight of spleens of male rats at the 25% level and a decrease in kidney weight of females at the same level. Span 60 at the 25% level produced a significant increase in kidney weights. Cecal enlargement and enlargement of the common bile duct occurred infrequently. With Tween 60 cecal enlargement was moderate at the 25% level and to a lesser degree at the 10% level. The increased size of the cecum was obvious at autopsy and the weight of the wall also proved to be greater for the test animals (mean of four representative ceca at the 25% level was 5.29 g) than for the controls (mean 3.59 g).

Pathology. The only gross change apart from liver enlargement in males on 25% Myrj 52 attributable to treatment with the compound was cecal distention, which ranged from slight to moderate at 25% down to very slight at 5%, and was absent in the 2% and control groups. The capacity of a few measured ceca varied from 3.5 to 6 cc (normal) to 19.5 cc (moderate to marked distention).

In the first experiment, bladder stones and tumors were found in two males fed 25% Myrj 45, and one other male at this level had stones without tumor in the bladder. A concretion, found in the distended bladder of one male fed 5% Myrj 45, was shown to consist of amorphous dense protein material, and those in the bladders of male rats at the 25% level proved on analysis to be composed of calcium oxalate. There were no bladder stones or tumors in any of the control rats nor in rats fed other stearate emulsifiers.

It was estimated that a study using 200 animals at each dosage level would show more accurately the level, or levels, in which the effect of bladder stones and tumors would be demonstrated. In this second test bladder stones and tumors were found only at the 25% level, and there were none in the controls. Twenty-five of the 150 animals had bladder stones (calcium oxalate) and of these, eleven also had bladder tumors. There were no instances of bladder tumors in which stones were not present also. All except one rat with bladder stones were males; the one female with a single stone had no bladder tumor.

Grouping together the bladder stones and bladder tumors found in both tests with Myrj 45 permits the following summarization. Stone formation was noted after 37 weeks of the experimental period. All but 5 of the 28 instances of stone were multiple. The largest number of stones was over 40, and the largest individual stone was 7 × 7 × 5 mm; the average number per bladder was around a half-dozen ranging from 1 to 3 or 4 mm diameter. Several of these bladders were markedly distended and some were hemorrhagic. In each instance included there was at least a 1-mm diameter stone. Among the 13 rats with bladder tumors there were several with more than one bladder tumor mass; among the individual animals the sizes of the sole or largest tumor mass ranged from 3 × 2 × 1 mm to 2.8 × 2.5 × 1.9 cm. The predominant location of the tumors was in the fundic area. One tumor had extended outside the bladder wall.

Of the 504 rats started on the original experiments, 423 were sent to the pathology laboratory, the remaining ones usually being discarded

(after a brief examination) because of pronounced postmortem autolysis. Of the 423 received, 339 were sectioned microscopically. In 177 instances, the microscopic sectioning was detailed and included lung, heart, liver, spleen, pancreas, stomach, small intestine, colon, kidney, adrenal, thyroid, leg muscles, leg bones with included marrow, testis (or uterus and ovary), and any tumors or other unusual conditions; parathyroid, prostate, and urinary bladder were often included. In the remaining 162 instances the sectioning was usually limited to liver, kidney, tumors, other unusual conditions, and in males the testis. Hematoxylin-eosin-stained paraffin sections of formalin-fixed tissues were routine. Of the 950 rats started on the second test of Myrj 45, only those 301 judged abnormal at autopsy were sent to the pathology laboratory for further examination. Microscopic examination in this special experiment was limited to 175 20μ blocks of tissue and included unusual tumors, common tumors (chiefly mammary) when the gross diagnosis was in doubt, and other unusual conditions.

Microscopic pathologic changes which were not equally present in the control groups and which could therefore be attributed to the emulsifiers were as follows:

Hepatic cell vacuolation interpreted as fatty change was slightly greater in the 25% group of Span 60 rats than at lower dosage levels or in controls (average slight as against very slight degree). With Tween 60 this increased hepatic cell vacuolation at 25% was less—not enough to be a distinct difference. With both compounds the enlarged ceca were normal microscopically.

Only one microscopic change could be attributed to Myverol, that being an increase in the number of calcified renal tubular casts or tubular segments over the small amount present in the controls. These casts (or segments) were located around the corticomedullary junction.

With Myrj 52, sections of the distended ceca were negative. The only microscopic effect attributable to treatment was the minor one of an approximate doubling in the two high dosage groups of the very small amount of focal adenomatoid hepatic cell hyperplasia present at the lower levels and in the controls.

With Myrj 45, the thirteen bladder tumors were all epithelial, and diagnosed as definite carcinoma (distinctly invasive) in two, early carcinoma (beginning invasion) in two, papilloma in eight, and "early tumor" in one. A minor microscopic effect attributable to Myrj 45 was a slightly

increased amount of hepatic cell vacuolation at 25% (of the type already mentioned for Span 60), the increase becoming less to absent as dosage was decreased.

In view of the unusually large number of rats per group in the second two-year Myrj 45 experiment, and of the careful study which is given the spontaneous tumors in our rats, a combined listing (Table 4) of the various types of these tumors in both experiments is given.

TABLE 4

BASIC DATA ON SURVIVAL AND TUMORS IN RATS FED MYRJ 45 FOR TWO YEARS

Percent of Myrj in diet:	0	2	5	10	25
Number of rats started in experiment	224	224	224	224	174
Number of males alive at end of first year	110	108	107	105	76
Number of females alive at end of first year	108	107	108	110	82
Total rats alive at end of first year	218	215	215	215	158
Number of males alive at end of experiment	63	50	56	44	33
Number of females alive at end of experiment	63	62	55	55	33
Total rats alive at end of experiment	126	112	111	99	66
Number of survivors without tumor	68	80	70	66	46
Number of nonsurvivors without tumor	82	95	91	107	91
Number of survivors with tumor	30	32	41	33	20
Number of nonsurvivors with tumor	19	17	22	18	18
Number of rats with tumors	49	49	63	51	38
Number of males with tumors	6	14	16	7	18
With bladder tumors only	0	0	0	0	12
With bladder and other tumor type(s)	0	0	0	0	1
With other tumor type(s)	6	14	16	7	5
Number of females with tumors	43	35	47	44	20
With mammary tumors only	36	28	35	32	10
With mammary and other tumor type(s)	4	2	4	5	3
With other tumor type(s)	3	5	8	9	7
Number of animals with tumors other than of mammary glands and urinary bladder	15	21	28	19	16
Number with more than 1 type of such tumor	2	2	3	1	2
Total number (by type) of such tumors	15	23	32	20	18
Pulmonary lymphosarcoma	7	8	7	7	5
Abdominal lymphosarcoma	1	2	5	3	2
Genital tract tumors	2	2	5	4	6
Subcutaneous connective tissue tumors	2	4	7	4	1
Liver tumors	0	1	3	0	1
Kidney tumors	1	1	2	1	0
Miscellaneous tumors	2	5	3	1	3

Table 4 shows the distribution of tumors among rats fed Myrj 45 for two years. In the male rats, urinary bladder tumors were outstanding because of their limitation to the 25% dietary level and their constant association with bladder stones. In the female rats, mammary tumors were much more frequent than other types, both in the controls and at every diet level. Among the diet levels, the only significant variation from the controls in the number of mammary tumors was a reduced incidence in the group fed 25%. At the 5% feeding level, the average total weight of mammary tumor tissue per rat (data not shown in the table) was over one and one half times as great as in the controls. The remaining major category, namely tumors of types which develop in both male and female, likewise is given. At the 5% feeding level, the number of this category is increased over that in the controls. There also was a general increase among some tumor types with the feeding of the test substance.

In regard to the effect of tumors on mortality it can be seen in Table 4 that the number of survivals without tumors decreased with each increase in dosage level of Myrj 45. These decreases are significant in each case except at the 2% level. There was no significance in the relationship of the number of survivors with tumors and of the dosage level of Myrj 45.

To supplement the information in Table 4 an analysis to determine the proportions of rats at each diet level which developed tumors, other than mammary and bladder, was made. Among female rats there was a regular increase in the proportion of animals which developed tumors as the diet level of Myrj increased, although the difference from the control was not demonstrated to be statistically significant. In contrast, the male rats fed Myrj 45 at both the 2% and at 5% dietary levels showed statistically significant increases over the control group in the proportion which developed tumors. In making the analysis just described, a life table technique was used (Sachs, 1959). In deriving the estimates of the incidence of tumors by this method adjustment is made for the number and time of occurrence of deaths within each treatment group. It is believed that this procedure therefore provides a truer estimate of the incidence of tumors than is given by simply dividing the number of animals which developed tumors, by those started in the experiment.

Dogs

One dog fed Myrj 45 was sacrificed 4 months before the termination of the experiment because of severe mange and general poor condition.

TABLE 5

WEIGHT AND FOOD INTAKE OF DOGS FED 5% MYRJ 45 AND 5% SPAN 60 IN A SEMISYNTHETIC DIET

Emulsifier	Dog no.	Sex	Age at earlier (months)	Time on experiment (months)	Food intake (g/kg/day)	Emulsifier intake (g/kg/day)	Weight in kilograms: Start	High	Finish
Myrj 45	1	M	48	21	20.1	1.09	7.3	8.5	8.0
				15		1.0	8.3	9.2	7.9
Myrj 45	2	F	56	21	13.9	1.09	5.8	7.5	6.0
				19		0.698	6.1	10.3	9.6
Myrj 45	3	M	35	19	12.7	0.664	19.0	20.2	17.8
Myrj 45	4	F	33	16	11.5	0.574	13.2	15.6	15.6
Span 60	5	M	36	20	13.0	0.648	17.0	17.0	16.8
Span 60	6	F	55	20	15.2	0.783	11.1	12.3	10.4
Span 60	7	M	35	20	12.7	0.636	13.4	15.3	14.7
Span 60	8	F	96	20	9.7	0.485	13.1	16.2	15.3
Control	9	M	35	19	15.1		14.3	14.3	14.3
Control	10	F	95	19	12.9		10.5	11.4	10.3
Control	11	M	34	19	14.9		12.1	14.4	13.3
Control	12	F	82	19	12.4		13.3	17.1	15.5

[a] Given in capsules; then changed to diet.

All others appeared normal and maintained body weight until the end of the experiment at which time they were sacrificed by chloroforming. The average food intake and emulsifier intake for each dog is shown in Table 5. Except for dog no. 2 whose food intake to body weight ratio is high and dog no. 8 in which the ratio is low, there is no appreciable difference between control dogs and those fed Myrj 45 or Span 60 in regard to food intake, weight maintenance, and longevity.

At autopsy the dogs showed nothing attributable to the feeding of either Myrj 45 or Span 60. The only major abnormalities seen were mange in three dogs (one severe and one slight with Myrj 45, and one of moderate degree with Span 60), excessive abdominal fat in a female on Myrj 45, and cataract (present four years) in a control female. In addition, there were a number of minor changes, but they were no more frequent than would be expected in a group of twelve dogs of this age, and there was no difference among the groups that could be attributed to either substance. The hearts and the completely opened intestines of these dogs were free from worms.

From each of the twelve dogs hematoxylin-eosin-stained paraffin sections of formalin-fixed tissue were made of lung, heart, liver, gall bladder, spleen, pancreas, stomach (fundic and pyloric), small intestine (three levels), colon, cervical and mesenteric lymph nodes, kidney, adrenal, thyroid, parathyroid, urinary bladder, testis (or ovary), prostate (or uterus), submaxillary salivary gland, thigh muscle, rib bone and bone marrow. Also, similar sections of Zenker-fixed tissue were made from liver, kidney, and adrenal of each dog, frozen sections of the same three organs from each dog were stained with Oil Red O, and a smear of rib bone marrow from each dog was stained with Wright-Giemsa stain. From the eight Myrj and control dogs the liver, kidney, and adrenal were additionally treated by the acid-ferrocyanide and, except one, the periodic acid-Schiff reactions, and routine sections were made of brain (four levels) and hypophysis. Skin was sectioned when indicated.

This detailed microscopic examination showed nothing attributable to the feeding of Span 60, and only a very slight and dubious effect on the liver from the feeding of Myrj 45. The liver from three of the four control dogs, and from three of the four dogs on Span 60, showed hemosiderosis varying from slight to moderate degree, with the pigment almost entirely in Kupffer cells and macrophages. Since such hemosiderosis is not seen in our dogs on the usual laboratory chow diet, it is attributed

in this experiment to the semisynthetic diet used. In the Myrj 45 dogs, the hemosiderosis was distinctly less, none showing more than a very slight degree. Also, the livers of two Myrj 45 dogs were the only ones of the twelve with hepatic cells which were slightly enlarged and (H-E stain) rarefied; with the periodic acid-Schiff reaction they were equally filled with reacting material. Taken together, these two minor differences, though they could be due to chance, do suggest a very slight effect on the liver. As to stainable fat in the liver, little difference between groups could be observed, since all livers showed from none to only small amounts except for two dogs with slightly larger amounts. One of these was the control dog with cataract while the other was the Myrj 45 dog with excess abdominal fat.

DISCUSSION

Some of the gross and microscopic pathologic effects described above, as well as effects on growth and mortality, may be due to nutritional inadequacy or to dietary imbalance when emulsifiers with nonutilizable polyol portions are fed at high levels in the diet, rather than to toxicity inherent in the compounds tested. Other changes may be due to a direct toxic effect of the compounds. Of particular concern is the significant finding of bladder stones and bladder tumors resulting from the feeding of Myrj 45. As all instances of bladder tumors in this study were accompanied by stones, and as there were bladder stones without tumors, it would seem that bladder tumor formation was secondary to bladder stones, presumably from mechanical irritation. This same situation occurred with the chronic feeding of diethylene glycol to rats (Fitzhugh and Nelson, 1946). It is not possible to say whether the 0.1% of diethylene glycol in Myrj 45 (Food Protection Committee, 1953) was responsible for the formation of the stones.

The increase in number of tumors other than of the urinary bladder in male rats at 2% and 5% Myrj 45 and the increase in total mammary tumor tissue in females at 5% indicate an effect on tumor production; however, a distinct effect is not shown convincingly in the experiment as a whole. The observed effects should not, in our opinion, label Myrj 45 as a weak carcinogen, but rather, in view of the known lability of rodent tumor production under various influences, should be considered a by-product of some effect on metabolism. Examples of such influences in tumor production are increase of certain fats in the diet or decrease with

caloric restriction. The decreased percentage of mammary tumors at the 25% level would also be in this category.

SUMMARY

Four emulsifiers were fed in concentrations ranging from 2% to 25% in the diets of rats for two years; two of these were also fed to dogs for 19 to 26 months at 5% of the diet.

Deleterious effects, largely limited to the higher levels of feeding, included increased liver weight with all substances at 25% except Myrj 52, plus the following for each substance.

Span 80 caused significant increase in mortality of rats at the 10 and 25% levels, growth depression and kidney enlargement at 25%, two instances of enlargement of the common bile duct at the 25% level, and slight fatty changes in some livers. The feeding of 5% had no effect on dogs.

Myrj 52 caused growth depression among male rats fed at 25% dietary concentration, diarrhea ranging from marked to slight in the 25% level dogs, and very slight to moderate enlargement of the rectum at the three higher levels, and very slight focal adenomatoid hyperplasia of the liver at 10% and 25%.

Tween 60 produced marked diarrhea at 10% level and less at 1% and 2% which was slight to moderate at the 25% level and less at 10%, and questionable fatty changes of a very slight degree in the livers of rats fed at 25%.

Myrj 45 produced bladder stones in about one-third of the 25% male rats (27 of 87) with associated bladder tumors in nearly half of these (13 of 27). One female also had bladder stones. Bladder stones did not occur below the 25% feeding level. The incidence of spontaneous tumors was affected in male rats at the 2% and 5% levels. Livers were significantly heavier at 5% and 10% and slight fatty changes occurred in some livers, the numbers increasing with dosage. Dogs exhibited no distinct effect due to the feeding of 5% Myrj 45.

Myverol 18-00 used as a control at 25% (the only level tested) caused a mild degree of renal tubular calcification.

ACKNOWLEDGMENT

We are indebted to Miss Rose Sachs of the Bureau of Program Planning and Appraisal for the statistical evaluation of tumor incidence by a life table technique.

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The Fate of Dietary Fat in the Body

Seventh Leverhulme Memorial Lecture

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(Paper presented to the Liverpool Section of the Society of Chemical Industry in Liverpool on April 6)

JUST over 110 years ago William Hesketh Lever was born. I did not have the privilege of knowing him, as I was still at school at the time of his death in 1925. Even at that time, however, like many other schoolboys, I knew something of what he had achieved at Port Sunlight and since then I have been fortunate enough to have had a closer association with the remarkable organisation that he founded. Horatio Ballantyne, in the first of these memorial lectures, quoted Emerson's aphorism "An institution is the lengthened shadow of one man". The present Unilever organisation, which has ramifications all over the globe, is a fitting memorial to a great industrial pioneer.

The first lecturer[1] dealt with the life and work of the first Lord Leverhulme, and subsequent lecturers[2-6] have taken as their theme some relevant aspect of chemistry or research. Two of these have dealt with fats, Professor Hilditch in the Third Lecture,[3] who discussed the historical development of the study of fat chemistry, and Sir Harold Tempany in the Fourth Lecture,[4] who spoke on "The tropics as sources of vegetable oils and fats". When I was invited by the President of the Society of Chemical Industry to give this seventh memorial lecture, which is an honour that I warmly appreciate, it seemed to me to be appropriate that I should give a further lecture on fats, since this was one of the major fields pioneered by the man whose name we commemorate today. While it is true that the first emphasis was on soap, an even greater contribution has been made since to the supply of fats for human consumption. It may, therefore, seem reasonable to enquire: what happens to these fats in the body after they are eaten? What effects different dietary fats may have on the body? What the important properties of a food fat may be from the health point of view? I hope in the course of this Seventh Memorial Lecture to attempt to answer some of these questions and to indicate where further research may seem to be desirable.

The Influence of the Properties of Dietary Fatty Acids on their Overall Absorption

The overall absorption of fats from the intestinal tract can only be assessed by the measurement of dietary fat intake and output of fat in the stools. Since a significant proportion of the fat in the stools may be endogenous, accurate assessment of absorption requires the use of a suitably labelled fat. Labelling of the fatty acids in a dietary fat with ^{131}I, ^{14}C or ^{13}C is commonly used; other methods involve the use of unusual fatty acids, such as erucic or elaidic acid. However, the iodine labelling may be insecure under certain circumstances, carbon labelling involves the choice of a particular fatty acid, which may complicate interpretation, and abnormal may be handled differently from normal fatty acids; thus, none of the methods is ideal and further work on fat labelling methods is needed. Present evidence indicates that 95-99% of the common dietary fatty acids are absorbed from a mixture of liquid fats. A number of the characteristics of the fatty acids fed appear to affect their overall absorption if they are fed individually.

Chain Length

It has been known for some years that the chain length of a fatty acid may affect its digestibility (Table I). Thus, long-chain fatty acids, such as palmitic and stearic acid, are poorly absorbed as compared with shorter-chain compounds. However, the addition of a liquid oil vehicle to the long-chain saturated fatty acid causes a marked improvement in absorption. This effect of chain length has been attributed to differences in melting point. As the melting point of the fatty acid rises well above body temperature (37° C) so the assimilability of the fatty acid falls. It has been thought that the long-chain saturated fatty acid of high melting point might come out of solution in the oil vehicle: stools containing an excess of fat often exhibit crystals of fatty acid, or perhaps more commonly of calcium soaps, which give a peculiar sheen to the surface of the stool. However, there are several difficulties about this interpretation. Thus,

Table I

Relationship between melting point and digestibility.
(Data adapted from Deuel's The Lipids.[7])

	Melting Pt. °C	*Digestibility coefficient* Rat	Guinea pig
Myristic acid	53	82	—
Palmitic acid	63	36	—
Stearic acid	69	16	—
Oleic acid	14	95	95
Linolic acid	51	96	56

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elaidic acid is equally as well absorbed as oleic acid in the rat, in spite of the difference in melting point, whereas there is the expected depression of absorption of elaidic acid in the guinea pig. A recent observation by Sammons and Gompertz[8] has thrown some further doubt on the simple explanation that the effect of chain length is due to difference in melting point. If erucic acid (melting point 33·5 °C), a monoethenoid fatty acid with a chain containing 22 carbons, is fed there may be no increase in the total amount of fat in the stools, but the proportion of erucic acid in the stool fat is significantly higher than the proportion in the fat fed. This suggests that there is selective rejection of this long-chain fatty acid, in spite of the fact that it is liquid at body temperature. It would seem, therefore, that chain length affects overall absorption, shorter-chain compounds being more completely absorbed. The high melting point of some of these long-chain fatty acids may be a factor in their relatively poor absorption, but it does not appear to be the whole story.

Degree of saturation

It can be demonstrated that unsaturated fats tend to be more readily and completely absorbed than more saturated compounds. The percentage absorption of an unsaturated fatty acid can be progressively reduced by hydrogenation. (Table II). This difference in assimilability of saturated and unsaturated fats has also been shown by van de Kamer and Weijers[9] in patients with defective fat absorption, in whom the stool fat can be shown to consist more of saturated than unsaturated fatty acids. When the steatorrhoea is aggravated in these patients for any reason, the saturated fatty acids show a much greater increase in the stools than the unsaturated group. The administration of more unsaturated fatty acids to a patient with defective fat absorption may cause a reduction in the amount of fat in the stools.

Table II

Effect of hydrogenation of cotton seed oil on digestibility.
(Data adapted from Deuel's 'The Lipids'[7]).

	Melting Pt. °C	*Digestibility coefficient in the rat* %
Cotton seed oil	38	91
	46	84
Hydrogenated cotton seed oil	53	69
	62	38
	63	24

These observations must be considered in relation to the peculiar properties of stool fats in normal individuals. The fat in the stools, both in the normal subject and in patients with defective fat absorption, contains a high proportion of stearic acid: this may amount to 30-40% of the fat present.[10] This is a higher proportion of stearic acid than is observed in any other naturally occurring animal fat. The source of this stearic acid is not yet satisfactorily explained. It may be partly due to the selective rejection of long-chain saturated fatty acid; it may also be partly accounted for by hydrogenation of unsaturated C18 fatty acids in the intestinal lumen; it has been suggested, but never adequately proved, that fats may be excreted from the intestine, finally fat synthesis might occur in micro-organisms, but such bacterial or yeast fats do not usually have such a high stearic acid content. So far as the influence of dietary fat on stool fat is concerned, the most important question is whether any significant effect is produced by the ingestion of increased amounts of more saturated long-chain fats. This may well have some effect, especially in patients with defective fat absorption. It is a point that might repay farther study.

Configuration

Unsaturated fatty acids may occur in either *cis* or *trans* configurations. The *trans* acids have higher melting points and may be expected to behave rather

similarly to the more saturated fatty acids. This has already been noted with reference to elaidic acid in the guinea pig, but not in the rat (Table I). However, Shorland *et al.*[11] have shown that there is no special difficulty in the absorption of *trans* acids; they pass into the body, may be deposited in fat depots, or excreted in the milk.

Substituted fatty acids

If hydroxy-fatty acids form the greater part of the fatty acid in a glyceride, as, for example, in castor oil, absorption may be affected. Castor oil is not well absorbed, mainly due to interference with fine dispersion. If a finely dispersible vehicle is added to the castor oil, so that the mixture is finely emulsified, the castor oil is well absorbed. Incidentally, it also loses its cathartic properties.[12] James and his colleagues[13] have demonstrated that hydroxy-fatty acids are formed in the intestinal lumen by the action of the intestinal flora. Such changes probably occur mainly in the large intestine, so that no significant effect on absorption would be expected.

Products of fat oxidation

Food fats may undergo oxidative changes with the formation of hydroperoxides, secondary oxidation products and polymers. Hydroperoxides, hydroxy-fatty acids and other products may be absorbed; details of their distribution and fate are not yet fully described. Polymerisation results in a decrease of assimilability. The possible biological effects of the ingestion of fat oxidation products are of considerable interest, and are being studied at the present time by several groups of investigators.

The Effect of the Composition of Dietary Fats on Digestion and the Mechanism of Intestinal Absorption

Dietary fat undergoes partial hydrolysis, and fine dispersion in the intestinal lumen. The fat is then taken up into the absorbing columnar cells on the surface of the small intestinal villi. The possible effect of differences in composition of the dietary fat on these various steps in the digestive and absorptive process must now be considered.

Pancreatic lipolysis

Pancreatic lipase does not rapidly and completely hydrolyse dietary glyceride to fatty acid and glycerol, as was at one time thought. The end-products are di- and mono-glycerides, fatty acids and relatively little free glycerol.[14] Desnuelle *et al.*[15] have shown that pancreatic lipase has a selective action on the fatty acids in a triglyceride, the fatty acids in the 1 and 3 position being preferentially liberated. It is apparent, therefore, that the composition of the dietary glyceride may affect the fatty acids set free and the nature of the residual monoglyceride left. Whether this has any metabolic significance is not known.

Fine dispersion

It is simple to demonstrate the fine dispersion of dietary fat in the small intestinal lumen by withdrawing a sample of intestinal contents by intubation. It can also be shown that the only emulsifying system present in the small intestinal lumen which is effective under the conditions that prevail is the triple combination of lower glycerides, fatty acids and bile salts.[16] More recently, Borgström[17] has shown that this same triple complex of monoglyceride, fatty acid and bile salt will, under suitable conditions, form micelles. Mono-olein has been shown to be particularly effective in micelle formation, but this may only be true under the peculiar circumstances studied *in vitro*. It is not yet certain how extensive micelle formation may be *in vivo*; it must be emphasised that the intestine during fat absorption contains a mass of glycerides and fatty acids in varying stages of dispersion. It seems likely that the particulate fat ranges from large fat globules down to extremely small emulsion particles and micelles. The precise point at which the particulate material becomes absorbable is not yet known. So far as present knowledge goes, all ordinary dietary fats are readily dispersed in the small intestinal lumen by partial hydrolysis in the presence of bile salts. As already mentioned, certain glycerides not commonly regarded as dietary fats, such as castor oil, are an exception.

Entry of fat into the intestinal cells

The absorbing cells in the small intestine have a characteristic "brush border". Study with the electron microscope has revealed that this consisted of many microvilli.[18] Particulate fat appeared to pass down between these microvilli and to enter the cell at the bottom of the inter-microvillous crypts.[19] It is possible, but not proven, that fatty material in molecular dispersion or in other invisible forms may enter the surface of the microvillus. If there are these two pathways, it is apparent that the composition of the dietary fat and possibly the position of the fatty acids in the glyceride may determine the pathway used. It is possible that the effect of chain length on absorption may be related to complex formation which might be a factor affecting the pathway of entry into the cell. In gluten-induced enteropathy, one of the more common forms of the malabsorption syndrome,[20] the microvilli of the intestinal cells may be markedly abnormal[21]; this may be one of the factors that cause the fat absorption defect usually observed in these patients. Much more information on the site of entry of fatty materials into the intestinal cell is needed before any final conclusions can be reached.

Changes in fat during absorption

The general nature of the changes that fats undergo during absorption can be clearly defined from consideration of the differences between fat in the lumen and fat in the chyle[22] (Table III). It is apparent that triglycerides are reformed and that long-chain fatty acids, with more than 10 carbons in the chain, are selected for this esterification. The shorter-chain fatty acids do not re-enter glycerides, nor is liberated glycerol re-utilised in the intestinal cell for glyceride synthesis to any appreciable extent. Fatty acids are also taken up into phosphatides

Table III

Differences between fat emulsion in the intestinal lumen and fat particles in the chyle

	Intestinal Lumen	*Chyle*
Composition	Triglycerides Diglycerides Monoglycerides	Triglycerides (containing long-chain fatty acids)
	Short-chain fatty acids	Phosphatidyl choline
	Long-chain fatty acids	Cholesterol esters
Surface film	Lower glyceride-fatty acid-bile salt complex	Lipo-protein
Behaviour with plasma protein at pH 7.4	Flocculation	No flocculation
Effect of phospholipase C	No effect	Flocculation and creaming

especially phosphatidyl choline, and into cholesterol esters. For the latter there is preferential selection of more unsaturated fatty acids. Two pathways for glyceride synthesis have been demonstrated in the intestinal cell. The pathway first described by Kennedy[23] involves phosphatidic acid as an intermediate; the other, more recently defined by my colleagues, Hübscher and Clark,[24] allows direct incorporation of fatty acids into lower glycerides. Since dietary fat is the main source of fatty acids in the intestinal cell, the composition of the dietary glyceride will affect the glycerides resynthesised. Further study of these pathways of resynthesis is necessary, especially a definition of the factors that determine which pathway is used during absorption.

Fat in the Chyle and in the Blood

The nature of fat in the chyle and in the blood

After a fatty meal the chyle and the plasma becomes loaded with fat; it consequently becomes lactescent. The main increase is in the glyceride fraction, although there is also a much smaller concomitant increase of cholesterol, phosphatide and fatty acid. The fat is intimately associated with protein. These lipo-protein complexes can be studied in various ways and can be divided into more or less arbitrary groups, such as by the ultracentrifugation techniques of Gofman.[25] After a fatty meal the main increase is in the lower density lipo-proteins (Sf 12–400, Density < 1·0019) and chylomicrons (Sf >400, Density < 1·0006). The composition of the glycerides in these lipo-proteins during absorption closely resembles those in the fat fed.

The particles of fat in the chyle and in the blood have been shown to have a different structure from the particles seen in the intestinal lumen (Table III). Whereas the particles in the intestinal lumen were negatively-charged particles flocculating in the presence of plasma protein at pH 7·4, the particles in the chyle had a surface film of protein and remained discrete in blood plasma at pH 7·4. Furthermore, phospholipid, mainly phosphatidyl choline, had become an essential part of the stabilising system. Destruction of the phosphatidyl choline by phospholipase D caused immediate clumping and coalescence of the fat particles in the chyle or in the blood.[26] This lipo-protein stabilising film of the fat particles in the blood may be an important factor in their subsequent metabolism.

There is no information on the effect of dietary fats on this stabilising surface film, but it has been shown that abnormalities may be present under certain circumstances. This has been observed occasionally in human subjects and it has also been induced in choline deficient rats.[27] The particles under these circumstances were found to be relatively resistant to phospholipase D and in the case of the human material studied a different protein fraction seemed to be involved. The possible significance of such changes in the stabilising surface film of the chylomicron will be further discussed later.

Alimentary hyperlipidaemia

The increase of particulate fat in the blood following a fatty meal usually reaches a peak in about 3–3½ hr. If large amounts of fat are ingested this may be delayed due to interference with gastric emptying time. With the moderate meal, giving a peak at 3–3½ hr., the blood fat levels will return to normal within 5–6 hrs. The level to which the blood fat rises is a function of the relative rates of transport of fat into the blood and removal of fat from it. A number of factors have been shown to affect the extent and duration of the alimentary hyperlipidaemia. Those that are of most interest in this discussion are differences between species, differences related to the nature of the fat ingested and effects of ageing. Man and dog tend to have a marked increase of blood glycerides after a fatty meal, the rat shows much less change and the guinea pig practically none. Unsaturated fats caused a hyperlipidaemia of shorter duration than more saturated fats.[28] In aged subjects the hyperlipidaemia was said to be of longer duration.[29] Administration of pancreatic lipase with the ingested fat reduced the amplitude of the hyperlipidaemia,[30] possibly due in part to a change in the rate of absorption. The hyperlipidaemia can also be decreased in amplitude by the administration of heparin and increased by protamine.[31] It is possible that the amplitude and duration of hyperlipidaemia may be of some significance in relation to deposition of lipid materials in blood vessel walls, further study of this field may yield more information of interest.

Factors concerned in the clearing of alimentary hyperlipidaemia

Hahn[32] accidentally observed that the administration of heparin hastened the clearing of alimentary hyperlipidaemia. The blood plasma from an animal treated with heparin will clear hyperlipidaemic blood *in vitro* under suitable circumstances. French, Robinson and Florey,[33] and Brown *et al.*[34] showed that this was due to the presence of a lipase in the blood plasma following heparin administration and that this lipase acted on the chylomicrons liberating fatty acids. One of the conditions necessary for effective *in vitro* clearing was the presence of an adequate fatty acid acceptor. Plasma albumin

readily fulfilled this function. The fatty acid-albumin complex is usually referred to as unesterified fatty acid (UFA), or better as non-esterified fatty acid (NEFA). Lipoprotein lipase has been demonstrated in the blood stream during fat absorption.[32-37] It is consequently tempting to conclude that hydrolysis of the blood glyceride is a necessary step in its removal. However, the lipoprotein lipase is not invariably present and it is difficult to account for all the fat handled on the basis of albumin-fatty acid complex turnover; furthermore, positive evidence exists that glycerides may penetrate into cells unhydrolysed. As in the intestinal lumen, therefore, hydrolysis appears to be partial rather than complete and glycerides as well as fatty acids are transported through membranes into cells.

However, there is evidence that lipo-protein lipase probably plays an important part in clearing alimentary hyperlipidaemia. Thannhauser *et al.*[38] showed that the alimentary hyperlipidaemia was cleared extremely slowly in some people. Consequently the blood fat from each meal added to the residual fat from previous meals, so that a cumulation of glycerides occurred in the blood. The blood fat level might reach 10 g./100 ml., or more. If such a patient was placed on a low fat diet, the blood glyceride level fell. This condition was called essential hyperlipidaemia and it seemed to be genetically determined. In one family with this inborn error of metabolism, Havel[39] has shown a deficiency of lipo-protein lipase in all members affected. However, a deficiency of lipo-protein lipase has not been demonstrated in other patients who appear to be suffering from the same disorder.

Theoretically, delayed removal of fat particles might also arise in the presence of lipo-protein lipase if the particle structure was abnormal. This possibility is supported by Korn's observation[40] that lipo-protein lipase acted much more slowly on a fat emulsion stabilised with soap than on the same fat emulsified with lipo-protein. Thus, the nature of the stabilising film was an important factor determining effective removal of the particle. In the family with essential hyperlipidaemia referred to above, the particle structure appeared to be normal and the particles were cleared at a normal rate by lipo-protein lipase obtained from other people. The production of abnormal particles which were relatively resistant to the action of phospholipase D in choline-deficient animals has already been mentioned.[2] These particles were also more slowly cleared by lipo-protein lipase. Mitchell[41] has shown that the fat particles reaching the blood at different times during the hyperlipidaemia may be cleared at different rates; those occurring early were cleared faster than those obtained later. Little is known about the effects of different dietary fats on the rate of clearing of alimentary hyperlipidaemia. At present, the matter must be left open, but more information is clearly desirable. A number of other dietary factors may affect the rate of clearance of the alimentary hyperlipidaemia and these in turn might materially affect the distribution and fate of the absorbed fat. It is possible that the effect of choline deficiency on the structure of the fat particle may be one of the factors responsible for the deposition of fat in the liver in such animals; it is certainly dietary fat that is deposited. It must also be noted that choline deficiency may interfere with the handling of fat by the intestinal and the liver cells.

The Effect of Dietary Fat on the Resting Blood Lipids

When the alimentary hyperlipidaemia has been cleared the blood fats return to a resting level. The amount of glyceride is usually low, but there are substantial quantities of phospholipids and cholesterol. The level of these blood lipids, especially cholesterol and β-lipoproteins, is affected by several factors, notably age, sex, genetic pattern and diet. With advancing age the level of cholesterol and β-lipoproteins in the blood increases. The levels in women are less in middle life, but progressive increase occurs after the menopause. Hypercholesterolaemia occurs as a genetically determined metabolic characteristic. The abnormality is a common one and genetic factors may play an important part in determining blood cholesterol levels. Superimposed on these inborn characteristics is an effect of diet. It was at one time thought that dietary cholesterol and overall fat intake were the important factors. However, it is now known that the dietary factor of greatest importance is the proportion of the fat ingested as polyunsaturated fatty acid. This tends to be high in many vegetable fats and low in a number of common animal fats, but these relationships are by no means invariable; thus coconut oil has a low unsaturated fatty acid content and pig fat may contain large amounts of linoleic acid. The effect of the poly-unsaturated fatty acids on blood cholesterol and β-lipoprotein levels has been elegantly demonstrated by Ahrens and his colleagues,[42] by Bronte Stewart *et al.*,[43] and many other investigators. The differences in blood cholesterol levels and possibly related cardiac disease in various racial groups so ably demonstrated by Keys[44] are due to dietary, rather than genetic differences.

Fats in the Portal Blood

The discussion so far has centred round fats in the systemic blood. These reach the blood stream as an intravenous injection into the great veins from the thoracic duct. This fatty material consequently short-circuits the liver first time round. The main factor that seems to determine the passage of particulate fat by the lymphatic route may be a peculiar property of the walls of lymphatic vessels, which unlike blood capillaries, do not seem to act as a barrier to negatively charged particulate material; negatively charged dyes also enter lymphatic vessels rather than blood capillaries. However, some fatty material passes into the blood capillaries in the villi and is transported in the portal vein direct to the liver. The fatty materials that have been proved by Chaikoff and his group[45] to pass by this route are the short-chain fatty acids (less than 10 carbons). The precise form in which they are transported is not known. The nature of the dietary fat will clearly determine this partition. About 10% by weight of the fat in cow's milk consists of short-chain fatty acids.

Biosynthesis and Interconversions of Fatty Acids

Unsaturated fatty acids

It is convenient at this stage to give some further consideration to the unsaturated fatty acids in the diet. One of the major dietary components is the monoethenoid fatty acid, oleic acid. There is, however, a number of other unsaturated fatty acids, monoethenoid, diethenoid and polyethenoid. Certain families of unsaturated fatty acids have been described by Mead[46] and these are grouped according to their interconversions in the animal body. Since this profoundly affects their metabolic functions, some consideration will be given to the three main families; that of oleic, that of linoleic and that of linolenic acid. All undergo essentially the same type of modifications which, therefore, result in different end-products. In each case the introduction of new double bonds proceeds between the existing double bonds and the carboxyl group. These double bonds are introduced in such a way that each pair of double bonded carbons is separated by a CH_2 group, as illustrated in the Figure. When the residual length of the carbon chain

Formation of Polyunsaturated Fatty Acids

R = Me·$(CH_2)_7$·INOLEIC ACID.
R = Me·$(CH_2)_4$·CH:CH·CH_2·INOLINOLEIC ACID.

R·CH:CH·$(CH_2)_7$·COOH (I)
R·CH:CH·CH_2·CH:CH·$(CH_2)_4$·COOH Me·COOH (II)
R·CH:CH·CH_2·CH:CH·$(CH_2)_6$·COOH (III)
R·CH:CH·CH_2·CH:CH·CH_2·CH:CH·$(CH_2)_3$·COOH (IV)

Figure

Formation of polyunsaturated fatty acids from oleic and linoleic acid. Linolenic acid gives rise to docosapentaenoic and docosahex-- aenoic acids by a similar series of reactions.

at the carboxyl end becomes too short, a further two carbons are added; this permits further double bonds to be introduced. These reactions lead to the formation of 5,8,11 eicosatrienoic acid from oleic acid, to arachidonic acid from linoleic acid, and docosapentaenoic and hexaenoic acids from linolenic acid. The linoleic acid family are probably the true essential fatty acids; they are extensively used in the structure of cell organelles. In essential fatty acid deficiency they are reduced in quantity and their place is taken by a trienoic acid, probably of the oleic acid family. This trienoic acid does not have the properties of arachidonic and changes in cell function and tissue permeability result. Interesting effects on skin permeability have been described by Sinclair.[47] A wider range of polyunsaturated fatty acids cause reduction of blood cholesterol and β-lipoprotein levels. This is due, at least in part, to increased excretion of cholesterol.

The effective linoleic acid for essential fatty acid function is the *cis cis* form. Linoleic acids with *trans* configuration are not effective and, indeed, may be antagonistic.

The body appears to be able to synthesise oleic acid in considerable quantities from non-fatty precursors. Linoleic acid and linolenic acid, however, cannot be synthesised at a sufficient rate to prevent deficiency of the polyunsaturated fatty acids normally derived from them. This is certainly true in the rat and several other species. It is not certain whether this is so in man; clearcut essential fatty acid deficiency is difficult to demonstrate in the human subject. Nevertheless, an adequate supply of *cis cis* linoleic acid is desirable in the human diet and care should be taken to keep a careful control on processes that might destroy or isomerise *cis cis* linoleic acid.

Saturated fatty acids

As with oleic acid, the saturated fatty acids can be readily synthesised in the body from two carbon fragments. The synthetic pathways have been clearly defined. Since our concern is with the fats of dietary origin, this aspect of fat metabolism need not be further considered here.

Deposition of Fat in the Tissues

The fats transported in the blood are deposited in most tissues. In the time available, however, we shall only be able to consider three sites of deposition; the liver, adipose tissue and blood vessel walls.

Deposition in the liver

From what has already been said it is apparent that absorbed fat may reach the liver either directly, in the portal vein, or secondarily, after it has passed once round the systemic circulation. It must be presumed that the shorter-chain fatty acids disappear into the fatty acid pool. Glycerides can also pass into the liver cells. The entry of glycerides into the liver parenchyma cells has been shown by Morris[48] and by Borgström[49] to occur without previous hydrolysis; the liver does not contain lipo-protein lipase and, indeed, destroys it. Once again the nature of the surface film on the fat particle may have some significance. If chylomicrons, stabilised with lipo-protein, are injected they apparently enter the liver parenchymal cells so that the liver shows a diffuse fat staining. If, however, a dextran-stabilised emulsion is injected, the picture is strikingly different, since the fat is concentrated in the reticulo-endothelial cells.[50] Thus, under normal circumstances, glycerides in the liver may be derived from two main sources; they may be formed from fatty acids which, in turn, may be derived from NEFA or biosynthesised from two-carbon fragments, or they may come directly from the blood glycerides. Normally only a small amount of glyceride is found in the liver; there must, therefore, be rapid removal. Present evidence suggests that the removal of fat from the liver also occurs largely in the form of glycerides. Accumulation of glyceride fat in the liver may result from an excessive intake or decreased output. Excessive fat feeding will result in an increase of glyceride fat in the liver, this becomes much more marked in animals with choline deficiency. The mechanism by which choline deficiency produces this effect is not known; it appears to be related to phospholipid formation. In both over-feeding and choline deficiency, it is dietary fat that is deposited in the liver. A great deal of further research is needed before the precise role of the liver in glyceride metabolism can be defined.

Deposition in adipose tissue cells

One of the earliest demonstrations made by Schoenheimer[51] with isotopic labelled materials was the uptake of dietary fat into fat depots. He was able to show that the fat in the fat depots of the rat had a half life of nine days. Subsequently it has been shown that various dietary fatty acids have different half lives. Oleic acid and palmitic acid are about the same at 12-17 days, but linoleic acid turns over more slowly; its half life may be as long as 68 days. This may be due to recycling of the linoleic acid in ester form, possibly in phosphatides or cholesterol esters. The adipose tissue cells can take up NEFA as well as glycerides; they are also able to synthesise fatty acids from 2-carbon precursors. Thus, the adipose tissue cells provide a rich store of potential energy. The fat appears to be liberated from the adipose tissue cells as NEFA: this is stimulated by ACTH; lipo-protein lipase has been demonstrated in the adipose tissue cells of several species, but not of all. Release of NEFA from the adipose tissue cells is stimulated in starvation and the diabetic state. Release appears to be decreased by glucose and by insulin. The effect of diet on the metabolism of adipose tissue is consequently complex.

Hilditch and Lovern[52] have shown that in many animals, other than ruminants, the fat in the adipose tissue strongly resembles the dietary fat. The higher the fat intake and lower the carbohydrate load, the greater is the similarity. The effect of dietary fat on the depot fat may be made use of in growing pigs with depot fat within a specified melting point range. In considering the difference in half life of the various depot fats, the effect of different types of fat feeding on the fat depots in man might repay further study.

Deposition of fat in blood vessel walls

Lipids are deposited in the walls of blood vessels from an early age. The amount of lipid tends to increase as age advances, so that eventually quite large plaques of fatty material may be seen in the walls of the blood vessels. These may undergo ulceration, secondary calcification and other changes. This process of atheroma must be regarded as part of the normal ageing process. It is thought that under certain circumstances it may be one of the factors concerned in myocardial infarction, a condition that seems to have become much more common in recent years. The evidence for this correlation is by no means conclusive and there is little justification for assuming any causal relationship.[53] The first point of importance in this discussion is whether there is a relationship between elevated blood cholesterol and atheroma. There seems to be no doubt that people with a high blood cholesterol level are likely to develop considerable deposition of lipids in the blood vessel walls. In some of these patients at least, the deposition of cholesterol and other lipids also occurs in other sites, giving rise to xanthomata. There may be some justification for regarding the lipid deposits seen in the blood vessels of patients with essential hypercholesterolaemia as being a specialised form of xanthoma.[54] It may be noted, however, that xanthomata can be significantly modified by a diet containing unsaturated fatty acids. Outside this and the diabetic group, there is much greater variation in the relationship between blood cholesterol levels and atheroma. However, if we assume that a high resting cholesterol level is likely to lead to the earlier development of atheroma, then a diet containing a high proportion of polyunsaturated fatty acids, which will lower the blood cholesterol level, might be used to control atherogenesis. This therapeutic use of unsaturated fatty acids seems a reasonable approach to this problem and it is certainly worthy of trial. Control of atheroma by this means has been demonstrated in animals; it is impossible to demonstrate in man, since there is no satisfactory method of measuring atheroma in a living human subject.

Even if atheroma were controlled, this is by no means a guarantee that there will be a dramatic decrease of myocardial infarction. Attempts are being made to see whether patients with this condition benefit from a diet containing a high proportion of unsaturated fatty acids. If they do not, this may be because blood cholesterol and atheroma do not play so great a part in the aetiology of myocardial infarction as some people have supposed. If they do, it may be because the unsaturated fatty acids also have other effects, such as modification of fibrinolysis.[55] A great deal more work is needed before any final answers can be given on these difficult problems. It is certainly unjustifiable to conclude that the increase in myocardial infarction in recent years can be clearly attributed to dietary factors. All attempts to demonstrate a deficiency of polyunsaturated fatty acids in the tissues of patients with myocardial infarction have so far been unsuccessful.[56] It is also unreasonable on present evidence to suggest that major dietary changes are needed for the population as a whole. There is no doubt that further long-term studies in both animals and man, designed to elucidate the significance of the various aetiological factors that may be involved in atherogenesis and myocardial infarction, are urgently needed. All concerned with the production of dietary fats must pay the closest attention to the results of these further studies; there is no doubt that the nature and quantity of fats consumed may have a profound influence on the metabolism and health of the consumer.

Utilisation and Excretion of Fat

Fat oxidation

One of the major uses of dietary fat is to provide energy. If fat is taken during starvation as much as 45% may be quickly oxidised; however, if glucose is taken at the same time the amount immediately used may be reduced to 5%. Fatty acids can be utilised by all cells, with the exception of the brain; heart muscle may use fatty acids selectively under certain circumstances. The pathways of fatty acid oxidation have been fully defined. The fatty acid combines with coenzyme A and then passes through a cycle of changes which eventually detaches two carbons from the chain as acetyl CoA. Oxidation proceeds in this stepwise

fashion until the whole fatty acid is broken down. The acetyl CoA is oxidised in the citric acid cycle. All the dietary fatty acids can be handled satisfactorily by these enzymes; it is immaterial whether they are even or odd numbered carbon chains, saturated or unsaturated, branch chained or otherwise. Some tissues, such as heart muscle, may have some difficulty in handling the propionyl CoA derived from oxidation of odd-numbered fatty acids, but it can be dealt with in the liver. It seems probable that NEFA is a particularly suitable form of fatty material for oxidation.

Fats in tissue structure

Glycerides are mainly concerned in fat transport and storage. However, fatty acids may be incorporated into phosphatides and cholesterol esters. These lipids form lipo-proteins that are the basis of cell structure. Cell membranes and the various cell organelles are largely built from lipo-protein units. As already mentioned, some of the organelles, such as mitochondria, are extremely rich in polyunsaturated fatty acids, such as arachidonic acid. A lack of essential fatty acids may interfere with phospholipid formation and result in fatty deposition in the liver. A supply of suitable polyunsaturated fatty acids is necessary in the diet of most animals, since the rate of synthesis of such compounds is too slow. They may be particularly important in growing animals and those in which considerable tissue regeneration is taking place.

Fat excretion

One of the most important fat excretions is milk and much of the fat in this may be synthesised from two carbon fragments.[57] Nevertheless, dietary fats do find their way into milk and, as already mentioned, this has been demonstrated in the case of *trans* acids. Polyunsaturated fatty acids are also likely to be largely of dietary origin. An adequate supply of suitable fats may be of some importance in lactation. There are several other forms of fat excretion, such as the sebum in the skin, the lipids excreted in the urine of the cat tribe and the excretion of lipids into the gastro-intestinal tract. I shall only have time to touch on two of these. A dry harsh skin is often seen in patients with a severe fat absorption defect and this is strikingly relieved when absorption improves; similar skin changes have also been described seasonally in Tanganyika which are said to be improved by administration of unsaturated fats. Further studies on the effect of dietary fats on skin fat excretion might be of interest. As regards fat excretion into the intestine, this remains largely an unsolved problem. However, as already indicated, there is evidence that the excretion of cholesterol into the intestine is increased by the ingestion of unsaturated fatty acids. This may partly account for the striking effect of polyunsaturated fats on the blood cholesterol level. Further studies on fat excretion into the intestine are required.

In conclusion, I hope that I have indicated that the nature of dietary fat may profoundly influence metabolism and that careful attention should be paid to the molecular structure of natural fats used as foods, as well as to changes that may be brought about during processing or storage. Great amounts of fat are now available for human consumption and there is no doubt about the nutritional benefit that this gives. Like most advances, however, it also carries potentialities for harm. It is our duty to ensure that our knowledge of the effects of dietary fats on the body is adequate to enable us to make the best possible use of this great opportunity for the betterment of mankind that the first Lord Leverhulme began.

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The Metabolic Effects of Glycerol Administered to Diabetic Patients

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Glycerol administered orally in amounts of 25 to 50 gm four times daily in place of isocaloric amounts of dextrose during partial insulin withdrawal to four ketosis-prone diabetic patients decreased the concentrations of ketone bodies in blood and alveolar air to within normal limits in all patients. The daily glycosuria in these patients and in the additional three diabetic patients who failed to develop ketosis after partial insulin withdrawal decreased by an average of 85 gm/day when glycerol was substituted isocalorically for dextrose. This suggested that glycerol is a carbohydrate which can be metabolized without requiring insulin. No consistent effect on fasting total serum lipids, free fatty acids (FFA), and hyper-pre-beta-lipoproteinemia was demonstrable under the conditions of the experiment. The disappearance of serum-pre-beta-lipoproteins after several days of a high-carbohydrate, hypo-caloric diet in one patient was sustained during glycerol administration.

The mechanism of ketosis in diabetes mellitus is still controversial although much evidence suggests that the central "key to ketosis is utilizable carbohydrate."[1] Insulin is the most important agent in the treatment of diabetic ketosis by making carbohydrates utilizable by cells. In cases of insulin resistance, the management of ketosis may present a serious problem[2] and the availability of a carbohydrate not requiring insulin may be a useful therapeutic agent. Even in milder cases of diabetes mellitus, supplementary therapeutic agents may have value in correcting biochemical changes associated with ketosis.[3] The potential advantage of the prevention of ketosis, abnormal lipid synthesis, and hepatic gluconeogenesis by administration of carbohydrates not requiring insulin (fructose and sorbitol) has been discussed by Renold.[4] In addition, there is recent evidence that ketones diminish glucose utilization when insulin release is either at its maximum or cannot be stimulated at all.[5] Unfortunately, the usefulness of orally administered fructose is limited because 60% of it is rapidly converted to glucose in the intestinal wall during absorption.[6] Sorbitol, being converted to fructose, is not effective in reducing ketosis and plasma free fatty acid (FFA) concentration in juvenile diabetics.[7] Also, because of slow absorption, it may cause diarrhea.

The antiketotic and protein-sparing effect of small amounts of various carbohydrates, including glycerol, in a diabetic patient on a high-fat, semi-starvation diet was first established by Hirschfeld in 1897[8] and, subsequently, by other investigators.[9] Thomas[10] found in 1924 that the substitution of isocaloric amounts of glycerol for other carbohydrates in the diet of two diabetic patients resulted in diminished glycosuria and acetonuria. It was known at the turn of the century, as reviewed by Deuel,[11] that up to one third of orally administered glycerol can be converted to glucose even in pancreatectomized animals. This indicates that insulin may not be necessary for the utilization of a portion of glycerol. The evidence documenting the lack of toxicity of long-term oral glycerol administration has been reviewed previously.[12]

It was the purpose of this preliminary investigation to determine whether glycerol has an antiketotic effect when administered orally to insulin-dependent patients. It is tentatively concluded that glycerol, when substituted isocalorically for other carbohydrates in the diet, decreased glycosuria, ketosis, and insulin requirements.

Materials and Methods

Seven patients were hospitalized in the Clinical Research Center, and physical activity was restricted to walking on the ward and was kept as constant as possible. The patients received a constant diabetic diet in four feedings daily throughout the entire period of study. In addition, four times daily one-half hour before each meal at 7:30 AM, 11:30 AM, 5 PM, and 8:30 PM, they ingested 200 to 250 ml of chilled water with 30 ml of lemon juice containing either artificial sweetener, 25 to 50 gm of dextrose, or 25 to 50 gm of glycerin. Upon their admission to the hospital, the daily dose of insulin was gradually reduced (Table 1) in order to induce glycosuria or ketosis or both. After a stabilization period, the glucose drinks were replaced isocalorically by glycerol.

Received for publication March 14, 1967; accepted Nov 3.

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General data on patients are listed in Table 2.

Fractional urine collections and pooled 24 urines were tested for glucose.[illegible] Blood glucose was determined (Autoanalyzer) daily before breakfast and at 5 PM just before the third daily dose of glycerin.[illegible] Fasting total serum lipids, fasting free fatty acids (FFA) in duplicate,[illegible] and serum lipoprotein electrophoresis[illegible] were determined by standard techniques. Acetone in end-expiratory air was measured at least four times daily within one-half hour of each glycerol administration by gas chromatography as described previously,[illegible] modified by the use of hexane, 10 ppm in nitrogen, (Matheson Corp., Rutherford, NJ) for repeated standardizations. The hexane, 0.5 ml, injected with a gas-tight syringe resulted in peak height of approximately 8 inches at range 1 and attenuation 2. Acetone and acetoacetic acid in serum were also determined by a gas chromatographic technique[illegible] daily one-half hour before breakfast and immediately before glycerol ingestion. When duplicate determinations of alveolar or plasma acetone resulted in peak height deviation of more than 5%, the analysis was repeated. The standard deviation (SD) of duplicate tests was 0.035μg/100 ml end-expiratory air in samples containing up to 1.40μg/100 ml. Due to doubling of attenuation for samples containing up to 2.80μg/100 ml, the SD of values in this range also doubled. The SD for duplicate acetoacetic acid and acetone determinations in serum was 0.08 mg/100 ml in samples containing up to 3.2 mg/100 ml and increased proportionately when the attenuation was doubled.

Results

Under the conditions of the experiment, glycerol was well tolerated and specifically caused no diarrhea, anorexia, or decreased food intake as observed under metabolic ward conditions.

Glycosuria (Table 1) in diabetic patients decreased when 100 to 200 gm of glucose per day was replaced isocalorically by glycerol. Patient 1 (Fig 1) was on a 1,500 calorie basic diet (80 protein, 55 fat, 165 carbohydrate) and 200 gm of carbohydrates as dextrose or glycerol (total, 2,300 calories), glycosuria decreased from 170 gm day to 60 gm while ingesting glycerol instead of dextrose. When artificially sweetened lemonade was substituted for carbohydrates, approximately 30 gm/day of glucose was eliminated in the urine. Comparable decreases of glycosuria in other patients are listed in Table 1. Glycosuria during the glycerol period was approximately one third of that during the glucose period. There was little change in fasting blood glucose concentrations. However, blood glucose concentrations at 5 PM tended to be lower during the glycerol period by an average of 80 mg/100 ml of blood (Table 1). This is illustrated in patient 1 (Fig 1).

Acetone concentrations in alveolar air were determined several times daily in triplicate in all patients. Patients 1, 2 and 7 (Table 1 and 2) never developed significant ketosis despite the reduction of insulin. Alveolar air acetone concentrations in patient 6 (Fig 2) ranged from 2.1 to 2.4μg/100 ml during the control period when this patient received a 1,400 calorie diabetic diet plus 100 gm of glucose (total, 1,800 calories), but no insulin. After the exchange of 100 gm of glycerol for the glucose, acetone concentrations in expired air decreased to 0.5 to 1μg/100 ml in the afternoons. After seven days glucose was resumed, and the acetone concentrations increased to 7μg/100 ml within two days and remained at that concentration. This rise above the acetone concentrations of the first control period may have been due to increasing effects of tolbutamide withdrawal at the time of admission.

The morning alveolar acetone concentrations of patient 4 (Table 1 and 2, Fig 3) ranged from 0.4 to 8.5μg/100 ml of air. On the days when fasting acetone concentrations were elevated, they decreased gradually during the course of each afternoon and reached normal values by evening. During the five-day period when patient 4 received 150 gm of glycerol daily, the acetone concentrations remained lower than 0.5μg/100 ml throughout each day. This suppression of ketosis persisted even though the late afternoon glucose concentrations were below 50 mg/100 cc on three occasions and high fasting FFA concentrations (approximately 1,500μEq/liter) developed several times following insulin reactions.

Patient 3 (Table 1 and 2), while receiving 20 units of insulin zinc suspension (Lente insulin) and 1,800 calories daily, developed hypoglycemia in the evenings after exchanging isocalorically, 100 to 150 gm of glycerol for glucose. She developed only a mild ketosis even though FFA concentrations increased from an average of 500μEq/liter to approximately 1,000μEq/liter following two insulin reactions.

The antiketotic effect of glycerol was demonstrated during two separate admissions of patient 5 (Table 1 and 2). After gradually decreasing

[illegible]

Patient	Daily Calories	Mean Daily Insulin: Baseline Requirement	Mean Daily Insulin: Experimental Reduction	Mean Daily Glycosuria (gm): Control Period	Mean Daily Glycosuria (gm): During Glycerol
1	2,280	30	20	170	60
2	2,400	30	[illegible]	100	35
3	1,320	50	25	105	50
4	2,000	38	20	8-78	5-27
5	(a) 2,200	50	15	110	30
	(b) [illegible]		25	85	40
6	1,800	20	[illegible]	120	90
				130	
7	2,300	50	20	210	60

* FFA ranges during total period of observation.

† Several hypoglycemic reactions in afternoon during isocaloric glycerol replacement; no insulin reactions while ingesting dextrose.

the daily dose of Lente insulin from 30 to 15 units, the fasting blood glucose concentrations increased from 90 to approximately 700 mg/100 ml, and alveolar acetone concentrations increased from 0.2μg/100 ml to 4μg/100 ml (Fig 4). When glycerol, 150 gm day, was substituted for dextrose, the alveolar acetone concentrations returned to normal within the first day and remained there during the following day even though fasting blood glucose remained near 700 mg/100 ml. After remaining at home for two months during which time he took 120 gm of glycerol daily in addition to his customary diet, this patient was readmitted to the hospital. He was then fed a 2,200 calorie diabetic diet, which included 150 gm of glycerol daily. Lente insulin was decreased from 40 to 25 units daily. During this six-day control period his blood glucose ranged between 250 to 380 mg/100 ml, and the alveolar acetone averaged 0.8μg/100 ml (Fig 5). During the subsequent seven-day period, glycerol was replaced by glucose with resulting increase of blood glucose to 680 mg/100 ml and a rise in acetone concentrations in alveolar air to 4μg/100 ml. When glucose was again replaced by glycerol in the diet, the blood glucose and alveolar acetone concentrations returned to the previous low values. The concentrations of acetone in alveolar air closely reflected

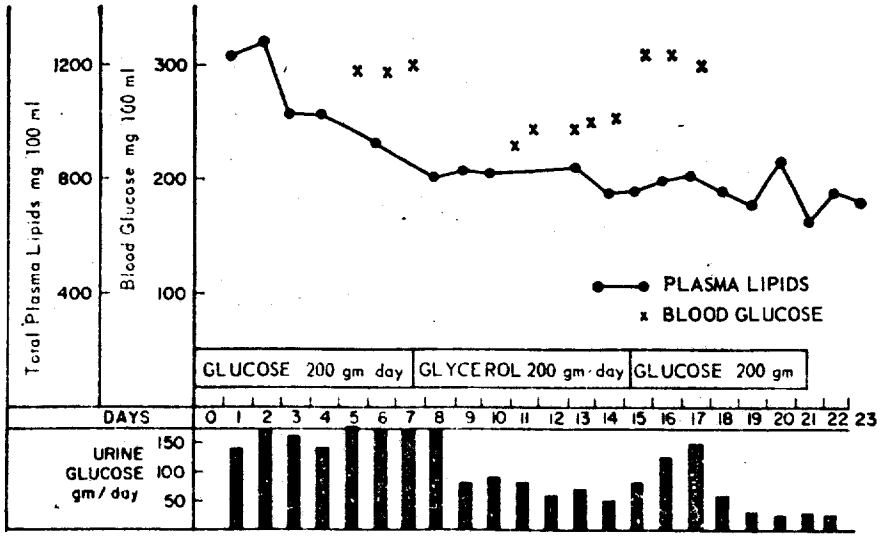

Fig 1.—Glycerol effect upon glycosuria and blood glucose (patient 1, Table 1 and 2). Blood glucose concentrations at 5 PM, was on a 1,500 calorie basic diet (80 protein, 55 fat, 165 carbohydrate) plus 200gm of glucose or glycerol. Insulin was reduced from 30 units before hospitalization to 20 units daily during the entire experimental period. No ketosis.

Table 2.—Clinical Characteristics of Patient's Severity Graded From 0 to + + +

Patient Age Sex	Weight kg	Height cm	Diabetes: Duration years	Retinopathy *	Nephropathy	Neuropathy	Cardiovascular Disease	Uric Acid mg/100 ml	Hyperpre-beta-lipoprotein
1 56 M	74	177	19	+ +	0	+ +	+ +	6.2	+ +
2 58 F	70	158	4	0	0	0	0	7.9	+
3 55 F	62	152	22	+	0	0	+	...	0
4 32 F	64	166	20	+	0	0	0	...	+ +
5 44 M	78	173	27	+ +	+ + +	+ +	+ +	7.1	+ +
6 58 F	65	162	18	+	0	0	0	...	0
7 30 M	62	165	10	+ + +	0	0	0	5.1	0

* Grading of retinopathy: + microaneurysms; + + microaneurysms, hemorrhages, and excudates; + + + proliferative diabetic retinopathy.

Table 1.—Biochemical Data

Mean Blood Glucose at 5 PM mg/100 ml: Control Period	During Glycerol	Mean End-Expiratory Acetone μg/100 ml: Control Period	During Glycerol	Mean Serum Acetone & Acetoacetic Acid mg/100 ml: Control Period	During Glycerol	Insulin Reaction During Glycerol Replacement	Serum FFA * μEq/liter (range)	Total Lipids mg/100 ml
300	230	<0.4	0.5	1.5	1.5	0	375-470	750-1,210
340	290	<0.3	0.3	1.5	1.5	0	640-920	1,055-1,480
190	146-<50 †	1.3	0.6	...	...		440-1,020	580-680
130	85-<50 †	0.4-8.5	<0.5	5.2	<1.2		700-1,600	1,200-1,470
720	620	4	0.2	6	1.4	0	430-890	810-1,300
...	...	5.2	0.9	3.8	1.7	+	475-960	1,050-1,290
250	180	2.4	0.9	2	<1.5	0	390-640	780-950
300		8		8.4				
220	...	<0.5	0.5	<1	<1	0	150-300	660-840

‡ Patient 5 admission (a) Fig 4 and (b) Fig 5. The only insulin reaction was on day of admission (b) to hospital.

§ Tolbutamide 1 gm twice daily before admission to hospital.

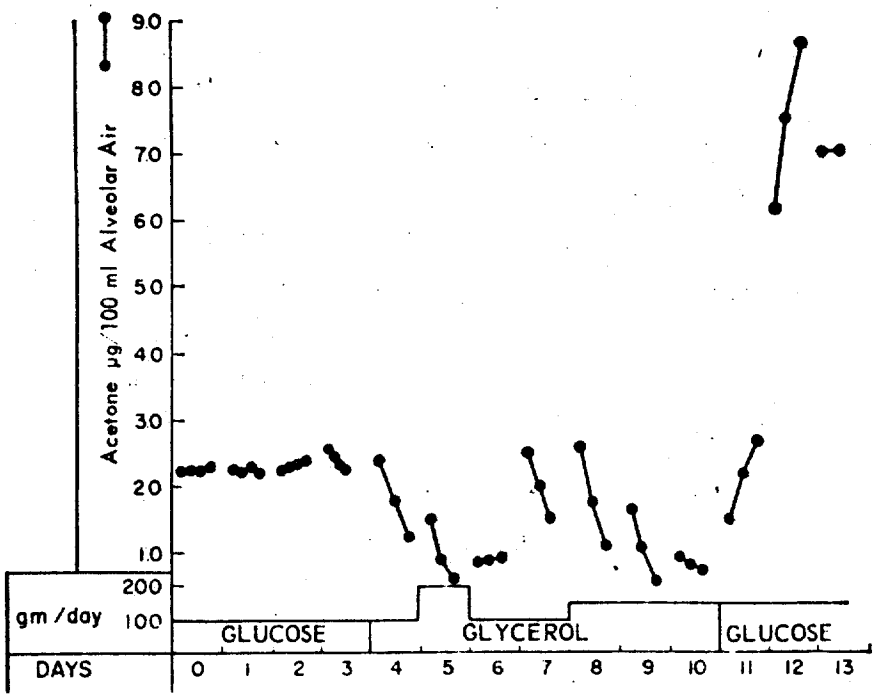

Fig 2.—Antiketotic effect of glycerol evident in afternoon. Patient 6 (Table 1 and 2) received a total of 1,800 calories daily and no insulin during the entire experimental period. Tolbutamide, 1 gm twice daily, discontinued upon admission. During the first control period before glycerol administration, acetone concentrations were lower than during the control period after discontinuation of glycerol probably because of some tolbutamide effect during the first control period.

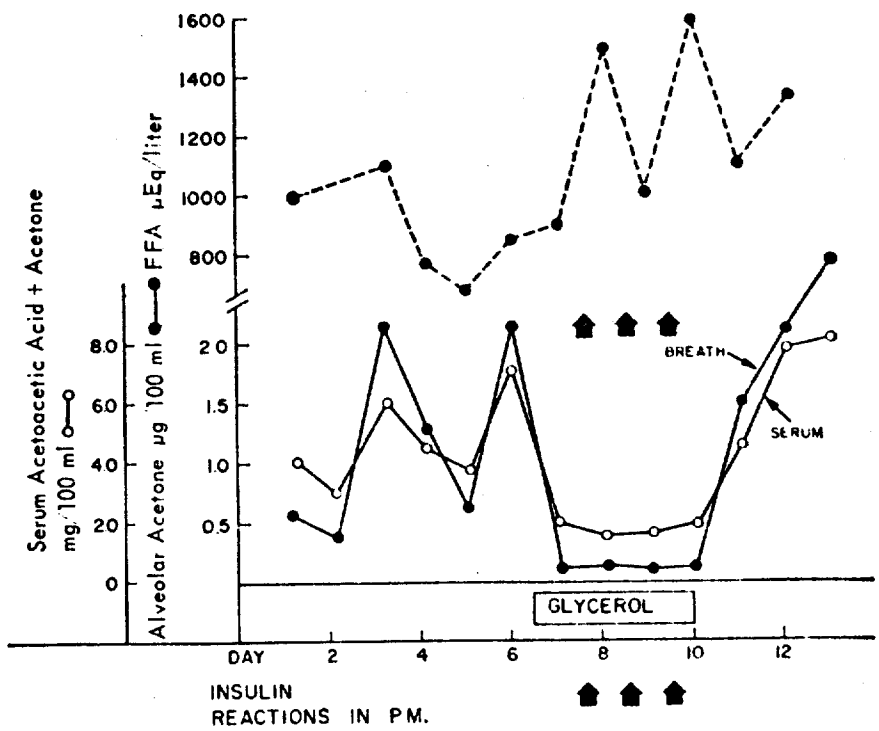

Fig 3.—Antiketotic effect of glycerol upon concentrations of acetone in alveolar air, as well as acetoacetic acid and acetone in serum collected simultaneously fasting at 7:30 AM. Patient 4 (Table 1 and 2) received a total of 2,000 calories, 20 units of Lente insulin, and 10 units of regular insulin daily during the entire experimental period. Insulin reactions in late afternoon ↑

the changes of acetoacetic acid and acetone in serum (Fig 5).

In general, there was no consistent effect of glycerol administration on fasting serum FFA concentrations and these concentrations varied markedly from day to day. Following insulin reactions, they were frequently elevated (Fig 3). However, in patient 7 (Table 2) fasting FFA concentrations were stable and decreased from an average of 280µEq/liter to 160µEq/liter when 200 gm of glycerol was substituted for glucose.

The serum total lipids likewise showed no consistent trend during these short-term experiments. However, the total plasma lipids of patient 1 (Table 2, Fig 1) gradually decreased from a maximum of 1,210 mg to a low of 750 mg/100 ml (normal 430 to 670 mg/100 ml) while he received glucose and a total of 365 gm of carbohydrates daily. This decrease was sustained while he drank glycerol. Concomitantly, a marked hyper-pre-beta-lipoproteinemia disappeared completely by the end of the first week of hospitalization. However, this patient lost 3.4 kg (7 lb 5 ounces) of body weight during his 25-day hospitalization. One month later after having regained his previous weight and while continuing to drink 120 gm of glycerol daily at home, the total lipids returned to 1,300 mg/100 ml, and the hyper-pre-beta-lipoproteinemia recurred.

Comment

The findings of previous studies[29] indicate that glycerol is a carbohydrate of which two thirds may be metabolized in the insulin-deficient organism and approximately one third is converted to glucose and excreted as such. Those findings are consistent with the observations reported here, which show that glycosuria during glycerol feeding was reduced to approximately one third

of that during glucose feeding. Glycerol is oxidized to carbon dioxide in liver slices from diabetic animals as efficiently as in liver slices of normal control animals.[21] However, the conversion of glycerol to glycogen is markedly decreased in liver slices from diabetic animals.[21] If ketosis in the insulin-deficient diabetic is partially the result of a lack of utilizable carbohydrates in the liver,[1,3] glycerol may be expected to have an antiketotic effect in vivo by supplying carbohydrate necessary for the oxidation of acetate via the Krebs cycle.

Recently Wieland demonstrated that glycerol has an antiketogenic effect greater than that of fructose in the perfused rat liver.[22] He suggested that glycerol may be antiketogenic by increasing triglyceride synthesis from acyl-coenzyme A or by interfering with acyl-coenzyme A formation by competing for adenosine triphosphate (ATP). Another mechanism of the antiketogenic effect of glycerol may be the restoration of normal utilization of acetate in the Krebs cycle. Glycerol apparently can supply those carboyhdrate intermediates which are necessary for the normal functioning of the Krebs cycle as is suggested by the work of Ashmore, et al.[21] In insulin deficiency, the glucose, which may be present in abundance in the liver cells, apparently cannot be adequately utilized because of a lack of insulin-induced glucokinase necessary for activation of glucose in the liver.[23,24] Glycerol may enter the

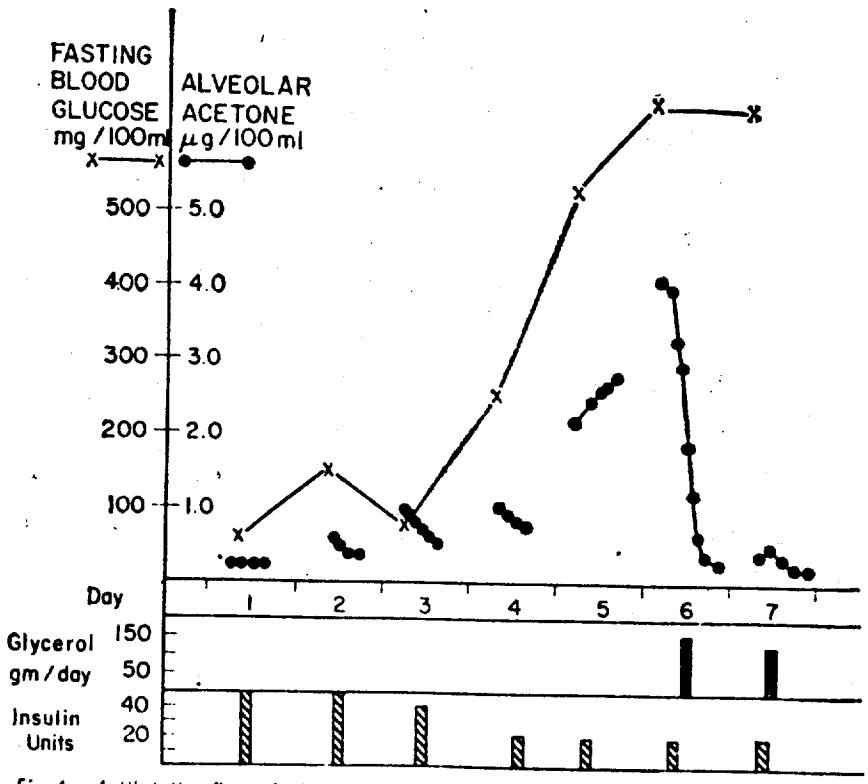

Fig 4.—Antiketotic effect of glycerol during ketosis induced by reduction of insulin. Patient 5 (Table 1) received a total of 2,200 calories daily during the entire experimental period. Acetone concentrations in alveolar air and fasting blood glucose concentrations.

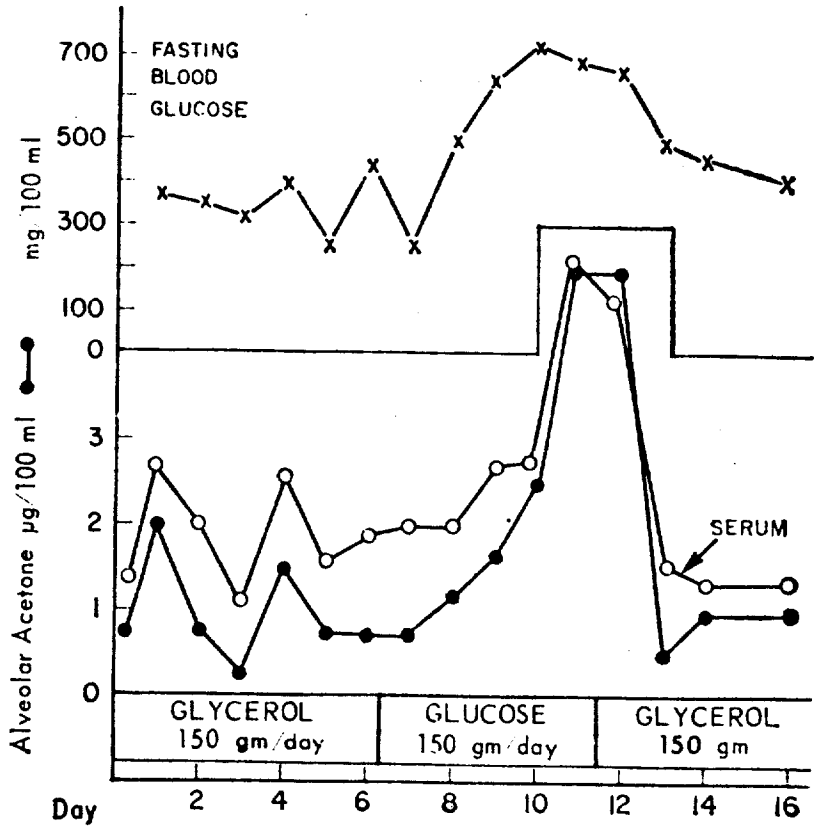

Fig 5.—Antiketotic and blood glucose lowering effect of glycerol (patient 5). Prior to data recorded, patient had received 120 gm of glycerol daily for two months at home. Severe insulin reaction upon admission to hospital. Total of 2,200 calories daily and 25 units of Lente insulin during entire experimental period. Fasting acetone concentrations in alveolar air and acetoacetic acid and acetone concentrations in serum determined simultaneously.

o—o indicates acetone µg/100 ml alveolar air, o—o, acetoacetic acid and acetone mg/100 ml serum.

glycolytic pathway at the triose level in the absence of insulin and may, thereby, become available for the oxidation of acetate in the Krebs cycle. Recent studies have demonstrated that insulin enhances postprandial glucose uptake by the liver.[25] Eighty percent of glycerol labeled with carbon 14 administered orally to the intact rat is oxidized to carbon dioxide[26]; therefore, only 20% maximally of the administered glycerol would be available for incorporation into triglycerides. This finding is more compatible with the hypothesis that in the intact animal, glycerol removes ketone bodies by accelerating their oxidation to carbon dioxide, rather than by converting them to fat.

Antiketotic Effect.—This study demonstrates that 100 to 200 gm of glycerol per day administered by mouth to insulin-dependent diabetics has an antiketotic effect. The isocaloric substitution of glycerin for glucose results in the lowering of ketones in alveolar air and blood. In a diabetic patient not receiving insulin, this antiketotic effect was demonstrable by a lowering of acetone concentrations in alveolar air from the elevated levels in the morning to normal concentrations by evening (patient 6). While the patient received glucose during the initial control period, the acetone concentrations in the afternoon remained the same as in the morning. When glucose was replaced by glycerol, the average concentrations of acetone decreased from 2.2μg/100 ml in the morning to an average of 0.8μg/100 ml of alveolar air in the afternoon. In patients receiving insulin in the morning, some decrease of ketones during the course of a day may occur concomitant with the action of exogenous insulin and with physical activity (patient 4). In both insulin-dependent and in slightly diabetic patients, elevated ketone levels were lowered during glycerol administration, and the diurnal decrease in the afternoon was further accentuated (Fig 2 to 5).

Glycosuria.—During substitution of glycerol for glucose, the daily urinary excretion of glucose was decreased to an equivalent of approximately one third of the amount of glucose substituted (Table 1, Fig 1). A further decrease of glycosuria when artificially sweetened lemonade was substituted for glycerol suggests that a small fraction (approximately 30%) of glycerol may be converted to glucose (Fig 1). This is in agreement with results in totally pancreatectomized animals who may convert approximately one third of administered glycerol to glucose[20] and utilize the remaining portion. Glycerol administration lowered slightly the fasting blood glucose concentrations and, to a greater degree, the afternoon blood glucose concentrations (Fig 1) even though the amount of insulin was not changed. The substitution of glycerol for glucose in patients 3 and 4 (Table 1 and 2) resulted in lowering of blood glucose sufficiently to cause evening hypoglycemic symptoms on several occasions (Fig 3). The observation that glycerol decreases glycosuria and lowers concentrations of blood glucose and of serum and alveolar acetone indicates that glycerol may be utilized by some insulin-deficient diabetic patients. This is in agreement with the animal studies cited above. The normal organism may convert a large portion of administered glycerol to glucose.[11] The insulin-deficient organism, however, appears to be able to utilize glycerol in the liver to reduce ketone body formation and to release only approximately one third as glucose into the systemic circulation.

Total Serum Lipids.—Glycerol had no consistent effect on total serum lipids in these short-term experiments. The decrease in total serum lipid concentrations in one patient (Fig 1) is possibly attributable to concomitant weight loss and occurred while this patient received glucose. It is of interest that in this patient the pre-beta-lipoproteins were no longer demonstrable after five days on a high-carbohydrate diet. One month after discharge from the hospital, while continuing to drink 120 gm of glycerol daily, this patient had regained his previous weight, total lipids had returned to 1,300 mg/100 ml, and the pre-beta-lipoproteins were again demonstrable. These findings continued essentially unchanged for the subsequent six months. It appears that in this particular patient the overall caloric restriction had a greater effect on total plasma lipids and pre-beta-lipoproteins than the composition of the diet.

Plasma Free Fatty Acids.—The increase of plasma FFA in insulin-deficient diabetic patients and animals has been shown to be a result of increased lipolysis.[27] While there appears to be a statistical correlation between elevated concentrations of FFA, blood ketones, and blood glucose, this correlation is frequently not demonstrable in serial determinations in a particular patient.[28,29] Perhaps a consistent relationship of fasting FFA cannot be expected in a particular subject because FFA concentrations may fluctuate markedly within a few minutes and because of the multitude of factors which may affect FFA concentrations.[30]

In this study the fasting FFA concentrations varied considerably from day to day in most patients. There appeared to be some increase in average fasting FFA concentrations when the insulin was decreased and a lowering of FFA concentrations when the initial dose of insulin was resumed. Low baseline FFA concentrations of 150 to 300μEq/liter were sustained during glycerol administration in patient 7 (Table 2). High FFA concentrations were observed in the absence of ketosis, and the

reverse was also true. These observations are in agreement with those of other investigators.[28] Free fatty acid concentrations had a tendency to increase markedly in the mornings after insulin reactions (Fig 3), as expected. It is possible that no consistent effect of glycerol on FFA concentrations was demonstrable because the time interval between the last glycerol administration in the evening and the determination of FFA was 12 hours.

This investigation was supported in part by Public Health Service grants MH-12574, AM-5172, FR-5362, and FR-82.

Waldo R. Fisher, MD, performed the serum lipoprotein electrophoresis and John I. Thornby, PhD, gave the statistical evaluation.

Generic and Trade Names of Drugs

Tolbutamide—*Orinase*.

Insulin zinc suspension—*Lente Iletin*

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J. Amer. Oil Chemists' Society
37: 589-590, 1960

• *Letter to the Editor*

Triglyceride Elution by Gas Chromatography

WE WISH TO REPORT a new development in gas-liquid phase chromatography (GLPC) analysis of fats and oils, not previously reported in the literature. We have successfully eluted triglycerides, through tristearin, with quantitative interpretation through trimyristin, and qualitative through tristearin, including most common edible oils (Figure 1).

Our initial studies gave us separation of the simple triglycerides, with both quantitative and qualitative interpretation, tributyrin through tripalmitin in polyester-packed columns ["Craig" polyester—ethylene glycol-succinate MW~1000, 30% on Chromosorb "W" (30/60 mesh), 18-in. column, operating at 250°C., 50 ml./min. He flow]. Because of the degradation of the polyester we have not attempted to operate these columns higher than 300°C.

We have extended the elutions through tristearin, using a silicone liquid phase at temperatures up to 350°C. The silicone used was fractionated from Dow Corning high-vacuum grease dispersed in ethyl acetate. A typical column used in this phase is an 18-in. stainless steel tube, packed with 30% silicone on Chromosorb "W" (30/60 mesh), operating at 300 to 350°C. with 50 to 200 ml. min. He flow. Table I shows a typical duplicate analysis of a synthetic mixture. Figure 2 shows typical log-retention plots for the columns used.

Despite what appears to be moderate degradation we have repeatedly reproduced the "fingerprint" of edible oils, and the curves given in Figure 1 appear as excellent qualitative elution curves.

TABLE I
Quantitative Interpretation of GLPC Elution. Tributyrin Through Trimyristin

Triglyceride	Standard Mixture		Curve I area %	Curve II area %
	Mole %	Weight %		
Butyric	14.9	8.11	8.15	12.2
Hexanoic	10.3	7.18	11.2	11.8
Octanoic	8.54	7.25	14.2	10.0
Decanoic	17.9	17.93	21.2	23.4
Lauric	22.3	25.71	29.7	26.0
Myristic	25.9	33.82	15.3	16.4

The edible oils containing a preponderance of lower-molecular-weight fatty acids (butter and coconut) give elution curves without degradation problems. It is interesting to note that butter oil shows 17 peaks, all reasonably symmetrical, and coconut 15 peaks, indicating 17 and 15 different molecular-weight triglycerides for these oils, respectively.

The triglycerides used in this work were prepared by standard $ZnCl_2$ catalyzed esterification, followed by distillation and/or crystallization.

The instrument is based on the Aerograph (Wilkins Instrument Company) circuitry with conventional oven and cell geometry. The cell is Gow-Mac TE111 Model 9230, with four tungsten filaments operating at ≈ 200 ma, 12 volts DC.

Column temperatures for the work ranged from 229 through 350°C., cell temperatures were kept at or slightly above column temperatures, injection and

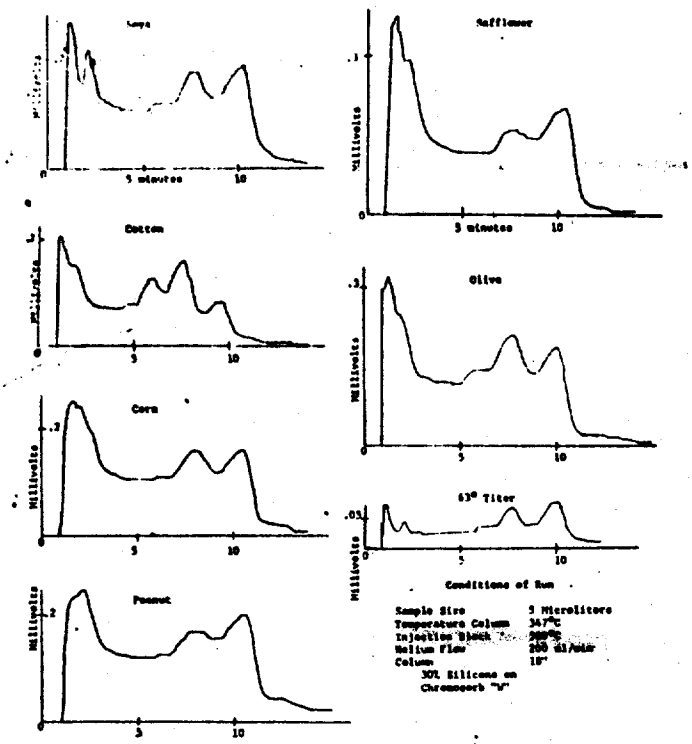

FIG. 1. Elution curves of some refined oils. GLPC runs.

exit heaters were held from 300 to 520°C. Helium flow rates were varied from 40 ml./min. to 200 ml./min. For any given run temperatures were held ± 2°C. and helium flow ± 1 ml./min.

The main problem in obtaining acceptable elution curves of the higher homologs is in vaporizing the oil without thermal decomposition. For triglycerides up to trimyristin this was obtained by increasing the injection block temperature. However the higher homologs do not respond as well, and increasing flow rate must be combined with the higher temperatures to obtain the desired result even though these changes have not yet given good curves for molecular weights above 800.

In making the quantitative estimates given in Table I, no corrections were made for the purity of the individual components although individual compound elution showed minor contaminants in every case. The area of each peak was compared as a percentage of the sum of the areas of all the peaks in the data given in Table I.

$$\% \, n = \frac{An}{\Sigma A_1 \ldots A_n} \qquad A_n = \text{Area of Peak n}$$

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[Received June 10, 1960]

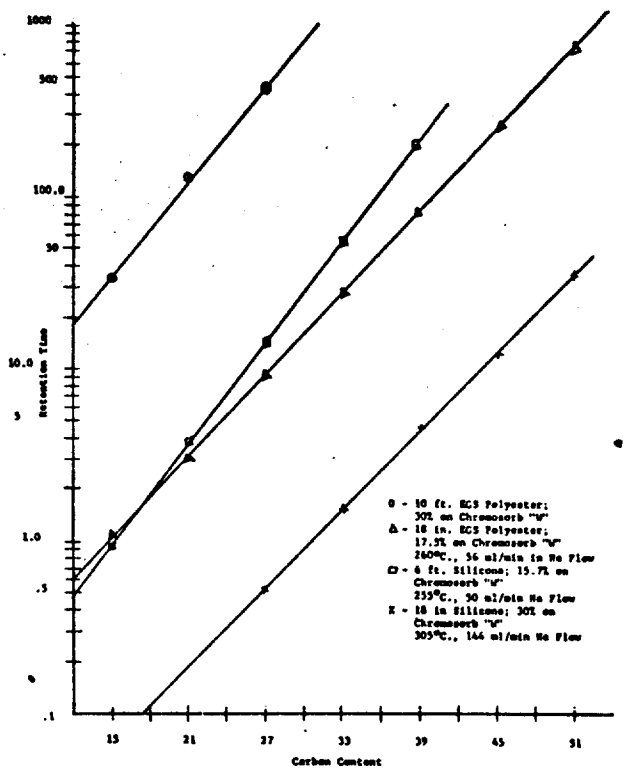

FIG. 2. Log retention-time *vs.* carbon content. Triglycerides GLPC.

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Fed. Proc.
22(2 Pt. 1): 368, 1963

SOME TOXICITY STUDIES WITH TRIACETIN. Joseph H. Gast* (spon: Arthur Furst). Inst. of Chemical Biology, Univ. of San Francisco, San Francisco, Calif.

Toxicity has been reported as 3.25 g/kg subcutaneously in rats. Twenty gram and adult Swiss mice were used. Four commercial preparations were used in 50% ethanol. Controls received glycerol and acetic acid. Intraperitoneally for male mice the LD_{50} was 1.7 g/kg and females 1.4 g/kg. Orally the LD_{50} was of the same order of magnitude being 1.8 and 1.1 respectively. On a limited number of adult rats intraperitoneal LD_{50} of 2.1 g/kg has been noted. Following administration, the animals hunched up, then acted paralyzed or lay flat on their backs with the hind legs moving as in running, or stretched prone, occasionally pawing at the litter. Later they lay semi-comatose with variable respiration. In most groups of animals death occurred within 15 minutes to 4 hours, however in some, survival as long as 96 hours was noted. No obvious defects appeared in survivors. Preliminary studies of chronic toxicity show growth depression, unkempt appearance but no effect on activity over 3 to 5 weeks. When triacetin was added, oxygen uptake of normal mouse liver homogenates were inhibited up to 17% with pyruvate substrate, cytochrome and succinoxidase were not inhibited. (Supported by NIH grant No. GM-08586-03.)

From: Journal for Food Testing and Research
138 (4): 216-220 (1968)

Contribution to the evidence of emulsifiers in foods

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Report of the State Institute for Dairy Farming Instruction and Reserch, Wangen in Allgäu.

Received on February 18, 1968.

The decrease of the milk surplus is a serious and much-discussed problem in EWG. A contribution to the solution of the problem consists in the development of new products, using milk and milk products. From the viewpoint of food legislation, these will have to be defined as "specific foods". Should a satisfactory keeping quality in the consistency be obtained when manufacturing said products, then the use of additives such as stabilizers and emulsifiers cannot be generally dispensed with. While no heterogeneous substances can be added to milk products according to 1. AVO of the Milk Law, this restriction does not apply to specific foods derived from milk products.

The following emulsifiers are considered for use in such foods: substances which do not qualify as(heterogeneous)foreign substances according to the LMG (Food Law), admissible according to the General Decree on Foreign (Heterogeneous)Substances for Foods. The above reasoning promted us to undertake tests for the purpose of obtaining evidence on the mono- and diglycerides of the higher fatty acids and of the edible glyceride acids (lactic acid, citric acid, tartaric acid and tartaric acid acetylate).

Legislation specifies the mono-and diglycerides as digestible fats and not as(heterogeneous)foreign substances.Accordingly, their use is permitted. However, the foreign substance character of the edible acid glycerides has been controversial for a long period of time (1). After the recent inclusion of the acetic acid-, tartaric acid- and tartaric acid acetylate glycerides into the General Foreign (Heterogeneous) Substance Decree (2) all edible acid glycerides will admittedly have to be regarded as foreign substances. Their use therefore would require the authorization of the legislator.

---

*) Mrs. D. ENKELMANN participated substantially in the implementation of the investigation, for which we wish to express our thanks here.

We undertook the analysis of said substances because they are approved in numerous countries and are regarded as harmless,except for the tartaric acid diacetylate glyxerides.Furthermore, they seem to be especially adequate as food emulsifiers on account of their physiologically useful effect.

We selected a cofee cream with a fat content of 15% as basic test substance, to which we added the above-mentioned emulsifiers in a concentration up to 0,5%. This was done in consideration of the idea that the use of emulsifiers could be desirable in condensed products with higher thickening rate as well, so as to obtain an improved keeping quality. The insulation and detection methods specified in literature(3,4) for emulsifiers of the above-mentioned type in other foods (for example: in meat and sausage products) could not be readily applied to our product. We therefore used other methods.

Description of the methods.

For our thin-layer chromatographic tests we selected as model substance a coffee cream having an approximate fat content of 15% and approximately 12% of dry matter; the additives under investigation (fatty acid monglyceride, lactic acid-, tartaric acid-, citric acid glyceride) were added in concentrations up to 0,5%. The following working rules then resulted after numerous tests:

Non-specific evidence concerning the mono- and diglyceries and the edible acid glycerides:

The fat extracted from a sample of approx. 10 g (for example: according to ROSE-GOTTLIER (5))is absorbed in 10 ml of chloroform. 0,01 to 0,02 ml of this solution is applied at the starting point on a siliceous gel G-layer which is impregnated with potassium hydrogen oxalate, and is chromatographed ascendingly with chloroform+ 1% methanol. After the evaporation of the solvent, spraying with 2',7' dichlorfluorescein follows. In filtered UV-light the single fat fractions appear as light spots and a dark base. If the chromatogram by then shows from $R_1$0 to $R_1$0,2 , this indicates the presence of strongly polar lipids--a class of substances which belong to the emulsifiers under investigation. Butter fat treated and extracted with the same method shows--when applied in a comparable concentration--no chromatographic evidence in this report: here the fractions become visible only from $R_1$0,5.

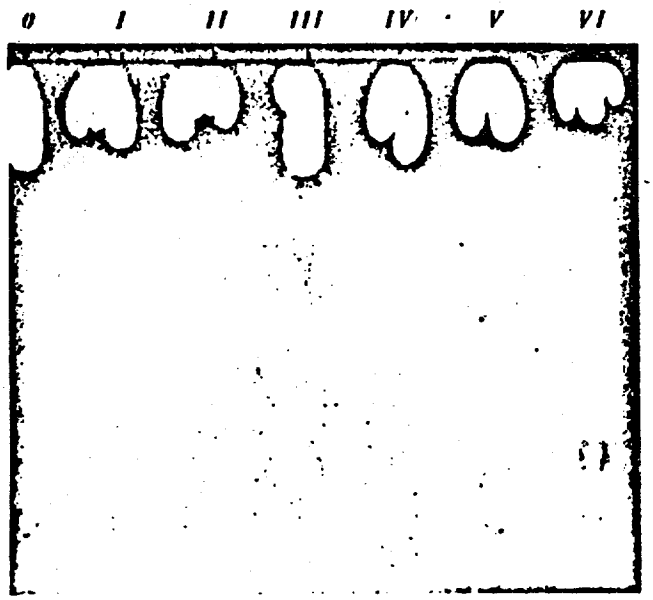

Illustration 1. Thin-layer chromatogram of the fat extracts of: 0-value (cofee cream); I) coffee cream + Na (K)-salts of the edible acids; II) cofee cream + 0,25% monglyceride; III) coffee cream + 0,25 lactic acid glyceride; IV) coffee cream + 0,25% diacetylate tartaric acid glyceride; V) coffee cream + 0,25% citric acid; VI) coffee cream + lactic acid-, tartaric acid and citric acid emulsifier (25% each). Adsorbent: siliceous gel G "Marck",impregnated*); flow medium: chloroform + 1% methanol; transit time: approx.40 min for 12 cm; application: 1,5 mg; spraying agent: 2',7' dichlorfluorescin.

---

*) Siliceous gel G for the preparation of the layer is stirred (instead of water) with an o,1 m potassium-hydrogenoxalate solution.

Specific evidence concerning the lactic acid-, tartaric acid- and the citric acid glycerides.

Since the isolation of the emulsifiers from the fat fraction is omitted, and since the investigation concerns only those acids (lactic acid, tartaric acid, citric acid) which are released during the saponification, the free hydroxy-acids must be eliminated from the fat.

Approximately 10 g(ml) of the substance under investigation is triturated with approx. 50 g annealed sodium sulfate (the sample should be powder-dry), and mixed with 20 ml of dimethylsulfoxide (DMSO), for the purpose of albumen denaturation and to achieve the precipitation of the fat-albumen complex. This is left standing for approx. 90 minutes at room temperature; then 50 ml of chloroform is added, and the mixture is heated to 60°C. Coolong is followed by decantation, and the residue is heated once more to 60°C, with the addition of 50 ml chloroform. Cooling and decantation of the residue follows, which is then rinsed with 25 ml of chloroform. The united DMSO-chloroform extracts (approx. 130 ml) are filteted into an evaporation container. To achieve the elimination of the DMSO and the leaching of the water-soluble substances such as lactic acid, tartaric acid or citric acid and their salts, the extract is brought into contact with large water volumes: the container or the beaker with the chloroform extract is placed into a 5-l vessel filled with water, at ½ elevation (for example: on a 600 ml beaker, high shape). The beaker is carefully tilted, so that the chloroform phase is barely retained. The set-up is left in this position overnight. The purified chloroform extract, together with an eventually formed film of the substances which are by then no longer soluble in water and chloroform, is then converted in a 250 ml round-bottom flask and freed from the solvent in a rotary evaporator. According to KRÜLLER (4b), the residue is hydrolysed for 90 min in boiling water, with 20 ml of a saturated barium-hydroxyl solution, 5 ml. of a concentrated ammonia solution and 3 ml of ethanol. Ba-citrate and Ba-tartrate, difficult to dissolve, will, among others, form thereby. These are subsequently separated with the aid of filtration after cooling, and the acids are released by adding a solution of potassium hydrogen-sulfate.

Filtration from the barium sulfate as well as from the fatty acids which are not water-soluble follows, and the clear solution which contains the acids under investigation is carefully condensed in the rotary evaporator almost to the stage of desiccation. The verification of citric acid and tartaric acid takes place after the recording of the residue in acetone-ether (1 : 1) with the aid of thin-layer chromatography. (DC).

Lactic acid, which does not form any Ba-salt difficult to dissolve, is identified from the clear filtrate of the saponification. For this purpose, the solution is converted to a strong tartaric acid condition (pH 4) and is shaken out three times with 25 ml of ether on each occasion. Lactic acid is then likewise identified with DC from the condensed dry ether solution.

Chemicals used

For the lipid reaction according to ROSE-GOTTLIER: see (5); for extraction according to ROHRLICH: sodium sulfate per anal., anhydrous , DSMO "Merck" for synthesis, chloroform DAB 6. For lipid hydrolysis: saturated barium hydroxid solution per anal., ammonia solution concentr. per anal., ethanol per anal. For the separation of the hydroxycarbon acids: potassium hydrogen sulfate-solution, 5% per anal., tartaric acid per anal., diethyl ether per anal., acetone per anal.

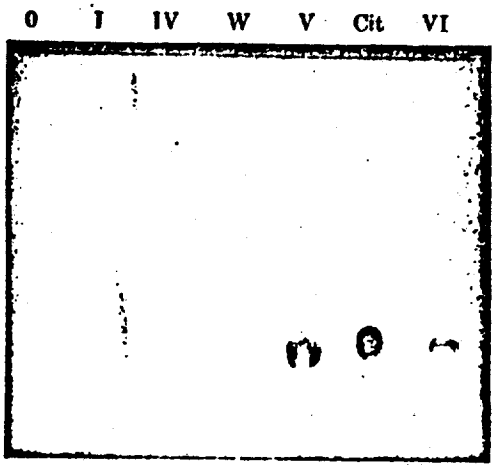

Abb. 2

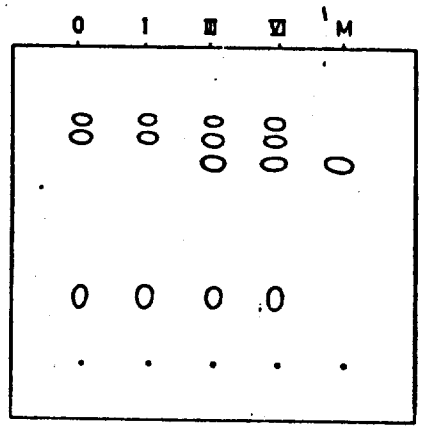

Abb. 3

Illustration 2. Thin-layer chromatogram of the hydrocarbon acids released by hydrolysis from the emulsifiers. Adsorbent: cellulose MN 300, air-dry; flow agent: Pentanol- (1)-formic acid (98 to 100%)-water (20+20+1); transit time: approx. 100 min for 12 cm; application: 0,03 ml of 2 ml acetone-ether solution; spraying agent: pyridine- acetic acid-anhydride (10+4). The tartaric acid shows a light blue, and citric acid a greenish-yellow fluorescence in the filtered UV, on a dark base.

W = tartaric acid; Cit = citric acid; 0,I,Iv,V,VI: see Illustration 1.

Illustration 3. Thin-layer chromatogram of the hydrocarbon acids released by hydrolysis; identification of lactic acid. Adsorbent, flow agent, transit time, application: see Illustration 2. Spraying agent: 8-hyroxy-chinoline of 0,05% in ethanol. The acids appear as dark spots in the filtered UV, after exposure the sprayed plate for a few seconds to an ammonia atmosphere.

M = lactic acid; 0,I,III,VI: see Illustration 1.

Discussion

During tests involving the application of methods described by KRÖLLER (4) for the isolation of emulsifiers of the edible glyceride acids to foods, the composition and consistency of which (foods) is approximately comparable to a 15% coffee cream, i.e. during the application of said method to the watery emulsions of approximately equal fat and albumen quantities, highly constant emulsions were frequently obtained with chloroform or with dichlormethane, as the best solvents. No phase separation occurred in said highly constant emulsions even after they were left standing for a prolonged period of time. Therefore the test material should be subjected to the chloroform extraction in as dry a condition as possible. Trituration with annealed sodium sulfate appeared to be the speediest and simplest desiccation procedure for small amounts of samples. Leaching of the chloroform extracts by repeated rinsing with water, with a 0,7% NaCl-solution or with water-alcohol mixtures likewise involved difficulties. Such difficulties do not arise with the method of ROHRLICH (6, page 227 of same) according to FOLCH and collaborators(7) which involves leaching of methanol-containing chloroform solutions in large volumes of water. Said method offers the advantage that chloroform- or dichlormethane solutions can be freed from hydrophilic components without special expenditures of energy and material. The formation of an emulsion is not possible thereby. We did alter the method somewhat insofar as we carried out the extraction of the lipids with DMSO-chloroform according to KAUFMANN (8). In this case, a water layer with a specific weight in excess of pure water will form during the leaching process at the phase limit DMSO/chloroform-water; said layer contains DMSO. When the container with the organic phase is placed into the large volume of water in a slightly elevated position, the DMSO water layer which is specifically heavier will fall over the rim to the bottom of the storage vessel, thereby making room for another layer of water. This process continues until all DMSO is transferred from the chloroform solution into the water and until the distribution balance is reached. Simultaneously with DMSO all other hydrophilic substances (in this case, for example: lactic acid or citric acid) are likewise washed out of the chloroform phase.

The result of the above-mentioned leaching method was tested with control analyises, whereby various quantities of lactic-, tartaric- and citric acid were added to the test samples. At the termination of the analysis procedure no corresponding acid could be detected in any of the investigated cases. The use of chromatography as specified by SCHWAIGER (9) for organic acids on cellulose layers for the identification of lactic acid, tartaric acid and citric acid from emulsifiers does not offer any noteworthy savings of time, as compared to the paper-chromatographic indetifications according to KRÖLLER (4).

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J. Biol. Chem.
206: 229–242, 1954

THE METABOLISM OF C^{14}-GLYCEROL IN THE INTACT RAT*

By L. I. GIDEZ† and MANFRED L. KARNOVSKY

(From the Department of Biological Chemistry and the Biophysical Laboratory, Harvard Medical School, Boston, Massachusetts)

(Received for publication, May 6, 1953)

Considerably more work has been carried out on the formation of carbohydrate from glycerol than on its incorporation into lipides. Thus, conversion of glycerol to glucose (1–4) and to glycogen (5–7) has been repeatedly studied *in vivo* and *in vitro* (6). More recently there has been work on the incorporation of C^{14}-glycerol carbon (8–10) and deuterioglycerol (11) into liver glycogen.

With regard to lipides, Karnovsky and Gidez (12, 13) and Doerschuk (8) have demonstrated the incorporation of C^{14}-glycerol into the neutral fats (acetone-soluble lipides) and phosphatides (acetone-insoluble lipides) of the rat. Glycerol has been shown by Kennedy to double the synthesis of phosphatides by isolated washed rat liver mitochondria (14). Similarly Balmain *et al.* (15, 16), Bloch (17), and Karnovsky (18) have reported a stimulation of acetate incorporation into fatty acids by glycerol.

The present paper contains the results of a study on the formation of CO_2, carbohydrate, and lipide from C^{14}-glycerol. In most of the data to be discussed the incorporation of glycerol into the various metabolic fractions has been followed with time. Preliminary results of this work have been reported previously (13).

EXPERIMENTAL

Synthesis of Radioactive Glycerol—The C^{14}-glycerol used in these experiments was prepared by the method described by Gidez and Karnovsky (19).

Normal male rats of the Wistar strain were used. All the animals were fasted for 24 hours prior to their use. During the first 12 hours they were given 5 per cent glucose *ad libitum*, and then water for the remainder of the time. Rats weighing 180 to 220 gm. were selected; their weights dropped to 160 to 200 gm. after the fast.

One series of experiments was carried out in a metabolism train. Respiratory CO_2, expired during definite periods, was collected for 6 hours after intraperitoneal, intravenous, or intragastric administration of varying amounts of α- or β-C^{14}-glycerol dissolved in 1 ml. of physiological saline. At the end of 6 hours the animals were injected intraperitoneally with 20 mg. of Amytal. When they reached light surgical anesthesia they were killed by decapitation and exsanguination. Blood and various organs were collected for isolation of lipides and determination of their specific activities. The amount and specific activity of the $C^{14}O_2$ expired in each period were also determined. The radioactivity of collected urine and feces was counted, and constituted usually about 1 to 5 per cent of the counts administered.

In a second series of experiments approximately 1 mM (92 mg.) of α-C^{14}-glycerol dissolved in 1 ml. of saline was injected intraperitoneally into each of several rats. The animals were put in individual wire cages placed in well ventilated hoods, without access to food or water. Prior to the injection, 0.1 ml. of tail blood was collected for blood glucose determination. At the end of 15 or 30 minutes, 1, 2, 4, or 6 hours, animals were stunned by means of a blow at the base of the brain, and then killed by decapitation and exsanguination. Blood was collected in heparinized tubes; the livers were removed immediately and one-half was used for glycogen determination, while the remainder was saved for lipide isolation. The lipides were fractionated, hydrolyzed, and oxidized with periodate as described under "Methods."

In a third series of experiments 1 mM of α-C^{14}-glycerol was administered to each of nine rats under the conditions above. Five animals were sacrificed after 1 hour, and the other four after 6 hours. The livers in each group were pooled and the total lipide extracted and fractionated. Pure tribenzoin and barium glycerophosphate were prepared from the neutral fat and phosphatide, respectively. A comparison was made of the activities of formaldimedon obtained after direct periodate oxidation of the aqueous hydrolysate fractions and those obtained after isolating the pure derivatives.

Methods

CO_2 Analyses—The $Na_2C^{14}O_3$-NaOH solutions from the metabolism train were diluted, and aliquots were used to determine CO_2 manometrically by the method of Van Slyke and Neill (20).

Glucose and Glycogen Analyses—Blood glucose was determined by the method of Nelson (21) and Somogyi (22). Liver glycogen was isolated by the method of Good *et al.* (23). After hydrolysis of the glycogen, the glucose formed was determined as above (21, 22).

Isolation of Lipides—The method of Bloor (24), with modifications, was employed for the extraction of tissue lipides. The tissue was ground with

* From a thesis presented by Lewis I. Gidez to the Division of Medical Sciences of Harvard University in partial fulfilment of the requirements for the degree of Doctor of Philosophy, 1953. This work was supported in part by grants from the Atomic Energy Commission, and the Eugene Higgins Trust through Harvard University.

† Predoctoral Fellow of the Atomic Energy Commission, 1950–52. Present address, Medical Department, Brookhaven National Laboratory, Upton, New York.

purified sand and alcohol, and the resulting mince was extracted with hot alcohol for 1 hour on a steam bath. After filtering, the tissue was reground and extracted twice with alcohol-ether (2:1) at 30°. The solutions were filtered and the combined filtrates were taken to dryness *in vacuo* on a water bath at 60°. The residue was extracted thrice with low boiling petroleum ether and filtered. This solution was washed with three successive portions of water (3 ml.) to remove any free glycerol that might have been present.

Lipides from plasma and whole blood were obtained by extraction with alcohol-ether (3:1) (25). Red blood cells were ground with $MgSO_4$ and extracted in a Soxhlet apparatus with chloroform.

The phosphatide content of the lipides was estimated by total phosphorus analyses (26, 27). The lipides were fractionated into acetone-soluble and acetone-insoluble components by the addition of 5 to 6 volumes of acetone and 4 to 6 drops of a saturated alcoholic solution of $MgCl_2$ to 1 to 3 ml. of a petroleum ether solution of the lipide (24). After standing in the cold for 1 hour or longer, the solution was centrifuged. The supernatant acetone was decanted, and the residue was washed with fresh acetone.

Phosphorus analyses were carried out on the acetone solutions. If the acetone-soluble material was found to contain more than 2 per cent phosphatides, a second fractionation was made. This process was repeated until there was 2 per cent or less phosphatide contamination.

Hydrolysis of Lipides

Neutral Fat—The neutral fat was saponified and the unsaponifiable fraction separated by the method of the Society of Public Analysts (28), modified to apply to samples of 30 to 50 mg. of fat. Alcoholic NaOH was used instead of KOH in cases in which periodate oxidation was later to be carried out. After removal of the unsaponifiable fraction, the aqueous solution was acidified with H_2SO_4, and the fatty acids were extracted with low boiling petroleum ether. The remaining solution was chilled, filtered, and treated with periodic acid (29), and the resulting formaldehyde steam-distilled. Formaldimedon was precipitated from the distillate by the addition of 2 ml. of 1 M sodium acetate and 1 ml. of an alcoholic solution of dimedon (5,5-dimethylcyclohexane-1,3-dione) (29). The precipitate was washed with water and recrystallized from aqueous alcohol.

Phosphatides—Samples of phosphatide (50 to 100 mg.) were refluxed for 2 hours with 1 ml. of 95 per cent alcohol and 5 ml. of 6 N HCl. Water was added and the fatty acids were extracted with four 15 ml. portions of low boiling petroleum ether. The aqueous fraction was chilled, filtered, and adjusted to pH 3, and then shaken with 4 ml. of Amberlite IR-100-H for 10 minutes. Preliminary tests had shown that this treatment effects the

complete removal of ethanolamine and serine from solution. The resulting solution showed a negative ninhydrin reaction on paper, and was filtered, made to pH 8, and treated with periodic acid. The formaldehyde was collected, precipitated, and recrystallized as above.

Preparation and Degradation of Lipide Glycerol Derivatives

In the third series of experiments described above, the neutral fats and phosphatides were hydrolyzed, tribenzoin was prepared from the aqueous fraction of the neutral fat hydrolysate, and barium glycerophosphate from the aqueous fraction of the phosphatide hydrolysate, after the ion exchange treatment described above. The methods of preparation of these derivatives were as used by Doerschuk (8). Tribenzoin was saponified, the benzoic acid separated by filtration after acidification and chilling, and the filtrate periodate oxidized. The formaldehyde representing the glycerol α-carbons was collected as above. The formic acid formed was oxidized with HgO, and the glycerol β-carbons obtained as barium carbonate (19). The barium glycerophosphate was burned and counted as $BaCO_3$. Samples were also converted to the sodium salt and were oxidized with periodate. Formaldehyde was collected as above.

Measurement of Radioactivity

All the samples to be counted were mounted on stainless steel disks which had a plating area of 1.54 sq. cm. A gas flow counter was used throughout the work (30).

Expired $C^{14}O_2$—The expired $C^{14}O_2$ was counted as $BaC^{14}O_3$.

Glucose and Glycogen—To the solutions of glucose from the blood filtrate and the hydrolyzed glycogen, 10 mg. of carrier glucose were added, and the phenylosazones were precipitated. These osazones were recrystallized from 50 per cent ethanol before being mounted for counting.

Lipides—The lipides were dissolved in $CHCl_3$ and drops of the resulting solution were put on disks of lens paper placed on the plating surface of the planchet. In some instances (unsaponifiable material and fatty acids) ether or petroleum ether solutions were added to the planchet. Solvents were removed *in vacuo* until the planchets reached constant weight.

α-Carbons of Lipide Glycerol—The formaldimedon from the α-carbons of the glycerol moiety of the triglycerides and phosphatides was plated and counted as such (19).

Self-Absorption—All the counts were corrected with appropriate self-absorption factors. $BaCO_3$ factors determined by Robinson were used.[1] In the case of the glucosazones, lipides, and formaldimedon, the appropriate corrections were determined experimentally and applied.

[1] Robinson, C. V., unpublished data.

Correction Factors to Counts As $BaCO_3$—Under the counting conditions employed in these laboratories, good agreement between activities obtained by counting organic substances, or by converting these to $BaCO_3$, and counting the latter were obtained. Results of the determination of the various small factors involved will be reported elsewhere.

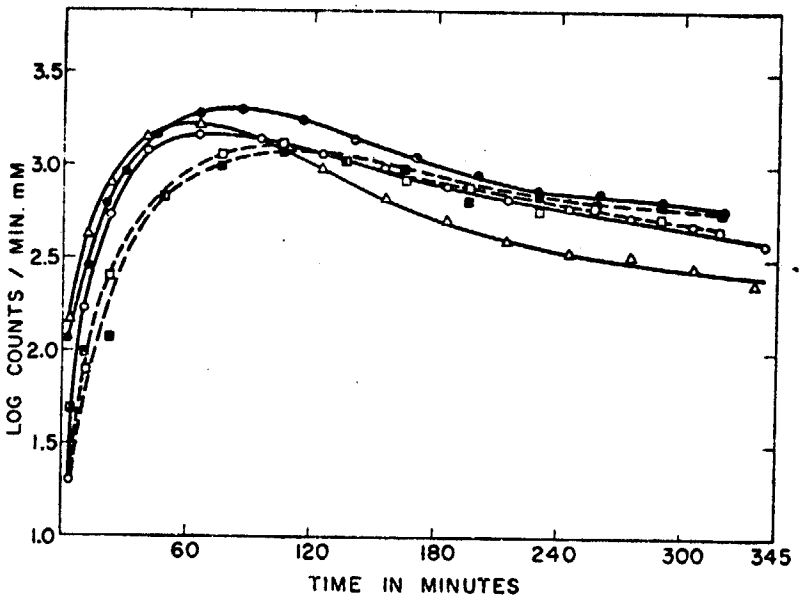

FIG. 1. Specific activities of expired CO_2 after administration of C^{14}-glycerol. 112.2 mg. of α-C^{14}-glycerol given intraperitoneally (●), intragastrically (○), and intravenously (△); 59.7 mg. of β-C^{14}-glycerol given intragastrically (■), and 177.2 mg. of β-C^{14}-glycerol given intraperitoneally (□). The specific activity of the α-labeled glycerol was 630,000 c.p.m. per mM, that of β-labeled glycerol 103,000 c.p.m. per mM. All the counts were normalized to 1×10^5 c.p.m. administered.

RESULTS AND DISCUSSION

Oxidation of Glycerol to CO_2—Three specific factors were studied with regard to their influence on glycerol oxidation *in vivo*, as reflected by the expiration of $C^{14}O_2$: (1) effect of the route of administration; (2) effect of the position of the label in the glycerol molecule; and (3) effect of the amount of glycerol administered.

Fig. 1 shows the changes in specific activity of expired CO_2 with time after the administration of α- or β-C^{14}-glycerol, given intraperitoneally, intragastrically, or intravenously. There are apparently no significant differences in the patterns observed.

Fig. 2 demonstrates the relationship of the amount of glycerol oxidized to the amount administered. Where less than 30 mg. of glycerol was given,

the proportion converted to CO_2 was greater than at levels above 30 mg., and bore an inverse relationship to the amount administered. At levels above 30 mg. an approximately constant proportion of the administered dose appeared to be oxidized. The mode of administration or position of label did not appear to affect the cumulative expiration pattern.

Synthesis of Blood Glucose and Liver Glycogen from C^{14}-Glycerol—In Table I are data on the formation of glucose and glycogen from glycerol. Net increases of blood glucose were demonstrable only during the first 30 minutes. On the other hand there was no net formation of glycogen until

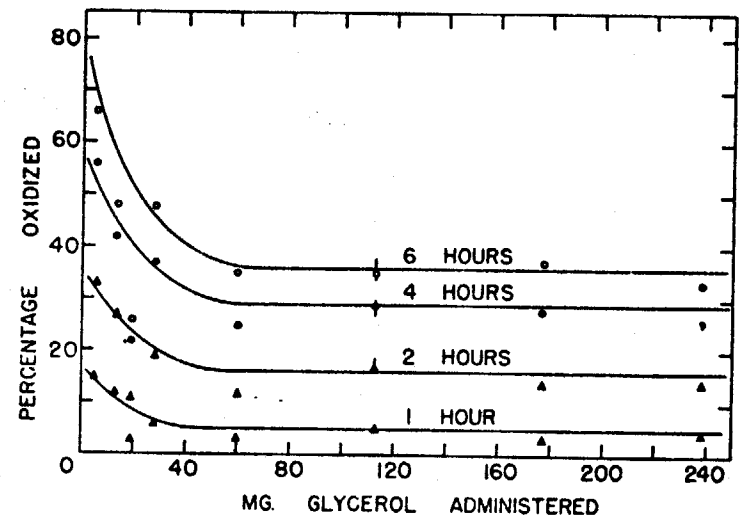

FIG. 2. Relationship of the percentage of glycerol oxidized to the amount administered. The percentage of glycerol oxidized was calculated as the percentage of administered counts expired. Three determinations were made at the 112.2 mg. level. The results of the experiment at 19 mg. were apparently atypical.

after 15 minutes, and maximal synthesis was not reached until after 1 to 2 hours.

Fig. 3 shows the percentages of total glucose and glycogen carbon derived from glycerol carbon. These data were obtained from specific activity measurements of the glucose phenylosazones derived from blood glucose and liver glycogen. Peak values for both carbohydrates occurred at 1 hour, one-third of the glucose and one-fifth of the glycogen carbon being derived from glycerol at that time. From the percentages in Fig. 3 and the chemical data in Table I, the values given in Fig. 7 for the percentages of administered glycerol converted to blood glucose and liver glycogen were obtained.

The values in Fig. 3 show the percentage of *total* carbohydrate carbons

derived from glycerol, but these data do not yield information on the percentage of *newly formed* glucose or glycogen arising from the administered glycerol. These figures may be calculated from the data of Table I and Fig. 3 for those times when there was *net synthesis* of glucose or glycogen. In the case of glucose, the data are for blood only, and not total extracellular glucose; for the calculations a blood volume of 4.5 ml. per 100 gm. of rat was used (31). In the case of glycogen, the initial levels were based on a fasting glycogen content of 0.046 per cent. The results are presented in Table II. The increases in glucose and glycogen are apparently greater

TABLE I

*Synthesis of Blood Glucose and Liver Glycogen at Varying Times after Administration of Approximately 1 mM of C^{14}-Glycerol**

Time	No. of rats	Blood glucose concentration		Liver glycogen, final†
		Initial	Final	
hrs.		*mg. per cent*	*mg. per cent*	*mg. per 100 mg. tissue*
¼	2‡	63 (±4.5)	94 (±14)	0.05 (±0.01)
½	5	73 ± 14	100 ± 9	0.19 ± 0.04
1	4	68 ± 12	65 ± 6	0.70 ± 0.18
2	5	66 ± 7	63 ± 4	0.71 ± 0.08
4	4	75 ± 1.3	66 ± 3	0.31 ± 0.002
6	2‡	47 (±14)	67 (± 9)	0.13 (±0.08)

* An accurately determined amount of α-C^{14}-glycerol (range 87.7 to 89.4 mg.) was administered; specific activity 1.085×10^8 c.p.m. per mM.

† The initial fasting level was 0.046 ± 0.007 mg. per 100 mg. of liver.

‡ The figures in parentheses represent the average deviation from the mean. In all other cases, since sufficient animals were available, the standard error of the mean has been calculated.

than can be accounted for by the glycerol that is converted to these metabolites. However, introduction of glycerol into the animal could presumably cause formation of new glucose and glycogen by direct conversion of C^{14}-glycerol to carbohydrate, or by a sparing action of oxidized C^{14}-glycerol on C^{12} intermediates, which may then be diverted for synthetic reactions.

Incorporation of C^{14}-Glycerol into Blood and Liver Lipides—The lipides isolated from whole blood were counted. Fig. 4 shows these activities and the activities of the corresponding liver lipides. As will be seen in Table V, probably all of the activity of blood lipides resides in the plasma since there were negligible counts in the red blood cells.

Incorporation of C^{14}-Glycerol into Liver Phosphatides and Neutral Fat—The specific activities of liver phosphatides and neutral fats counted as lipide are plotted in Fig. 5.

The lipides that were counted to give the curves of Fig. 5, *i.e.* phosphatides and neutral fat, were, however, not homogeneous fractions. The several chemical species in each fraction might have incorporated C^{14} from C^{14}-glycerol at different rates. Determination of the changes in activity of a defined chemical entity would be of greater interest. Table III represents such data obtained for the glycerol moieties of the neutral fat and

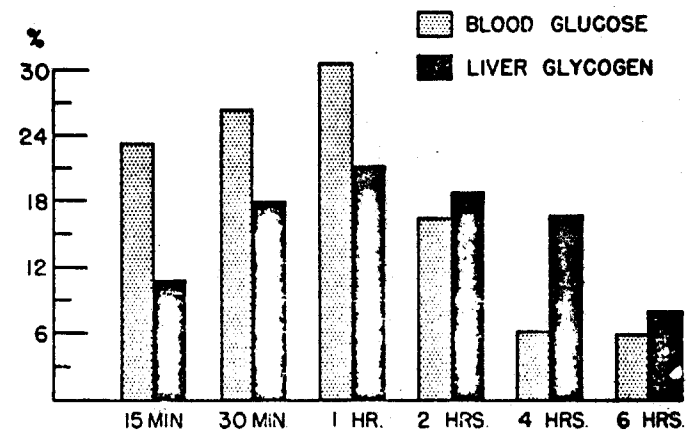

FIG. 3. Percentages of blood glucose carbon and liver glycogen carbon derived from glycerol carbon. The number of animals in each time period and the other conditions were as in Table I.

TABLE II

Newly Formed Blood Glucose and Liver Glycogen Derived from Administered C^{14}-Glycerol

The values are given in per cent.

	¼ hr.	½ hr.	1 hr.	2 hrs.	4 hrs.	6 hrs.
Blood glucose	70	100				
Liver "		39	23	21	21	15

phosphatide above. The activities of formaldehyde obtained by direct periodate oxidation of aqueous hydrolysate fractions agree reasonably well with results obtained after passing through the defined derivative. Statistical analysis of the results indicated that the differences were on the border line of significance. Table III indicates a rapid drop in the activity of neutral fat glycerol α-carbons from 1 hour to 6 hours, and a much slower decline in the activity of phosphatide glycerol α-carbons.

Determination of the activity of the β-carbon of neutral fat glycerol, carried out on the tribenzoin glycerol, showed randomization of activity into this carbon to the extent of 2 per cent of the total glycerol activity at

1 hour, and 5.5 per cent at 6 hours. Randomization is thus small, and, at least in 1 hour, of questionable significance (19). Doerschuk (9) obtained a value of 18 per cent for rat carcass neutral fat glycerol 24 hours after ad-

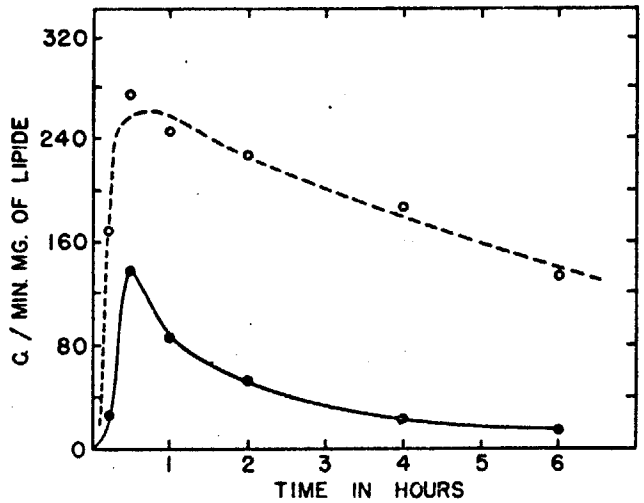

FIG. 4. Specific activities of liver lipides (○) and whole blood lipides (●) after administration of approximately 1 mM of α-C¹⁴-glycerol. All the points are the means of three experiments, except at 15 minutes and 6 hours, when only two experiments were carried out. Other conditions were as in Table I. All the results were normalized to 1 × 10⁶ c.p.m. administered.

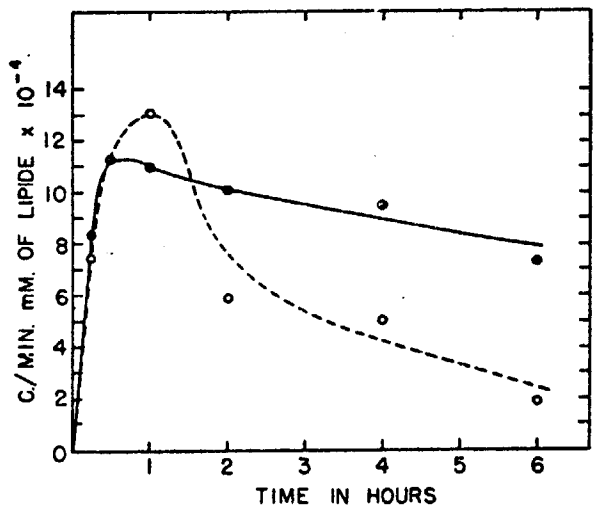

FIG. 5. Specific activities of liver phosphatides (●) and neutral fat (○), after administration of approximately 1 mM of α-C¹⁴-glycerol. The number of animals in each group and the other conditions were as in Table I. All the counts were normalized to 1 × 10⁶ c.p.m. administered. Assumed molecular weight for phosphatide is 744; for neutral fat 840.

ministration of α-C¹⁴-glycerol, and it has been shown (32) that 12 to 14 per cent of the activity of glycerol of the hen's egg resides in the β-carbon, after administration of acetate-1-C¹⁴.

TABLE III

Specific Activities of Formaldehyde Obtained on Periodate Oxidation of Hydrolysates and Pure Glycerol Derivatives

85.2 mg. of glycerol, specific activity 1,443,333 c.p.m. per mM administered to each rat; specific activity (c.p.m. per mM).*

	Neutral fat		Phosphatide	
	1 hr.	6 hrs.	1 hr.	6 hrs.
Formaldehyde, after direct periodate oxidation	118,100 ± 610	31,200 ± 294	37,800 ± 1320	26,150 ± 570
α-Carbon of tribenzoin glycerol†	114,000 ± 1640	27,500 ± 700		
α-Carbon of Ba-glycerophosphate‡			33,800 ± 582	24,250 ± 1715

* The values are the means of three to five planchets. The standard error of the mean is given. All counts were normalized to 1 × 10⁶ c.p.m. administered.

† Melting point and mixed melting point with authentic tribenzoin 71.5–72°.

‡ Per cent carbon = 11.4 and 11.8; theory 11.7 per cent.

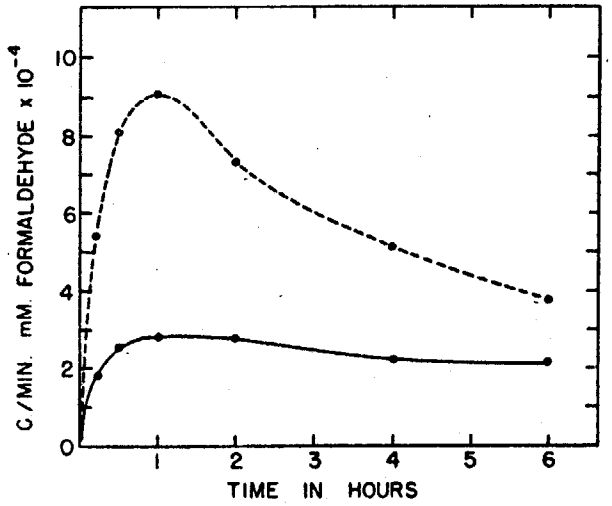

FIG. 6. Specific activities of the formaldehyde obtained on direct periodate oxidation of hydrolysates of liver phosphatides (●) and triglycerides (○). All the conditions were as in Table I. The counts were normalized to 1 × 10⁶ c.p.m. administered.

An attempt was made to calculate the randomization of activity in the barium glycerophosphate from the phosphatide by using the values for the whole molecule, obtained by combustion, and those of the α-carbons. The differences fell within the counting error, and no figure could be assigned.

TABLE IV

Specific Activity of Liver Fatty Acids and Unsaponifiable Fractions after Administration of Approximately 1 mM α-C^{14}-Glycerol

The conditions are the same as in Table I.

Time	Neutral fat		Phosphatide
	Unsaponifiable fractions	Fatty acids	Fatty acids
hrs.	*c.p.m. per mg.*	*c.p.m. per mg.*	*c.p.m. per mg.*
¼	0.3	0.3	1.4
½	16.5	9.3	2.6
1	2.1	6.8	1.8
2	1.8	2.6	1.8
4	3.3	2.6	2.4
6	1.7	0.9	1.0

TABLE V

Incorporation of C^{14} into Lipides of Liver and Extrahepatic Tissues 6 Hours after Administration of C^{14}-Glycerol

In all cases the counts have been normalized to 1×10^6 counts administered, and liver lipide then arbitrarily set at 100.

Tissue	Lipide	Given counts incorporated
	c.p.m. per mg.	*per cent*
Liver	100	5.9
Brain	13	0.26
Kidney	13	0.16
Spleen	12	0.06
Whole blood	10	
Heart	10	0.07
Lung	9	0.09
Stomach	6	0.05
Mesenteric fat	3	0.09
Testes	3	0.07
Small intestine	2	0.04
Depot fat	1	0.16
Pancreas	0	0
Red blood cells	0	0
Total		6.95

The randomization is, however, of the same order as that in the neutral fat glycerol.

The data of Table III and the low degree of randomization suggest that Fig. 6 represents a reliable picture of changes in neutral fat and phosphatide glycerol activities. At the time of peak incorporation, about 20 per cent of triglyceride and 7 per cent of phosphatide glycerol α-carbons were derived from the α-carbons of administered glycerol. The fact that the curves of Fig. 6 do not satisfy the precursor-product criteria of Zilversmit

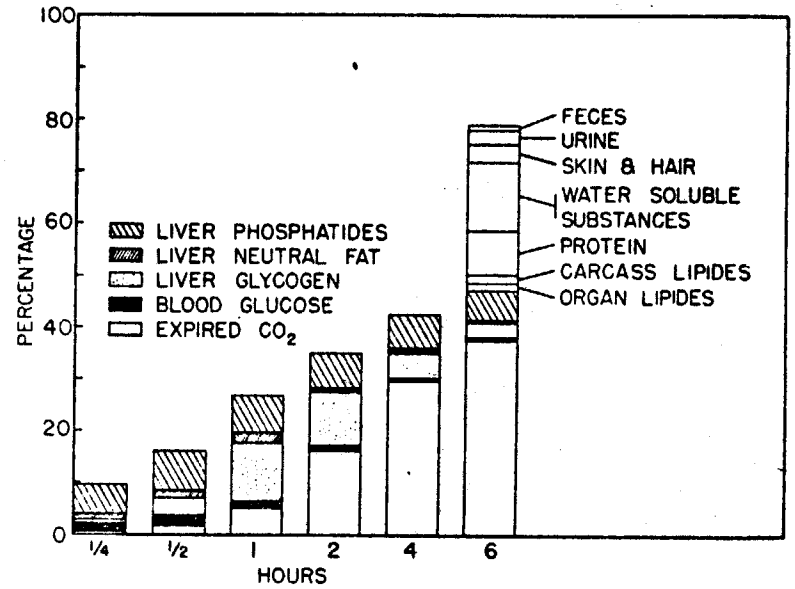

FIG. 7. The percentage of administered counts in different metabolites at various time intervals. The CO_2 values and the counts of organ lipides (other than liver), urine, feces, protein, "water-soluble substances," skin, and hair, and carcass lipides are from one typical animal. All other data are the means of two to six animals.

et al. (33) might indicate that phosphatide glycerol and neutral fat glycerol are independently derived from the administered free C^{14}-glycerol (*cf.* (34)).

The fatty acids and unsaponifiable material of the liver lipides were also counted. The data appear in Table IV. There appeared to be maximal activity in the three fractions at 30 minutes. The specific activities of the neutral fat fatty acids were slightly higher than the activities of the phosphatide fatty acids, a finding in keeping with the results of Pihl and Bloch (35).

Incorporation of C^{14}-Glycerol into Extrahepatic Lipides—Table V shows the specific activities of lipides from various tissues. In lipides from extrahepatic tissues the phosphatide fractions were 3 to 5 times as active as the neutral fats.

Radioactivity Balance

In the case of one rat killed 6 hours after injection of C^{14}-glycerol, an attempt was made to strike a radioactivity balance, even though the experiment had not been specifically designed for the purpose. The results appear in Fig. 7. It will be noted that approximately 80 per cent of the activity was recovered, even though the "water-soluble fraction" of the liver and other organs (containing amino acids, carbohydrate intermediates, etc.) was not obtained, since these organs were used for isolation of glycogen and lipides. This fraction probably constituted an important percentage of counts, as did the same fraction obtained from the carcass. Fig. 7 also serves to summarize the disposition of radioglycerol carbon in different fractions at varicus time intervals after administration.

SUMMARY

1. The metabolism of C^{14}-glycerol in the intact rat has been studied with the aid of α-C^{14}- and β-C^{14}-glycerol.

2. Insignificant differences were found when the glycerol was given intraperitoneally, intragastrically, or intravenously.

3. From 30 to 238 mg. of glycerol administered, an approximately constant proportion was converted to CO_2.

4. As a result of glycerol administration, there were net syntheses of blood glucose and liver glycogen. 70 to 100 per cent of the new glucose was derived directly from glycerol, whereas only 15 to 39 per cent of the glycogen was newly formed from glycerol.

5. The specific activity-time curve of blood lipides paralleled that of liver lipides; however, the C^{14} content of the latter lipides was several times as great.

6. Liver triglyceride glycerol rapidly reached a specific activity about 3 times that of phosphatide glycerol, and declined in activity much more rapidly than the latter. Randomization of activity was low.

7. Activity was found in the lipides of most tissues examined. It is of interest that brain lipides were rather active.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
FOUNDED BY CHRISTIAN A. HERTER

PUBLISHED BY

THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC.

ROBERT A. HARTE, MANAGER
9650 ROCKVILLE PIKE
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Serial 3354
13 December 1972

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METABOLISM OF FREE GLYCEROL IN THE ANIMAL ORGANISM

F. N. Gil'miyarova

Trihydric glycerol alcohol plays an important role in the organism and its metabolism, (being) closely related to the exchange of fats and carbohydrates. It appears as an essential compositional faction of triglycerides, and also of many other lipids, and may be synthesized in the organism from intermediate products, particularly (from) carbohydrate exchange.

The consumption of glycerol by the tissues takes place only after its conversion into alpha-glycerophosphates in the liver and kidneys under the influence of a glycerokinase catalyst (5). M. D. Kurskiy (1) and Gulyy (2), studying the effect of free glycerol on fat production, also found that it is preliminarily phosphorylized into alpha-glycerophosphate and then converted into fats. Besides this, it may be converted into glucose and glycogen or oxidized to (form) CO_2 (6, 7).

Bespalov and Gurovich (3), in tests on rabbits, demonstrated the possibility of anabolizing glycerol and converting it into monosaccharides, without a side effect, during internal introduction as a 25% solution in a dosage of 10 ml. Materials (on) increasing the level of sugar in the blood following the introduction of glycerol into an organism tell about this (15). Similar data on humans has also been produced (8). The toxic effect of glycerol appears only under large doses (4, 9).

According to the data of Eymer, Kopetz, and Schwarz (10), the liver plays a chief role in the anabolism of free glycerol.

The content of free glycerol in the blood of rabbits, according to the materials of Strack and Biesold (11), is equal to an average of 3.9 mg. %, and in the blood of humans, 4.4 mg. % (12).

Despite the presence of a series of works on the metabolism of glycerol in the organism, enough attention is not yet being paid to this problem. In particular, there are no data in the references on its content in the tissues and organs. Moreover, A. T. Cameron (13) indicates that glycerol in (its) free form is not generally contained in the organs and tissues of the animal organism.

In connection with this, we placed in front of ourselves the assignment of studying its content in the blood and tissues under various conditions of the feeding and upkeep of animals.

Procedure

The determination of free glycerol in the blood and tissues was carried out according to the method of Bailey (14). (This) method is based on the conversion of glycerol by means of oxidizing it with sodium periodate in formaldehyde. Finishing (this product) with phenylhydrazine and potassium ferricyanide forms a complex compound, red in color. In our performance of (this) task according to specified procedure, several modifications are introduced, as presented below.

To a centrifugal test tube, containing 0.9 ml. of doubly distilled water, 0.1 ml. of blood or tissue homogenate is added. For the condensation of protein, 0.2 ml. of a 0.3 M (molarity) solution of barium hydroxide, 0.18 ml. of a 5% solution of zinc sulfate, and 0.25 ml. of a 4% solution of magnesium sulfate are added. The test tubes are centrifuged (for) 15 minutes at 5000 rpm. To the 1 ml. (of solution) produced after centrifugation of (this) supercharged fluid, 0.4 ml. of subnormal sulfuric acid and 0.1 ml. of a 0.2 M solution of sodium periodate are added for oxidation of the glycerol, which is continued over a course of 10 minutes, whereupon (the process) is stopped (for) the addition of 0.2 ml. of a 1 M solution of sodium arsenite. Allowing 5 minutes for the formation of a tinted phenylhydrazine-ferricyanide

complex, 1 ml. of a 0.4% solution of phenylhydrazine and 0.1 ml. of a 5% solution of potassium ferricyanide are added. For the full development of this tint, the mixture is left (standing) for 20 minutes at 20 degrees. In order for the tint to stiffen, and to make it more durable, 2.3 ml. of a 4% solution of hydrochloric acid is added. The optical density of the tinted solution is determined on a FEK (photoelectrocolorimeter) at a wave length of 450 millimicrons.

Test results and their discussion

Rabbits weighing 2.5-3.0 kg., placed in similar conditions and on a normal nutritive regimen, served as test animals.

At first, the content of free glycerol in the blood was studied. Tests on 137 rabbits determined that its content in the blood, on an empty stomach, normally amounts to an average of 3.81 mg. %.

Next, the fluctuations of glycerol in the blood under a normal nutritive regimen (oats, root vegetables) was studied.

Table 1

Content of free glycerol in the blood of rabbits under a normal nutritive regimen over the course of a day

No. (of tests) in sequence	On an empty stomach	Glycerol content in mg. % Following food intake		
		After 4 hours	After 6 hours	After 24 hours
1	3.8	5.0	6.0	3.1
2	3.8	7.3	7.5	3.6
3	3.1	4.1	7.8	3.8
4	3.7	4.8	6.1	3.7
5	3.1	6.8	6.3	3.8
6	3.6	7.4	5.8	3.8
7	3.3	5.2	2.4	3.5
8	2.1	6.1	3.1	2.6
M ± m	3.3±0.3	5.8±0.43	5.6±0.68	3.4±0.1

As seen from the data presented in table 1, glycerol content following the intake of food (oats, root vegetables) is increased after 4 hours, remains increased for the ensuing 2

hours, and after 24 hours, practically lapses to normal.

Inasmuch as we didn't find (any) information in the references on the independent fluctuations of glycerol, we (also) studied its content in the blood of rabbits over the course of several days in a week (see table 2).

Table 2

Fluctuations of glycerol in the blood on an empty stomach over the course of a week

No. (of tests) in sequence	Rabbit's number	Glycerol content in mg. %			
		Days of the week			
		Monday	Tuesday	Wednesday	Thursday
1	9	4.5	4.6	4.3	4.7
2	10	3.8	4.8	5.8	3.7
3	11	4.9	4.2	4.3	4.5
4	12	5.0	4.5	4.8	4.2

These studies showed that the level of free glycerol is varied in the comparatively smaller ranges, in the same animal, over the course of (several) days in a week, depending on the potentialities of its method of determination.

It was also interesting to determine the effect of a charge of glycerol on its content in the blood. For this purpose, blood was taken from the auricular vein of a rabbit with an empty stomach and, thereupon, a 5% solution of glycerol was injected into the vein of the other ear, estimating 100 mg. for (each) kg. of the rabbit's weight. The extraction of blood was carried out 30 minutes, 2 (hours), and 24 hours after the intravenous injection of glycerol. The results received in this series of tests are presented in table 3.

Table 3

Effect of the intravenous injection of glycerol on its content in the blood

No. (of tests) in sequence	Before (injection)	Glycerol content in mg. %		
		30 minutes	2 hours	24 hours
1	3.5	13.5	3.1	4.0
2	-	14.6	6.5	5.0
3	3.8	12.2	6.8	2.8

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Table 3

4	2.8	15.8	3.6	2.9
5	3.1	12.7	3.5	4.5
6	5.4	21.7	5.5	5.6
7	3.6	8.8	7.4	3.5
M ± m	3.7±0.37	14.2±0.5	5.2±0.68	4.0±0.40
R		0.0013	0.015	0.10

Analyzing the results of the series of tests in question, it is not difficult to note that the content of free glycerol in the blood sharply increases after 30 minutes and still remains increased after 2 hours. Averaged data is correspondingly equal to 4.2 mg. % (before injection), 14.2 mg. % after 30 minutes, and 4.0 mg. % after 24 hours.

Glycerol content in the blood following the oral introduction of a glycerol charge was explained in subsequent tests. For this purpose, a group of rabbits were given glycerol in (their) food one time, estimating 100 mg./kg. The extraction of blood was carried out at 1, 2, 4, and 24 hours after food intake (table 4).

On the basis of the data established in table 4, it is apparent that a small oral charge of glycerol does not produce a sharp increase of its content in the blood, however, in the absolute majority of tests, its level is increased 1-2 hours after the charge (is introduced). A certain discontinuity of results is apparently demonstrated by its non-uniform absorbability, due to the fact that we added the glycerol to root vegetables. On the other hand, the insignificant increase of glycerol content in the blood following the charge is partially explained by the fact that it is exposed to the intestinal wall, (and) quickly absorbed and used, (thereby) not producing a significant increase in the blood.

Disregarding (the fact) that glycerol is a component of reserve fat, there naturally arose a need to study its content in the blood as a result of malnutrition, because it is known that during malnutrition, fat is first consumed.

Table 4

Effect of food charged with glycerol on its content in the blood of rabbits

No. (of tests) in sequence	Before (intake of food)	Glycerol content in mg. %			
		After 1 hour	2 hours	4 hours	24 hours
1	3.3	5.3	6.2	2.8	3.5
2	2.1	3.1	5.0	3.1	4.0
3	3.6	4.7	6.1	3.1	3.5
4	3.5	5.0	6.4	7.6	3.9
5	5.0	3.9	4.2	7.0	4.0
6	2.1	3.8	4.2	7.0	4.0
7	2.8	4.1	4.0	3.7	3.8
8	3.8	3.8	4.0	2.0	4.4
9	4.4	4.6	3.1	3.4	3.6
10	2.5	5.4	5.0	5.0	4.5
11	3.0	4.0	5.2	4.7	3.1
M ± m	3.3±0.27	4.2±0.31	4.8±0.53	4.5±0.53	3.8±0.12
R		0.05	0.012	0.055	0.06

As a result of this (study), we conducted a series of tests on 9 rabbits subjected to starvation over the course of two 24-hour periods. During this time they received only water. Blood was withdrawn on the morning prior to the commencement of the starvation (period), on empty stomachs, and thereafter, at 1- and 2-day (intervals).

Table 5

Glycerol content in the blood of rabbits during starvation

No. (of tests) in sequence	Weight before testing	Weight after testing	Glycerol content in mg. % Blood extraction periods		
			Before (testing)	After 24 hours	After 48 hours
1	2.700	2.250	4.0	3.7	4.4
2	2.600	2.530	3.4	3.8	5.2
3	2.300	1.810	6.2	4.1	1.9
4	2.300	2.150	3.0	4.6	3.0
5	2.000	1.680	3.9	6.0	3.0
6	2.200	1.880	3.0	5.0	5.0
7	3.000	2.770	3.0	5.4	3.5
8	2.700	2.650	3.4	4.3	3.8
9	2.300	2.030	2.1	5.0	5.2
M	2.450	2.190	3.55±0.38	4.65±0.25	3.8±0.38
R				0.014	0.11

As apparent from the data presented in table 5, the content of free glycerol in the blood is increased in the majority of instances (7 of 9) following a 24-hour period of starvation, and then, after two 24-hour periods of starvation, this increase is noted in a fewer percentage of cases. This may be explained by the fact that, at this time in the organism, in line with the decomposition of fats and the release of glycerol, its intensified oxidation is observed.

As is known, the metabolism of substances, which depends not only on factors related to feeding, but also on other causes, in particular hormonal (ones), is subject to marked changes in relation to the time of the year.

This is why we studied the content of free glycerol in the blood and tissues of animals in spring and fall. For determining glycerol in the tissues, (some) animals were killed with an aeroembolism and pieces of the liver, heart, muscles, and intestine were quickly removed from them, desiccated, freed from traces of blood with the help of filter paper, weighed, (and) homogenized with water. For the (actual) determination, 1 ml. of (this) homogenate was taken and further processed, as described above.

The averaged data we obtained is presented in table 6.

Table 6

Glycerol content in the organs and tissues of rabbits

Number of tests		Blood	Liver	Heart	Muscles	Intestines
Fall	9	4.3±0.37	99.4±5.1	19.7±3.2	10.2±1.6	17.5±1.5
Spring	8	4.8±0.31	95.9±3.9	10.6±1.6	9.7±1.6	9.7±1.8

On the basis of the data presented in table 6, it is apparent that, in spring, the glycerol content in all investigated elements, with the exception of the blood, is less than in fall. The most significant differences in the content of free glycerol, outside the bounds of a mistake in testing, is

observed in the cardiac muscles, the intestine, and, to a lesser degree, in the skeletal muscles.

The tests already presented above showed that the content of free glycerol in the individual [severed] organs is varied. In the liver its level is rather high, (but) at the same time, its content in the blood and muscles is relatively low. Apparently the various organs take different parts in the exchange of glycerol. In connection with this, there arose a need to study the effect of the prolonged introduction of relatively small doses of glycerol on its content in the blood and tissues. Tests were set up, as earlier, on rabbits which received glycerol in the form of a 50% solution mixed with root vegetables (carrots, beets) over the course of 5 months, estimating 50 mg. per kg. of (rabbit's) weight. After this, the animals were killed and a determination carried out as in the preceding series (of tests). The corresponding results are established in table 7.

Table 7

Glycerol content in certain organs and tissues of the rabbit following the prolonged introduction of glycerol

No. (of tests) in sequence	Glyerol content in mg. %				
	Blood	Liver	Heart	Muscles	Intestines
1	3.9	104.0	19.6	9.2	17.4
2	4.5	126.0	37.6	36.2	42.2
3	-	108.0	28.8	12.0	56.8
4	3.1	144.0	-	12.0	71.0
5	3.0	-	15.2	3.6	14.6
6	3.5	99.2	20.8	13.8	24.6
7	7.9	141.6	14.4	6.2	16.8
8	-	120.0	24.8	10.0	35.8
9	8.8	151.2	24.8	10.0	25.0
M ± m	4.8±0.8	123.0±6.1	26.0±3.7	12.5±3.1	33.8±6.5
Mk ± m	4.3±0.37	99.4±5.1	19.7±3.2	10.2±1.6	17.5±1.5
R		0.0017	0.085	0.20	0.014

On the basis of these tests, it is apparent that glycerol content is increased in the organs and tissues: by 11.0% in

the blood, by 23.0% in the liver, by 32.0% in the cardiac muscles, by 22.0% in the skeletal (muscles), (and) by 92% in the intestines. In other words, free glycerol introduced into an organism is poorly absorbed by the tissues of a normal rabbit, inasmuch as even 24 hours after the termination of a glycerol charge (the determination was carried out 24 hours after the next lot of glycerol) its increase in all investigated organs and tissues of the rabbit is observed.

Finally, the effect of glycerophosphate on the level of free glycerol in the blood and tissues during its prolonged introduction with food was studied. We ascertained from this that glycerophosphate, appearing as an intermediate product of the synthesis of fats within the animal organism, may be a source of free glycerol in the blood and tissues. With this aim, a series of tests was set up for the introduction of calcium glycerophosphate (a mixture of alpha beta isomers) into rabbits in a dosage of 50 mg./kg. of body weight, together with food (root vegetables), over the course of 5-6 months. Seventeen rabbits were (placed) under testing, 9 of which were controlled.

Results of the conducted studies are set (forth) in table 8.

Table 8

Glycerol content in certain organs and tissues following the prolonged introduction of glycerophosphate

No. (of tests) in sequence	Glycerol content in mg. %				
	Blood	Liver	Heart	Muscles	Intestines
1	5.4	131.2	11.2	-	19.6
2	7.1	224.0	26.4	9.2	25.6
3	6.6	120.0	-	5.2	22.2
4	7.7	120.0	10.4	7.6	20.8
5	4.1	96.0	-	4.0	14.4
6	9.2	134.4	30.4	18.2	19.4
7	8.4	144.0	24.0	10.0	14.4
8	5.8	88.2	24.8	5.4	17.2
M ± m	6.8±0.59	129.9±5.1	21.2±3.1	8.5±1.0	19.2±1.3
Mk ± m	4.3±0.37	99.4±5.1	19.7±3.2	10.2±1.6	17.5±1.5
R	0.0016	0.0014	0.15	0.045	0.13

As the data obtained and set (forth) in table 8 show, glycerophosphate, and also glycerol, causes an increase in free glycerol, usually in the blood and liver only, during its prolonged intake with food. In contrast to a glycerol charge, glycerophosphate causes a decrease of free glycerol in the skeletal and cardiac muscles and has practically no effect on (its) level in the intestines. It seems these data may be explained by the fact that glycerophosphate introduced into the intestines is not subjected to conversion, and is dephosphorylized in the liver, from whence it passes into the blood.

Summing up the conducted studies, it may be ascertained that the investigation of free glycerol content in the organism must be given serious attention. Our data shows that exogenous glycerol is desired and appropriated by the organism, but rather slowly. The suggested conclusion is that if the accumulation of free glycerol in the organism will result in metabolic perturbation - this may lead to serious disturbances, which are apparently observed at times, although concrete instances of (these) disturbances are not yet recorded.

On the basis of the experimental data we obtained, the following conclusions may be drawn:

1. The content of free glycerol in the blood under normal conditions is equal to 3.81 mg. % on the average, being subject to insignificant changes over the course of a week in certain animals.
2. Food intake reflects (very) little on the level of glycerol in the blood. A single charge of glycerol in food, in a dosage of 100 mg./kg., likewise does not reflect in a significant manner on its quantity in the blood. However, the intravenous injection of glycerol in that exact dosage leads to its sharp increase in the blood and at 30 minutes (after injection) amounts to 14.2 mg. % on the average. (This) increment remains increased for 2 hours, which is explained by the organism's slow consumption of glycerol.
3. Seasonal fluctuations of glycerol in the blood and tissues

take place, whereupon its level is higher in fall than in spring.

4. The prolonged introduction of glycerol into rabbits as (a part of their) food, over the course of 5-6 months, leads to an increase of its level in the blood, the liver, the skeletal and cardiac muscles, and the intestines 24 hours after the last intake of food with glycerol.

5. During the prolonged introduction into animals of glycerophosphate in food, in a dosage of 50 mg./kg. of body weight, its increase is observed only in the blood and liver, which may be explained by the presence of catalysts in these organs, (which) break down glycerophosphate.

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Metabolism of free glycerol in the animal organism. F. N. Gil'miyarova. *Tr. Kuibyshevsk. Med. Inst.* 29, 10-18(1964). The av. content of free glycerol (1) in the blood of fasted rabbits was 3.81 mg. %. After feeding it increased slightly and reached normal within a day. A single dose of 100 mg. I/kg. taken internally had little effect on I content in the blood. Within 30 min. after intravenous injection of 100 mg. I/kg., I content in the blood had risen to 14.2 mg. % and remained elevated for 2 hrs. Daily starvation increased I concn. to 4.65 mg. %. The I content in the autumn was 99.4 in the liver, 19.7 in the heart, 17.9 in the intestines, and 10.2 mg. % in the muscles. In the spring these indexes were somewhat lower (esp. in the heart and intestines). Prolonged (5-6 months) intake of I with the food in a dose of 500 mg./kg. increased I content by 92% in the intestines, 32% in the heart, 22% in the skeletal muscles, 23% in the liver, and 11% in the blood. Prolonged intake of the same dose of glycerol phosphate with the food increased I concn. only in the

BULLETIN

OF THE

NATIONAL FORMULARY COMMITTEE

Volume XV November-December, 1947 Nos. 11-12

CONTRIBUTED ARTICLES

RESPONSE OF RATS TO DIETS CONTAINING VARYING AMOUNTS OF GLYCEROL AND OF PROPYLENE GLYCOL*

By N.B. Guerrant, G.P. Whitlock, M.L. Wolff and R.A. Dutcher

Glycerol has long been used in foods, beverages, flavoring extracts, and as a vehicle in pharmaceutical preparations. While glycerol is a normal constituent of both plant and animal fats and in this respect cannot be regarded as a toxic material, all writers are not in complete accord regarding its potential toxicity when ingested in large amounts. Deichmann (1) has published an excellent review relative to the behavior of glycerol in the animal organism and cites conditions under which excessive doses of glycerol might possess harmful effects. On the other hand, Johnson, Carlson and Johnson (2) described experiments with rats, dogs and men in which relatively large amounts of glycerol were ingested without apparent harmful effects.

The extensive use of glycerol has been due primarily to its unique physical properties and to its relative freedom from toxicity. Among its many desirable physical characteristics are: its sirupy consistency, its hygroscopic nature and its characteristic solvent properties. Owing to the temporary scarcity of glycerol for domestic uses during and following the World War II, glycerol substitutes have aroused increased interest. Of the various glycols investigated to date, propylene glycol (propanediol-1,2) has received, perhaps, the most favorable consideration as a glycerol

*Received September 26, 1947 from the Department of Agricultural and Biological Chemistry, Pennsylvania State College, State College, Pennsylvania.

substitute. This is due to the fact that propylene glycol is apparently the least toxic of the more commonly produced glycols (3) and possesses physical characteristics somewhat similar to those of glycerol.

While certain similarities in the physical properties of glycerol and propylene glycol have been well established, there remains a variance of opinion regarding the pharmacological properties of the two compounds. The literature pertaining to their relative toxicities and to the potential dangers of glycerol and propylene glycol in relation to their chemical constitution and their fate in metabolism has been adequately reviewed by von Oettingen (4). In an attempt to summarize the scientific literature bearing on this question, Laug and associates (5) state that ".... It is concluded that ethylene glycol, diethylene glycol, ethylene glycol monoethyl ether, diethylene glycol monoethyl ether and dioxan should be entirely omitted from foods and drug preparations and that propylene glycol must be avoided except in very small concentrations." The studies reported do not, however, appear to have answered satisfactorily several pertinent questions which arise when the possible consequences to animals of ingesting materials of questionable toxicity are considered. Among these questions are:

(a) What is the maximum daily intake of glycerol or of propylene glycol for a given species of animal which may be tolerated without evidence of biological injury?

(b) Are there any harmful effects arising from ingesting submaximum daily intakes of these compounds over an extended period and are there any cumulative effects which become evident when several generations of experimental animals have subsisted on diets containing these compounds?

The investigations reported in the present paper were undertaken with the view of throwing further light on the above questions as they concern glycerol and propylene glycol.

Experimental

Growth studies. - The studies reported at this time consist of two phases: growth studies and reproduction studies. The first phase of the investigation was concerned with a study of the effects of varying intakes of glycerol and of propylene glycol on the growth rates of young rats, the glycerol or the propylene glycol each being provided as a partial constituent of the energy component of a basal diet which, prior to supplementation, had proved adequate for growth. The basal diet consisted, in parts per 100, of purified casein 20, salts mixture (McCollum 185) 4, Cell-U-Flour 2, filtered butter fat 6, medicinal grade cod liver oil 2, dehydrated brewers' yeast 6, and corn starch 60. This basal diet was modified by replacing corn starch with glycerol and with propylene glycol, respectively, at the levels of 1, 3, 6, 10, 15, 20, 30, 40, 50 and 60 parts per 100 Gm. of total diet (see Table I). In consequence of these substitutions, this series of

TABLE I. - Composition of Diets Employed in a Series of Comparative Growth Studies Conducted Over a Period of 20 Weeks Using Young Growing Rats as the Test Subjects

	Ingredients - Per cent									
Diet No.	Casein	Salt Mixture	Cell-U-Flour	Butter Fat	Cod Liver Oil	Yeast	Corn Starch	Glycerol	Propylene Glycol	Total
Control	20	4	2	6	2	6	60	--	--	100
1	20	4	2	6	2	6	59	1	--	100
2	20	4	2	6	2	6	59	--	1	100
3	20	4	2	6	2	6	57	3	--	100
4	20	4	2	6	2	6	57	--	3	100
5	20	4	2	6	2	6	55	5	--	100
6	20	4	2	6	2	6	55	--	5	100
7	20	4	2	6	2	6	50	10	--	100
8	20	4	2	6	2	6	50	--	10	100
9	20	4	2	6	2	6	45	15	--	100
10	20	4	2	6	2	6	45	--	15	100
11	20	4	2	6	2	6	40	20	--	100
12	20	4	2	6	2	6	40	--	20	100
13	20	4	2	6	2	6	30	30	--	100
14	20	4	2	6	2	6	30	--	30	100
15	20	4	2	6	2	6	20	40	--	100
16	20	4	2	6	2	6	20	--	40	100
17	20	4	2	6	2	6	10	50	--	100
18	20	4	2	6	2	6	10	--	50	100
19	20	4	2	6	2	6	0	60	--	100
20	20	4	2	6	2	6	0	--	60	100

growth studies involved 21 dietary groups, each group consisting initially of ten young rats. All rats constituting the series were from the same genetic source and were of approximately the same age and weight when placed on experiment. Efforts were made to distribute males and females equally among the several experimental groups. When high mortality of test animals was experienced among certain groups during the early stage of the feeding trials, additional animals were subsequently added to those groups.

Except during the course of certain special tests, all animals of the series were maintained in individual all-metal cages of the Wahmann Lc-28 type, which were provided with raised screen floors preventing access to excreta. The test diets were fed ad libitum and fresh distilled water was accessible to each animal at all times. The animals were observed daily and body weight changes and food consumption were recorded at weekly intervals. Hemoglobin determinations were made at regular intervals on bloods from representative animals from each group by the Newcomer method and blood glucose was determined by the Shaffer-Somogyi method (6). Other animals were transferred to special exercise cages for definite periods in order to observe the comparative effects of the composition of the diet on the inclination of the animals to exercise. Likewise, representative animals from the respective groups were transferred to special metabolism cages at specified intervals for the purpose of determining the effect of glycerol and propylene glycol intake on water intake and urinary output. Samples of urine were analyzed for glycerol or for propylene glycol by the method of Lehman and Newman (7).

Animals which survived the dietary treatments were maintained under observation for at least 20 weeks. Animals which succumbed, as well as representatives of those which survived the growth tests, were subjected to pathological examination.

In order to study the effect of this series of diets on reproduction and lactation in the rat, typical animals, which survived the 20-weeks experimental period, were maintained on the respective diets and were mated. Other animals were slaughtered for the purpose of determining the effect of diet on dry matter, fat and liver glycogen. Dry matter was determined by desiccation under partial vacuum, fat by Soxhlet extraction of the desiccated material with ethyl ether, and glycogen was determined by the method of Good, Kramer and Somogyi (8). The iodine number of the extracted body fat was determined by the method of Wijs (9). All remaining animals were submitted to pathological examination.

In a second series of feeding tests, groups of young rats were fed, respectively, four basal diets identical in composition

TABLE II. - Composition of the Various Diets Employed in a Series of Reproduction Studies in Which Rats Were Continued Under Observation Through Three Successive Generations

	INGREDIENTS - Grams															
Diet Number	Yellow Corn Meal	Rolled Oats	Linseed Meal	Wheat Bran	Meat Scrap Meal	Skim Milk Powder	Sodium Chloride	Calcium Carbonate	Ferric Citrate	Alfalfa Leaf Meal	Ground Whole Wheat	Wheat Bran, Germ, etc.[1]	Corn Starch Commercial	Glycerol	Propylene Glycol	Total
31 (Stock)	680	196	320	100	200	400	40	60	4	200	1800	---	--	--	---	4000
32 (Control)	680	196	320	100	200	400	40	60	4	200	--	540	1260	--	---	4000
33	680	196	320	100	200	400	40	60	4	200	--	540	1162	98	---	4000
34	680	196	320	100	200	400	40	60	4	200	--	540	1186	--	74	4000
35	680	196	320	100	200	400	40	60	4	200	--	540	1064	196	---	4000
36	680	196	320	100	200	400	40	60	4	200	--	540	1113	--	147	4000
37	680	196	320	100	200	400	40	60	4	200	--	540	966	294	---	4000
38	680	196	320	100	200	400	40	60	4	200	--	540	1040	--	220	4000
39	680	196	320	100	200	400	40	60	4	200	--	540	868	392	---	4000
40	680	196	320	100	200	400	40	60	4	200	--	540	966	--	294	4000
41	680	196	320	100	200	400	40	60	4	200	--	540	476	784	---	4000
42	680	196	320	100	200	400	40	60	4	200	--	540	672	--	588	4000
43	680	196	320	100	200	400	40	60	4	200	--	540	83	1177	---	4000
44	680	196	320	100	200	400	40	60	4	200	--	540	378	--	882	4000

[1] From 1800 Gm. of ground whole wheat.

females were examined at frequent intervals for indications of pregnancy in order that all pregnant rats could be removed to individual cages before the litter was delivered. When litters arrived, the date, the number of young born, and the average weight of the young were recorded. If the number of young in a litter exceeded six, the less thrifty young were discarded on the fifth or sixth day. Litters were weighed at weekly intervals until they were weaned. When the young animals reached a suitable size and age for weaning, the female was weighed and returned to the mating cage. Nine choice young (3 males and 6 females) were retained on the same diet as second or subsequent generation animals. These were carefully selected and, so far as possible, were chosen from first litter animals. The remaining young were either submitted to pathological examination or were discarded.

In this series of reproduction studies as in the preceding series, an attempt was made to obtain at least two weaned litters from each female. Where reproduction failures were experienced, the hitherto unreproductive females were mated to other males which were subsisting on the corresponding diet. As a last resort, the remaining unproductive females were mated to normal males from the breeding colony. At the completion of each of the several reproduction tests, the mature males and females were subjected to pathological examination. This latter series of reproduction studies was continued through three successive generations.

During the course of the studies with the first three generations there were observed some marked differences in the biological responses of different groups of animals which apparently were attributable to dietary origin. Attempts were made, therefore, to ascertain whether these differences would become more markedly manifested in succeeding generations and whether the differences would disappear or become less marked as the result of maintaining the progeny on more favorable diets. Three groups of young rats (each consisting of 9 males and 18 females) were selected from the third generation young of Groups 9, 10 and 12, (see Table III) respectively, for use in these studies. The animals of each of the groups were divided into three subgroups, each subgroup containing 3 males and 6 females. (See groups 15-23 in Table III). The animals of one subgroup were continued on the original diet. The animals of the second subgroup were changed to the corresponding glycerol or propylene glycol-containing diet. The animals of the third subgroup received the control diet (Diet No. 32). These experiments were continued through three additional generations.

Thus at the end of these tests, data had been obtained on effect of feeding glycerol or propylene glycol-containing diets through six generations, glycerol or propylene glycol-containing diets through three generations followed by the control diet for three generations, propylene glycol-containing diets for three generations followed by the corresponding glycerol-containing diet fed through three generations followed by the corresponding propylene glycol-containing diet for three generations.

TABLE III. - Composite Responses of Three Generations of Female Rats Produced on Each of Several Diets

Group No.	Diet[1] of Previous Three Generations	Diet[1] During Test-Period (Three Generations)	Substitution[2] in Test-Period Diet Per cent	No. of Females Involved	No. of Females Producing Litters	No. of Unproductive Females	Weight of Females at 100 days Gm.	Weight of Females at 200 days Gm.	Av. Age of Female at First Litter days	No. of Litters Born	No. of Young Born	Av. Wt. of Young at Birth Gm.	No. of Young Weaned	Av. Wt. of Young at 21 Days Gm.	Av. No. of Young Per Female	Av. No. of Young Born Per Litter	Total Young Weaned Percent
1	--	31	Stock	18	18	0	183	233	115	54	418	6.2	275	31.2	23.1	7.7	66
2	--	32	Control	36	36	0	183	242	117	91	689	6.0	422	29.9	19.1	7.4	61
3	--	33	G* 2.5	18	17	1	187	217	118	35	253	5.9	165	29.8	14.9	7.2	65
4	--	34	PG* 2.5	18	16	2	176	218	124	38	260	5.5	147	29.3	16.3	6.8	57
5	--	35	G 5.0	18	18	0	202	234	111	43	325	5.4	212	30.2	18.1	7.6	65
6	--	36	PG 5.0	18	18	0	192	233	120	40	315	5.4	193	26.5	17.5	7.8	61
7	--	37	G 7.5	18	18	0	221	249	103	39	287	5.5	165	31.3	16.0	7.4	58
8	--	38	PG 7.5	18	18	0	175	230	121	40	229	5.8	144	27.9	12.7	5.7	62
9	--	39	G 10.0	18	15	3	178	242	131	49	349	6.0	170	30.3	23.3	7.1	49
10	--	40	PG 10.0	18	16	2	160	242	160	46	280	5.8	158	29.6	17.5	6.1	54
11	--	41	G 20.0	18	18	0	169	228	130	51	392	5.9	228	27.7	21.8	7.7	58
12	--	42	PG 20.0	18	16	2	159	204	159	38	204	6.0	120	27.8	12.7	5.4	59
13	--	43	G 30.0	18	17	1	165	227	132	51	361	6.0	239	25.2	21.2	7.1	66
14	--	44	PG 30.0	18	9	9	150	215	175	18	113	6.0	77	23.9	12.5	6.3	68
15	39	32	Control	18	17	1	184	253	109	34	267	5.7	179	30.2	14.8	7.8	67
16	39	39	G 10.0	18	18	0	168	200	101	37	278	5.6	200	28.5	15.4	7.7	62
17	39	40	PG 10.0	18	18	0	155	201	150	36	234	5.5	157	27.1	13.0	6.4	67
18	40	32	Control	14	14	0	177	206	98	32	226	5.5	158	29.5	16.1	7.1	69
19	40	39	G 10.0	16	16	0	167	198	114	34	236	5.6	153	28.8	14.8	7.0	65
20	40	40	PG 10.0	16	16	0	158	204	121	35	223	5.4	158	28.1	14.0	6.4	68
21	42	32	Control	18	18	0	203	241	111	39	361	5.6	197	31.1	20.6	9.3	54
22	42	41	G 20.0	18	18	0	200	260	100	40	333	5.5	191	30.4	18.5	8.3	57
23	42	42	PG 20.0	18	18	0	178	218	124	35	237	5.6	154	28.6	13.2	6.8	65

[1]See Table II for composition of diets.

[2]Calculated on the basis of an isocaloric amount of starch.

*G = glycerol; PG = propylene glycol.

Data

Since space does not permit a full presentation of all the data obtained in the course of this investigation, only typical data are presented. Inasmuch as the investigation involved female responses primarily, the data presented concern particularly this sex. The data presented in Table III and Figures 1-5 inclusive, typify some of the findings of this investigation. Some of these data have been subjected to statistical analyses and where the findings were statistically significant this has been pointed out.

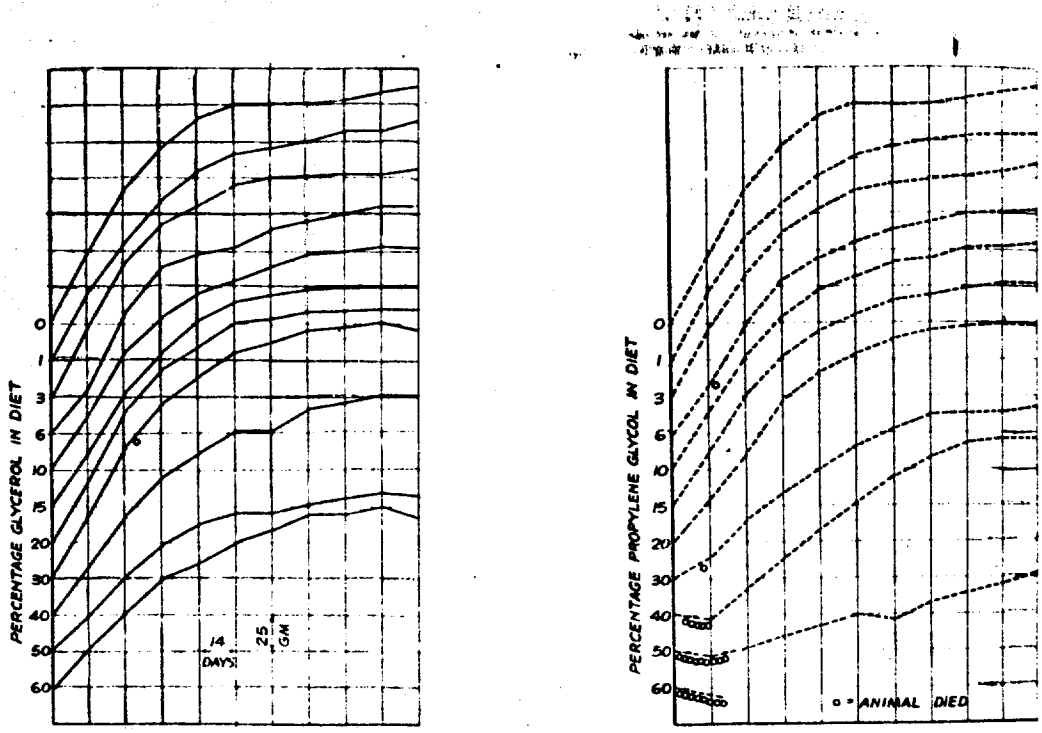

Figure 1 - The effect of replacing varying percentages (by weight), of the carbohydrate component of the diet by equal weights of glycerol or propylene glycol on the rate of growth of young female rats (for composition of diets, see Table I).

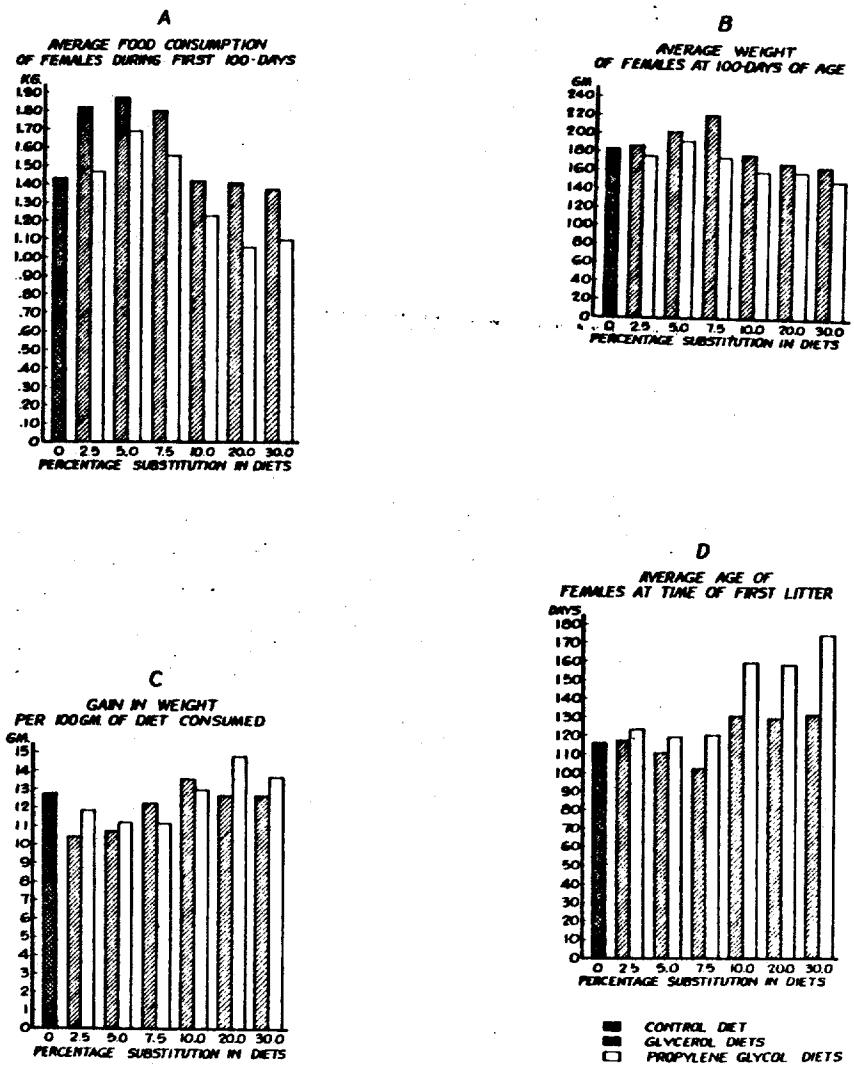

Figure 2. - The relative effects of the glycerol and of the propylene glycol content of the diet on the food intake, the rate of growth and the reproductiveness of young female rats. (The glycerol and propylene glycol were incorporated in the respective diets in equal caloric amounts. See Table II).

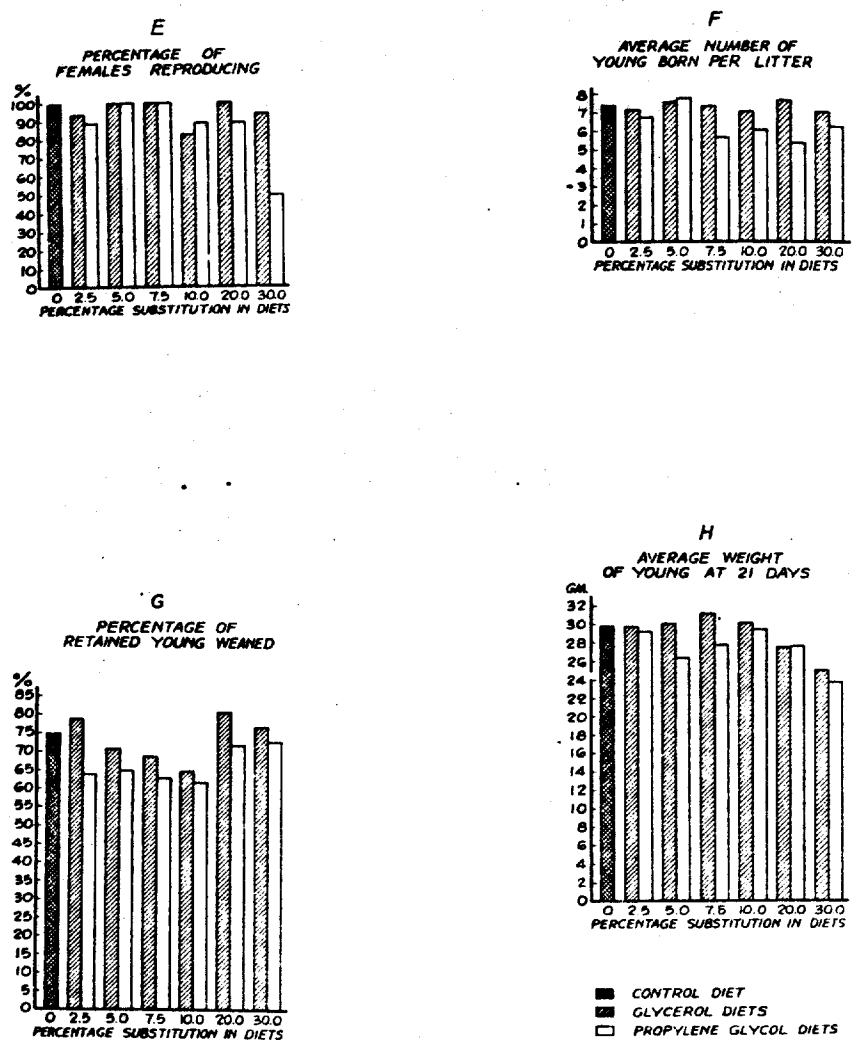

Figure 3. - The relative effects of the glycerol and of the propylene glycol content of the diet on reproduction by young female rats, with special reference to the number of litters born, the size of litters, the percentage of young weaned and the rate of growth of young. (See Table II for the composition of the various diets employed).

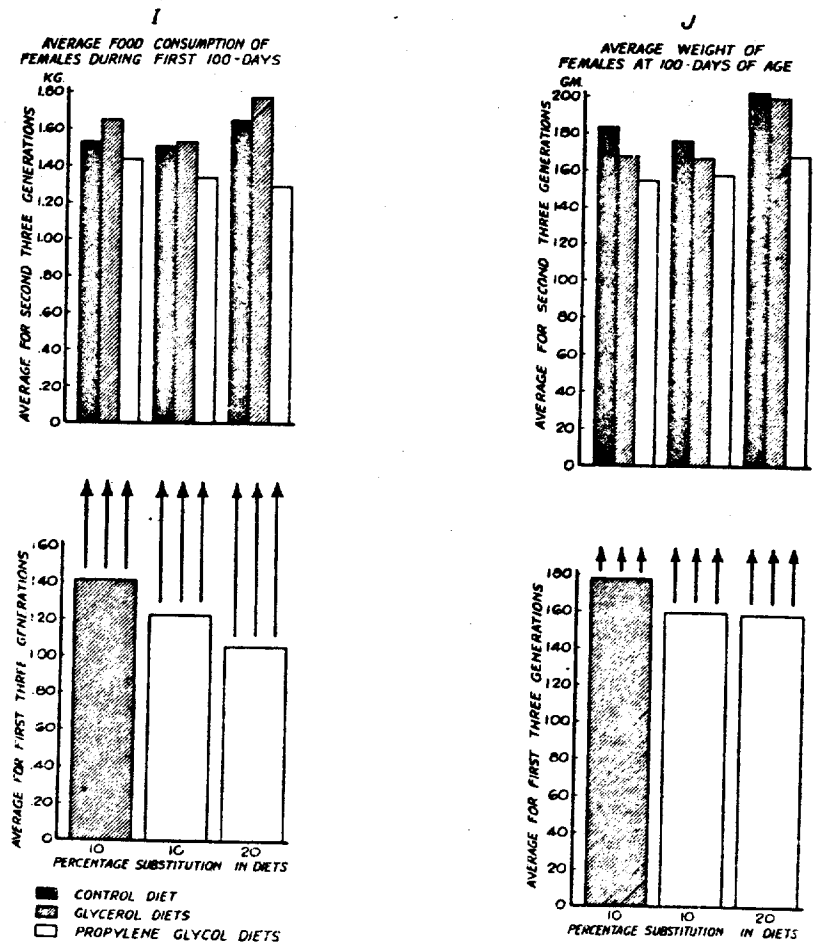

Figure 4. - The effect on food consumption and on growth of transferring growing rats which had been reared for three generations on glycerol or on propylene glycol-containing diets to the control diet and to the corresponding glycerol or propylene glycol diet, and continuing them under observation for three additional generations. (See Table II for the composition of the various diets employed).

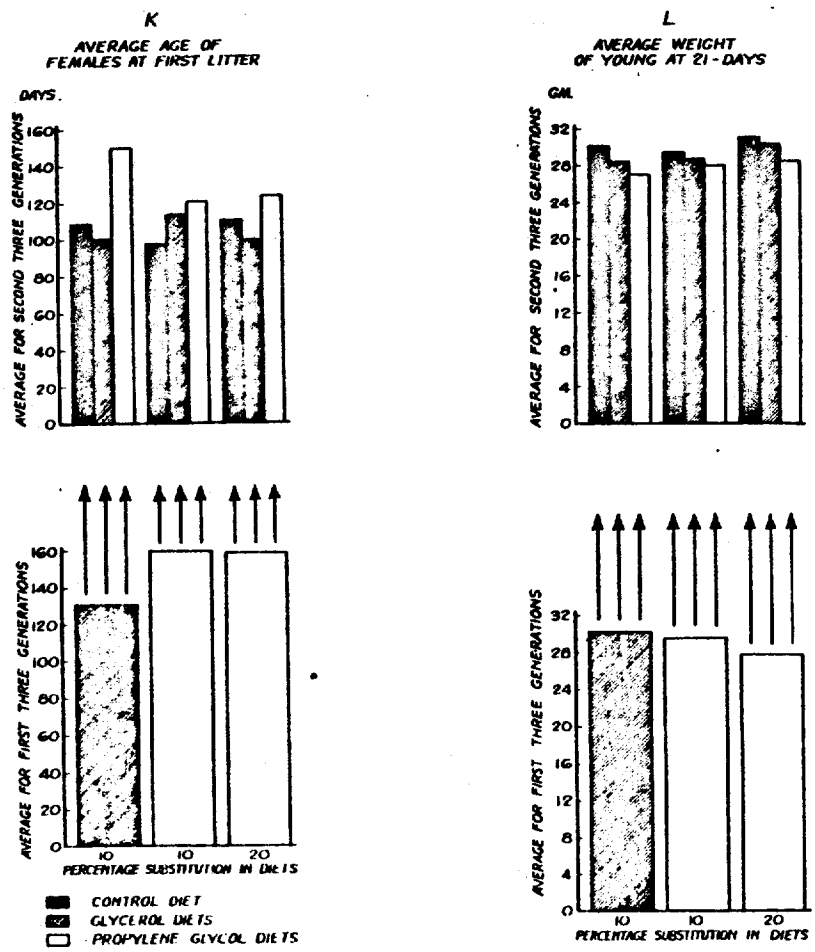

Figure 5. - The effect on reproduction and on growth of the progeny of transferring growing rats which had been reared through three generations on glycerol or on propylene glycol-containing diets to the control diet and to the corresponding glycerol or propylene glycol diet and continuing them under observation for three additional generations. (See Table II for the composition of the various diets employed).

Discussion

Some of the more important findings of the early phases of the investigation have been reported briefly in a previous publication (10). These findings, together with those obtained through subsequent experimentation, will be discussed more fully at this time.

Food Intake and Growth

In the initial experiments in which a series of purified diets containing ten different increments of glycerol and propylene glycol were fed to growing rats (males and females) no marked differences in the growth responses could be detected until the alcohols were fed at the 30 per cent level. At this level the glycerol animals grew at approximately normal rates, while the propylene glycol animals grew less rapidly (see Figure 1). Weanling rats invariably died within a few days after being placed on diets containing 40, 50 and 60 per cent of propylene glycol. When comparable groups of young rats were fed diets containing similar percentages of glycerol, growth was depressed somewhat but practically all of the test animals survived a 20-week feeding period.

A statistical examination of the growth data revealed that where the concentration of the respective alcohols in the diet was 7.5 per cent or more, the glycerol-fed rats made significantly greater gains in body weight ($P = .01 - .05$)[1] than did the corresponding propylene glycol-fed rats (Figures 1 and 2B). An examination of the growth data of the young females used in the reproduction studies (Figure 2B, also see Table III) reveals that first generation rats made somewhat greater gains in body weight during the first 100 days than did second and third generation females but the differences were not statistically significant. This was likewise true at 200 days of age. Older rats (half grown) showed greater tolerance of the diets containing the higher percentages of propylene glycol than did young rats, but their growth rates were not equal to those of animals receiving diets containing comparable percentages of glycerol. Even when the older rats were employed, none of the test animals receiving the diets containing 60 per cent of propylene glycol survived the 20-week test period.

While only the growth responses of female rats are given (Figure 1), the growth responses for corresponding groups of male rats were of the same relative magnitude.

When young rats were fed diets containing 30 per cent of glycerol and 30 per cent of propylene glycol during alternate weeks, growth responses invariably increased during glycerol periods and were depressed during propylene glycol periods. When groups of young rats were fed purified diets containing 30 per cent of either

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[1]Where P is greater than .01 indicates a probability of less than 1 per cent that the difference is due to chance.

corn starch, dextrin, glucose or sucrose in combination with 30 per cent of glycerol or propylene glycol, growth rates were not affected by the type of carbohydrate employed, but in each instance the glycerol-fed animals made greater gains than the propylene glycol-fed animals. When rats were fed diets containing isocaloric quantities of glycerol and propylene glycol (approximating 2.5, 5.0, 7.5, 10, 20 and 30 per cent of the diet)[1] through three generations, the average weight of the females at 100 days of age was always less for the propylene glycol-fed rats than for the glycerol-fed animals (Figure 2B). In the case of both glycerol and propylene glycol-containing diets, third generation females weighed somewhat less at 100 days of age than first generation animals. However, the decrease in weight was more pronounced among the third generation propylene glycol-fed animals.

In every comparison where glycerol and propylene glycol diets were fed ad libitum to groups of rats, the glycerol diets were consumed in greatest quantities (see Figure 2A). This was true regardless of whether the alcohols were incorporated in the diets on equal percentage or equal caloric basis. When groups of young rats were permitted to choose between glycerol and propylene glycol diets during a five-week test period, the diets were consumed at the rate of approximately 20 Gm. of glycerol diet to 1 Gm. of propylene glycol diet. When the diets were changed during alternate weeks, over a 12-week period, the rats consumed approximately 25 per cent more food during the week in which the glycerol diet was fed than during the following week in which the propylene glycol diet was fed.

When starch, glycerol and propylene glycol diets were fed on the restricted basis but in equal caloric amounts (approximately 30 per cent of glycerol or propylene glycol) over an 8-week period, the rates of growth of three groups of rats were essentially the same, indicating no marked differences in the efficiency of the utilization of the three sources of energy. Where diets containing isocaloric amounts of glycerol and propylene glycol (approximately 30 per cent of each) were fed ad libitum to young growing rats, the propylene glycol animals made slightly greater gains in body weight per unit of food intake than did the glycerol-fed animals. Likewise, during the reproduction studies it was found that in four out of six possible comparisons, the young female rats receiving the propylene glycol-containing diets made greater gains in body weight per unit of food intake than did the glycerol-fed animals (see Figure 2C). Apparently these differences may be attributed to the more efficient food utilization by the slower growing animals.

Water Intake, Urine Volume, Urinary Excretion

Comparisons relative to water intake, fecal elimination and general activity were not possible at the higher levels of alcohol

[1]For composition of diets, see Table II.

intake (40, 50 and 60 per cent) owing to the high mortality among the rats receiving the propylene glycol diets. Where comparisons were possible, the glycerol-fed animals consumed less water (17 per cent), eliminated a greater number of fecal pellets and were considerably more active as indicated by the number of rotations of the wheel of the exercising cage than were comparable groups of propylene glycol-fed rats. Although there was considerable variability in the amounts of water consumed by the different animals constituting the various experimental groups, the water intakes of those animals receiving the diets containing the higher percentages of glycerol were especially large, the average daily intake being 21 ml. for the control group as compared with 62 ml. for the 60 per cent glycerol group. These increases in water intake were reflected in greater urine output. Since the animals receiving the diets containing the higher percentages of propylene glycol had died during the early days of the feeding tests, no comparison of daily urine volume was possible for these glycerol and propylene glycol groups.

An examination of the urinary excretions of representative rats from the various groups employed in the growth studies revealed that those animals receiving the high propylene glycol diets (40, 50 and 60 per cent) eliminated considerable quantities of propylene glycol in the urine. The amount eliminated seemed to depend on the amount of propylene glycol in the ingested diet and frequently ranged as high as 25 per cent of the intake. On the other hand, the amount of glycerol in the urine never exceeded 15 per cent of the amount ingested. It appears that these differences in urinary elimination may partially explain the relatively poor growth obtained with diets containing high percentages of propylene glycol.

Before death, many of the young rats subsisting on diets containing the higher concentrations of propylene glycol manifested symptoms which seemed to be characteristic of thiamine deficiency although the diets were shown to contain ample quantities of this vitamin. As a result, some of the urines collected were assayed for thiamine by the thiochrome method. The results showed that the propylene glycol-fed rats were excreting considerably larger quantities of thiamine than were the glycerol-fed rats. This was true particularly of those rats receiving diets containing 30 per cent or more of propylene glycol. It is not possible to state whether the differences were due to differences in metabolic requirement for thiamine or to an abnormal kidney function associated with propylene glycol metabolism.

Blood Glucose and Hemoglobin

The data do not show that blood glucose or the hemoglobin values bore any definite relationship to the diet consumed. All glucose and hemoglobin values were within the range of those usually reported for rats subsisting on normal diets. A second series of determinations made on the bloods of the same rats at a later stage of the experiment yielded somewhat higher hemoglobin values. This would indicate that neither glycerol nor propylene

glycol exerts any marked depressing effect on normal hemoglobin formation.

Body Composition

The composition of the diets ingested by this series of growing rats did not appear to have any effect on the water content of the body tissues, the fat content or on the iodine number of the fat. The values obtained were in accord with those obtained on the control animals and were in agreement with the values reported in the literature for normal rats.

The concentration of glycogen in the livers of glycerol-fed rats was higher than that of livers from rats receiving comparable propylene glycol diets. The concentration of liver glycogen found was somewhat higher for the glycerol fed rats than the values reported by Deuel and co-workers (11).

Pathological and Bacteriological Findings

Pathological studies[1] frequently revealed changes in the tissues of rats from certain dietary groups. These changes were found to be more characteristic and pronounced as the concentration of dietary glycerol or propylene glycol increased. No consistent pathological changes were observed in the tissues from animals which had received diets containing 7.5 per cent or less of glycerol or propylene glycol. Tissues from animals which had received diets containing 10 per cent of these substances frequently showed pathological changes. However, tissues from animals which had subsisted on diets containing 20 per cent or more of these alcohols were found to be more frequently involved and the extent of involvement was more pronounced. In general, the most marked pathological changes found in the glycerol-fed animals were found in the liver (marked hydropic and fatty degeneration of parenchymatous cells), while the kidneys were most severely involved (degeneration of tubular epithelium, accompanied by interstitial hemorrhage and edema, acute glomerular nephritis and with calcified areas in the cortex) in the propylene glycol-fed rats.

Tissues from mature animals reared and maintained on diets containing glycerol and propylene glycol (approximately 10, 20 and 30 per cent, respectively) showed essentially the same pathology as did the younger animals. While these pathological changes appeared to be somewhat more severe in the tissues from those rats which had received diets containing the higher percentages of polyhydric alcohol, tissues from second and third generation animals reared and maintained on such diets did not show an increased pathology over the tissues from the first generation animals.

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[1]We are indebted to Dr. W. T. S. Thorp, Experiment Station pathologist, for his generous cooperation during the course of the investigation.

Growing rats subsisting on purified diets exhibited no marked differences in either the type of the number of bacteria found in the intestinal tract of the glycerol or the propylene glycol-fed rats, except on those diets containing the higher concentrations of these substances. Under the latter conditions, proteolytic anaerobes and Lactobacillus acidophilus organisms were reduced to the point where difficulties were encountered in obtaining suitable cultures for study.

Reproduction and Rearing of Young

Reproduction and rearing of young were entirely unsatisfactory among all groups of animals subsisting on the purified diets, due in part, to a high degree of sterility among the animals, to weakness of females, to lack of care for the litter, and to poor growth and high mortality among the young. Reproduction and rearing of young were slightly more satisfactory among the animals receiving the glycerol diets. Attempts to improve reproduction and rearing of young by supplementing the purified diets with vitamins and minerals were without marked success. Consequently, further attempts to carry on reproduction studies with purified diets were abandoned.

Reproduction studies with diets composed of the breeding colony diet (70 per cent) and glycerol (30 per cent) or propylene glycol (30 per cent) resulted in some very striking differences. Rats which received the glycerol diet were carried through seven successive generations with a considerable degree of success while similar groups of rats receiving the propylene glycol diet failed to produce the third generation of young. The females not only failed to breed normally but when they did breed and gave birth to five litters, they were unable to feed the young satisfactorily.

Since the protein, mineral and vitamin content of the stock diet was diluted appreciably by the incorporation of 30 per cent of glycerol or 30 per cent of propylene glycol, it was impossible to state whether the poor reproduction experienced on the above diet was due to the inclusion of propylene glycol or to the diluting effect of this substance on certain essential dietary ingredients which existed in the original diet at or near threshhold levels. Consequently, a more extensive series of reproduction studies were initiated in which the diets employed contained protein, minerals and vitamins from natural sources and in which part of the energy was furnished on an isocaloric basis as glycerol or as propylene glycol (see Table II). Since diet No. 31 of this series is the breeding colony diet, the results obtained from feeding this diet are not included in this report.

As a whole, the reproduction studies with this series of diets were relatively successful in that animals from each dietary group were continued under observation through three successive generations. Figure 2D shows the effects of dietary treatment on the average age of females at the time of the birth of the first litter. These effects were obtained in spite of the fact that male and female animals were caged together from the time of wean-

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ing until the pregnant females were removed from the cages (61 - 167 days). Those animals receiving the starch diet (control group) were among the first in their respective generation to produce litters. The youngest female to give birth to a litter was 78 days old and was from the third generation animals.

While no significance could be attributed to a specific generation in respect to the average age of the females at the time of dropping the first litter, the glycerol-fed females were younger at the time of the first litter than the corresponding propylene glycol-fed animals, the difference being statistically significant (P = 0.4) where the alcoholic intake was 7.5 per cent and above. All females (12 from each generation) receiving the control diet produced litters, and the average age of the 36 females at the time of their first litter was 117 days. Propylene glycol-fed rats were, on the average, somewhat older at the time of birth of the first litter than were the glycerol-fed animals. The difference was rather striking. The average age of the propylene glycol females was greater in all six comparisons, the differences being 6, 9, 18, 29, 29 and 43 days, respectively, for the six concentrations of propylene glycol employed. Here again, the effect of the diet on the age of the female at the time of the first litter was neither amplified nor decreased through three successive generations.

In view of the limited data available (Figure 3E) it is difficult to state with any degree of certainty what effect the composition of the diet had on the ability of the females to reproduce. This is especially true of those groups of animals from which one or more females died before reaching the reproduction age. All females receiving the control diet were fertile and produced one or more litters of living young. The same was true of the 18 females receiving the diet containing 5, 7.5 and 20 per cent of glycerol and 5 and 7.5 per cent of propylene glycol. One of the females (third generation) receiving the 2.5 per cent glycerol diet died giving birth to the first litter while one female (first generation) receiving the 2.5 per cent propylene glycol ration died as a result of an injury and another (third generation) was found to be sterile.

Of the 18 females originally placed on the 10 per cent glycerol diet, one died before reaching reproductive age (first generation), another died while giving birth to a litter (third generation), and a third was apparently sterile (third generation). Likewise, of the 18 females subsisting on the 10 per cent propylene glycol diet, one died while giving birth to a litter (third generation) and a second was sterile (second generation). Two of the 18 females receiving the 20 per cent propylene glycol diet (second generation) and one of the 18 females that received the 30 per cent glycerol diet (third generation animals) proved incapable of producing litters.

Nine of the 18 females placed initially on the 30 per cent propylene glycol diet failed to give birth to litters. Two of the nine unproductive females were first generation animals, two were

second generation animals and five were from the third generation. Since attempts to mate the unproductive females with other males from the respective dietary groups and finally with normal males from the breeding colony met with failure, it seemed reasonable to conclude that their unproductiveness was due to sterility. However, subsequent pathological examination of reproductive organs failed to reveal any marked abnormality.

The total number of litters produced by the three generations of females while receiving the various diets is shown in Table III. It is to be noted that the average litter production by the females receiving the various diets was not markedly different except where diets containing high concentrations of propylene glycol (20 and 30 per cent) were fed. This does not reflect the true reproductiveness of the different groups of females since it was the general practice to discard females after they had successfully weaned two or three litters, regardless of age.

The contrast in the efficiency of reproduction of the females receiving the 30 per cent glycerol diet as compared to those receiving the corresponding propylene glycol diet was particularly striking. These particular females also showed statistically significant differences (P greater than .05) between the reproductive efficiency of the first, second and third generation females. To this group of 18 females, 18 litters were born, 11 from the first generation females, 6 from the second generation females and one litter from the third generation females.

An examination of the data relative to the number of young born per litter (Figure 3F) reveals that, in general, larger litters were produced by the females receiving the glycerol diets than were produced by the females subsisting on the propylene glycol diets. In five out of six possible comparisons, the females receiving the glycerol diets produced, on an average, larger litters than were produced by females receiving the corresponding propylene glycol diet. However, the size of the litter did not appear to bear any definite relationship to the concentration of the alcohol in the diet. The average birth weight of the young from the several dietary groups ranged from 5.4 Gm. to 6.2 Gm. and did not appear to bear any consistent relationship to composition of diets fed (see Table III).

Since all litters were reduced to six young on the fifth or sixth day after birth, it is obvious that larger numbers of young were discarded from those groups producing the largest litters. Of the 689 young born to the females receiving the control diet, 28 per cent were discarded. Of those young which were produced on the 2.5, 5, 7.5, 10, 20 and 30 per cent glycerol diets, 25, 28, 24, 25, 28 and 22 per cent, respectively, were discarded. The percentage of young discarded from the litters produced by the females receiving 2.5, 5, 7.5, 10, 20 and 30 per cent propylene glycol diets were 16, 28, 14, 14, 13 and 15 per cent, respectively.

Of those young which were retained, the glycerol-fed mothers weaned a larger percentage than did the propylene glycol-fed

mothers (Figure 3G). This was found to be true in all six comparisons. The concentration of the glycerol or of the propylene glycol in the diet did not appear to have any marked influence on the female insofar as her capacity to care for the young was concerned. When the young were weaned at 21 days of age there was found to be a definite relationship between the higher concentration of the polyhydric alcohols in the various diets and the weight of the young.

Young produced by the females receiving the high glycerol and the high propylene glycol diet (30 per cent) weighed, on an average, 20 per cent less than the young from the control group or from groups receiving the diets containing the lowest percentage of the alcohols (Figure 3H, Table III). In five out of six comparisons, the progeny of glycerol-fed mothers were heavier at 21 days of age than those from propylene glycol-fed mothers, the difference being statistically significant at the 5, 7.5 and 30 per cent levels ($P = .03 - .05$.) These data indicate that the growth rate of suckling rats is affected more severely by lower concentrations of dietary glycerol and propylene glycol (20 and 30 per cent) than that of more mature animals (Figure 1).

When the progeny of third generation rats were continued on the glycerol or the propylene glycol-containing diets for three additional generations, and when similar young were transferred to the corresponding alcohol-containing diets and carried through three additional generations, and when additional young were transferred to the control diet and continued on experiment for three generations, additional data were obtained (see Figures 3 and 4, also Table III) which substantiate those obtained in the previous reproduction studies.

Female rats which had been maintained for three generations on a 10 per cent glycerol diet and then transferred to a 10 per cent propylene glycol diet and carried through three generations consumed less of the propylene glycol containing diet, grew at a slower rate, reproduced their first litter at a more advanced age, and weaned smaller progeny than did similar animals which had been maintained on the glycerol diet or which had been transferred to the control diet and continued under observation for three additional generations.

Likewise, when young female rats which had been weaned by third generation mothers subsisting on the propylene glycol-containing diets (10 and 20 per cent) were transferred to the control diet or to corresponding glycerol diets and continued under observation for three additional generations, they consumed more food and grew at a faster rate during the first 100 days, produced litters at an earlier age and weaned larger young than did comparable females continued on the propylene glycol diets for three additional generations. During this period of observation, the glyc-

erol-fed rats produced somewhat larger litters and weaned a higher percentage of the retained young than did the corresponding propylene glycol-fed rats.

Taken as a whole, the findings of these studies do not substantiate the recent report of Lakritz (12) which states: "Many toxicity tests have been conducted on propylene glycol by several laboratories; it was found to have a low systemic toxicity, in fact much lower than glycerin".

It is to be noted that the concentration of glycerol and of propylene glycol in the various diets employed in this investigation ranged from 1 to 60 per cent of the diet. It is also to be noted that no measurable effect on growth, on reproduction or on the general health of the rat was observed unless the diet contained 7.5 per cent or more of the polyhydric alcohols. The concentrations in the diet at which these alcohols were found to have a deleterious effect on the general health, the growth and the reproduction of the rat were far in excess of that which may reasonably be expected in human diets. Furthermore, it is possible that man and rat may react differently to those substances when they are ingested.

Summary

Growth and reproduction studies with rats subsisting on diets in which a portion of the carbohydrate component had been replaced by varying amounts of glycerol or propylene glycol yielded the following results:

Young rats, receiving purified diets which contained varying percentages of glycerol and propylene glycol showed marked difference in growth response. These differences were particularly marked where the diets contained 30 per cent or more of the alcohols. At the 30 per cent level of intake, the glycerol-fed animals grew at an approximately normal rate, while the propylene glycol animals grew less rapidly. Young rats lost weight and frequently died within a few days when the diets contained 40, 50 and 60 per cent of propylene glycol, whereas other young rats receiving diets containing comparable amounts of glycerol grew at a subnormal rate, but the majority of the animals survived a 20-week test period. Older rats were able to tolerate the higher intakes of propylene glycol more effectively than younger rats, but their growth response was always depressed.

When growing rats were fed glycerol-containing diets and propylene glycol-containing diets during alternate weeks, growth responses increased during glycerol periods and decreased during propylene glycol periods. The glycerol diets seemed more palatable in that they were consumed in greater quantities than were the corresponding propylene glycol diets. Without exception, the animals excreted the greatest volume of urine, the greatest percen-

tage of the thiamine intake and the greatest percentage of the alcohol intake during the weeks in which the propylene glycol-containing diets were fed. At the highest level of intake, more than 25 per cent of the ingested propylene glycol was accounted for in the urine, while in no instance was more than 1.5 per cent of the ingested glycerol accounted for.

Reproduction and subsequent rearing of young was definitely unsatisfactory with all groups of rats receiving the purified diets. However, those groups of rats receiving the glycerol-containing diets gave birth to a greater number of litters and to larger litters, and they also succeeded in weaning a higher percentage of the retained young than similar groups of rats receiving comparable propylene glycol-containing diets. Seven generations of rats were reared without particular difficulty on a diet composed of 70 parts of a breeding colony diet and 30 parts of glycerol, while other rats subsisting on a comparable diet containing 30 parts of propylene glycol failed to wean third generation young. Furthermore, six generations of rats were reared on a well-balanced diet containing 10 per cent of glycerol or 10 and 20 per cent of propylene glycol, respectively. Under comparable conditions, growth and reproduction were more satisfactory among the glycerol-fed animals.

In a more extensive investigation in which a series of diets was used which contained proteins, minerals and vitamins from natural foods and in which part of the energy was furnished as glycerol or as propylene glycol, data were obtained which show that female rats receiving the glycerol-containing diets consumed more food, grew faster, reproduced at a younger age, produced, on the average, larger litters, weaned a greater percentage of the young and weaned larger young than did other female rats receiving corresponding propylene glycol-containing diets. The effect of these diets on subsequent generations was reflected most markedly in the number of litters born, the size of the litters and in the weight of the young at weaning.

There appeared to be no marked difference in either the frequency of observation or in the intensity of the pathological conditions observed among the glycerol and the propylene glycol-fed rats, the chief difference being that the livers of the former animals were more generally adversely affected while the kidneys of the propylene glycol animals were more usually abnormal. The frequency of observations of pathological conditions and the severity of the pathology appeared to bear some relation to the concentration of the alcohols in the diet, although these differences were not always clearly defined.

It is to be noted that the concentration of glycerol and of propylene glycol in the diets employed in these growth and reproduction studies with rats were, perhaps, in most instances, greater than will ever be encountered in human diets.

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Food Res.
19(2): 257-262, 1954

COMPARISON OF THE NUTRITIVE VALUES OF MONO-, DI-, AND TRIGLYCERIDES BY A MODIFIED PAIR-FEEDING TECHNIQUE[a,b]

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(Manuscript received October 10, 1953)

It is many years since mono- and diglycerides were shown by Lewkowitsch *(8)* to be normal constituents of certain natural fats and oils. The great interest in these simpler glycerides which has developed during recent years is due primarily to the fact that they are being used extensively in manufactured shortenings and margarines *(9)*, and that they are formed as intermediate compounds during the digestion of triglycerides by higher animals and by man *(2, 3, 5, 10, 13)*.

Tidwell *(12)* reported that the monoglycerides of olive oil fatty acids were absorbed by rats at a faster rate than olive oil alone or fatty acids alone. Ames *et al. (1)* concluded that there is no significant difference in the growth response, reproductive ability, or lactation performance of rats fed the monoglycerides of cottonseed oil as compared with cottonseed oil. These two lipids were observed to have the same coefficient of absorption.

Mattson *et al. (9)* fed mono-, di-, and triglycerides of the same fatty acid composition to weanling rats *ad libitum* as 25% of a synthetic diet, and reported that, except for slight inherent differences in caloric values, these three lipids were nutritionally equivalent. When their data were calculated in terms of caloric efficiency (gain in body weight per 100 calories of diet eaten) the weight increases were identical.

It is likely that growth response to food calories is not an arithmetic function. Furthermore, the nutrient intake of animals fed diets differing in caloric value is not the same. The present series of experiments was designed to meet these objections, for rats of comparable groups were fed the same intake of food calories day by day and the intake of nutrients was essentially the same.

In the pair-feeding method, individual animals are offered a test diet *ad libitum*, and comparable animals are fed a control diet in a restricted amount. Since this method is useful only when the animals have a greater appetite for the control diet than the test diet, the animals on the control diet exist in a condition of mild starvation during the test period. The pair-feeding procedure has been criticized on this account. It is quite possible that the comparison would be more accurate if both the animals on the test and those on the control diets were pair-fed at levels which are less than their desires.

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[a] Publication No. 174 from the Department of Food Technology, The Massachusetts Institute of Technology, Cambridge.

[b] Presented before the Thirteenth Annual Meeting of the Institute of Food Technologists, Boston, June 23, 1953.

The pair-feeding technique cannot be used to compare two diets which are equally appetizing to the test animals, for the *ad libitum* ingestion of the test diet sometimes exceeds that of the animals on the control diet. It is possible, however, to conduct a pair-feeding experiment on diets with similar appetite appeal by restricting slightly the food intake of the test group, and pair-feed the control diet at that level. In this paper this procedure will be referred to as the "restricted pair-feeding technique."

The restricted pair-feeding was carried out on a group rather than on an individual rat basis. Since the animals were carefully randomized between all groups there was no significant difference in the *ad libitum* requirements of the groups within a series and the comparisons are valid.

In the three series of experiments reported here, weanling rats were offered mono-, di-, and triglycerides and a mixture of these three fats, in a synthetic-type diet at approximately 15% and 25% levels, and in a diet of natural foodstuffs at approximately 15% levels. Within each series the fatty acid content of each diet per 100 calories was the same and the total calories offered each day to each group within each series were identical. Moreover, the protein/caloric ratio of the diets was the same in each experimental series.

EXPERIMENTAL

Preparation and characterization of diet fats. The triglycerides were prepared[5] from a mixture of partially hydrogenated soybean and cottonseed oils. The mixture

[5] The fats used in this study were prepared under the direction of Dr. F. V. Baur, Chemical Division, The Procter & Gamble Co., Cincinnati, Ohio.

TABLE 1

Characteristics of test fats[4]

	Monoglycerides	Diglycerides	Triglycerides	Mixture of mono-, di-, triglycerides
Glycerides				
Complete melting point	54.2	48.6	47.0	47.6
Saponification value	160.2	181.8	191.1	174.4
Acid value	9.9	1.7	<0.1	4.7
Hydroxyl value	303.0	95.0	5.0	165.0
Iodine value	62.3	71.7	74.8	68.1
Peroxide value (freshly opened cans)	0.0	0.10	0.51	0.28
Free glycerol	1.26%	0.03%	0.01%	0.71%
Monoglycerides	86.4	3.5	0.9	32.8
Diglycerides	6.6	92.3	2.2	52.8
Triglycerides	0.7	3.3	96.9	11.3
Free fatty acids	5.0	0.9	<0.1	2.4
Total fatty acids	79.3	90.7	95.5	86.6
Fatty acid composition				
Saturated acids	18.1%	17.4%	18.0%	17.8%
Oleic acid	76.8	77.5	77.0	77.1
Linoleic	4.9	4.8	4.7	4.9
Linolenic	0.04	0.06	0.06	0.05
Conjugated dienoic acids	0.20	0.21	0.19	0.18
Conjugated trienoic acids	....	<0.01	....	....

[4] Data supplied by the Technical Committee of the Institute of Shortening and Edible Oils, Inc.

TABLE 2

Composition of diets

Diet Group	Experiment A				Experiment B				Experiment C			
	224	225	226	227	228	229	230	231	232	233	234	235
Components	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Casein	26.2	26.8	27.0	26.6	26.2	26.8	27.0	26.6				
Sucrose	40.2	41.3	41.5	41.0	50.6	51.4	51.5	51.1				
Soybean oil	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0				
Salt mixture (6)	3.9	3.9	4.0	3.9	3.9	3.9	4.0	3.9				
Celluflour	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0				
Vitamin mixture*	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5				
Skim milk powder									21.6	21.6	21.6	21.6
Whole wheat									55.60	57.00	57.40	56.50
Meat powder (71% protein)									4.0	4.0	4.0	4.0
NaCl									1.2	1.2	1.2	1.2
$CaCO_3$									0.77	0.77	0.77	0.77
Vitamin A									1200IU	1200IU	1200IU	1200IU
Vitamin D									120IU	120IU	120IU	120IU
α-tocopherol												
Monoglycerides	27.2				16.8				16.8			
Diglycerides		25.5				15.4				15.4		
Triglycerides			25.0				15.0				15.0	
Mixture of mono-, di-, and triglycerides				26.0				15.9				15.9
Calories/100 g	509.1	521.0	524.6	517.3	464.2	469.8	471.4	468.5	416.9	421.4	423.0	420.4

*Vitamin mixture (mg. 100 g. diet): thiamine HCl, 0.4; riboflavin, 0.5; pyridoxine HCl, 0.4; niacin, 2.0; Ca pantothenate, 2.0; inositol, 200.0; biotin, 0.03; folic acid, 0.25; choline chloride, 300.0; ascorbic acid, 10.0; menadione, 0.3; α-tocopherol, 10.0; p-aminobenzoic acid, 10.0; also 1250 USP units vitamin A and 125 USP vitamin D 100 g. diet.

of mono-, di-, and triglycerides was prepared from this hydrogenated triglyceride by interesterification at 180°C. with glycerol using sodium hydroxide as a catalyst *(4)*. The monoglycerides and diglycerides were prepared from the mixture of mono-, di-, and triglycerides by molecular distillation *(7)*. The characterizations of these 4 fats are given in Table 1.

Experimental procedure and composition of the diets. Weanling albino rats of the Sprague-Dawley strain were distributed into 12 groups of 15 rats each so that the average and the mean weights of each group were the same. The groups of animals were offered distilled water and the experimental diets outlined in Table 2.

In Experiment A the 4 test fats were fed at approximately 25% levels in a synthetic-type diet which was rich in high quality protein and contained liberal amounts of all minerals and vitamins known to be required in rat nutrition; in addition, other nutrients which are not required by rats when living on natural diets were included. At the beginning, each of the 4 groups was offered approximately 90% of its *ad libitum* demands as determined by a preliminary trial on a diet (No. 226) fed to rats of similar age. As the experiment progressed various groups failed to consume all the diet offered during each 24-hour period, and it was necessary to change the daily feeding levels to 80%, 70%, and finally 75% of the estimated *ad libitum* requirement. During the 70 days of this experiment each group consumed essentially all of the diets offered. Account was taken of the small differences in the caloric content of each diet and feedings were conducted on an equi-caloric basis, rather than on an equi-weight basis.

In Experiment B the 4 fats were fed at approximately 15% levels in the same synthetic-type diet as was fed in Experiment A. The initial feeding level was at 90% of the expected *ad libitum* demand; later the feedings were adjusted to 80%, to 75%, and then to 80% of this level. Feedings to the 4 groups were conducted on an equi-caloric basis.

In Experiment C the 4 fats were fed at approximately 15% levels in a natural diet similar to the Sherman B diet *(11)* but modified by replacing the whole milk powder by equivalent amounts of skim milk powder and test fats. These diets were first offered at a level estimated to be 90% of the *ad libitum* demand of weanling rats. Later it was changed to 75%, to 80%, and finally to 75% of *ad libitum* demand. All 4 groups were fed on an equi-caloric basis day by day.

TABLE 3

Summary of body weight and food consumption data

Diet No.	Diet characteristic	Rats/group	Weight gain/rat	Length of tibiae	Diet consumed /rat/day	Calories consumed /rat/day	Wt. gain /calorie consumed
				Experiment A. 25% test fats in synthetic-type diet			
			gm.	*mm.*	*gm.*	*cals.*	*gm.*
224	Monoglycerides	15	214 ± 3	39.1 ± 0.1	10.79	54.9	0.056
225	Diglycerides	15	211 ± 2	39.1 ± 0.1	10.43	54.3	0.055
226	Triglycerides	15	207 ± 2	38.8 ± 0.2	10.49	55.0	0.054
227	Mixture of mono-, di-, triglycerides	15	214 ± 2	38.9 ± 0.1	10.64	55.0	0.056
				Experiment B. 15% test fats in synthetic-type diet			
228	Monoglycerides	15	219 ± 2	38.7 ± 0.1	11.84	55.0	0.057
229	Diglycerides	15	220 ± 2	38.9 ± 0.2	11.60	55.1	0.058
230	Triglycerides	15	219 ± 2	38.9 ± 0.1	11.69	55.1	0.057
231	Mixture of mono-, di-, triglycerides	15	221 ± 3	39.0 ± 0.1	11.64	54.5	0.058
				Experiment C. 15% test fats in natural diet			
232	Monoglycerides	15	200 ± 2	37.8 ± 0.1	11.99	49.9	0.057
233	Diglycerides	15	201 ± 2	38.4 ± 0.2	11.78	49.6	0.058
234	Triglycerides	15	202 ± 2	38.4 ± 0.1	11.76	49.7	0.058
235	Mixture of mono-, di-, triglycerides	15	201 ± 2	37.9 ± 0.1	11.74	49.4	0.058

In all 3 experiments the rats were housed individually in wire mesh cages with raised flooring. The diet to be fed each group was divided equally between the 15 rats in the group and placed in feeding jars which were designed to minimize scattering. The food remaining in the feeding jars, and the small amount that was scattered during each 24-hour period were weighed. An amount of fresh diet equivalent to that scattered and uneaten was added to the ration to be fed during the following 24-hour period. Since very little food was scattered and since the several groups were fed less than their desires, this carry-over feeding of the diets proved to be an unimportant refinement in procedure. All animals were weighed twice weekly.

At the end of 70 days the rats were autopsied, a tibia from each rat was photographed by x-ray and the length of the tibia was measured on the photographic negative.

DISCUSSION

The data in Table 3 indicate that there were no significant differences in the increases of the body weights of groups fed the different fats in the same diets on an equi-caloric basis, under conditions of slightly restricted food intake. This observation was independent of the type of diet (natural or "synthetic") or the quantity of fat (15% or 25%) fed in the "synthetic" diet.

The average lengths of the tibiae of the rats receiving these diets were approximately the same, indicating that the skeletal size of the rats in each group was the same. The tibiae of the rats fed the natural diets were shorter than the tibiae of the rats fed the synthetic type diets. This would be expected for the animals on the natural diet consumed approximately 10% less calories and gained less body weight during the experiment.

The restricted pair-feeding technique employed in these experiments was designed to meet the most serious criticism which has been leveled against the original pair-feeding procedure. The groups in each series were maintained in a state of mild hunger which was essentially the same in all groups.

The animals in these 3 series of experiments showed a weight increase which was 60-70 g. less than was observed in a preliminary experiment with rats on the same diets fed on an *ad libitum* basis for 70 days. The comparisons within each experimental series are valid, nevertheless, because all groups received the same treatment.

SUMMARY

Male weanling rats were fed mono-, di-, and triglycerides and a mixture of these fats as 15% and 25% of a synthetic-type diet, and as 15% of a diet of natural foods, for 70 days on an equi-caloric, restricted, pair-feeding basis.

No significant difference in body weight increase or caloric-efficiency was observed and these fats appear to be nutritionally equivalent, calorie for calorie.

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TOXICOLOGY AND APPLIED PHARMACOLOGY 4, 107-115 (1962)

Pleural and Pericardial Effusions Associated with Daily Intravenous Administration of Acetylated Tartaric Acid Esters[1]

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Received June 19, 1961

Daily intravenous administration of two fat emulsions, Lipomul I.V. and SR emulsion, to dogs on occasion produced such undesirable side effects as anemia, gastrointestinal bleeding, ulceration, and pleural-pericardial effusion (Hartwig *et al.*, 1961). In an effort to establish the role of the emulsified-stabilizer system of the fat emulsion in the production of these side effects, dogs were infused with a suspension of one of the stabilizers of the SR emulsion, TEM (diacetyl tartaric acid ester of mixed mono- and diglycerides).

Two of the side effects associated with the infusion of the whole SR emulsion, anemia and pleural-pericardial effusions, developed. This report describes these and other changes that developed following daily infusions of commercial (as received) and purified lots of TEM.

MATERIALS AND METHODS

The thirteen mongrel dogs used were fed Purina Dog Chow and water throughout the infusion period. The TEM[4] preparation was infused

[1] This study was supported by Contract no. DA-49-007-879 from the Research and Development Division, Office of the Surgeon General, Department of the Army, Washington, D. C.

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TABLE 1
OBSERVATIONS ON DOGS INFUSED WITH TEM

Dog number	Weight (kg)	Dosage (ml/kg)[a]	Number of infusions	Outcome
1	16.1–14.4	30(U)	18	Sacrificed
2	10.0– 9.6	50(P)	22	Died
3	7.2– 6.4	50(P)	22	Sacrificed
4	12.4–10.2	50(P)	20	Died
5	11.0–10.3	45(U)	18	Sacrificed
6	9.6–10.0	50(U)	4	Died
7	11.0–11.7	45(U)	16	Sacrificed
8	9.1– 8.5	55(P)	8	Sacrificed
9	9.2–11.7	55(P)	14	Sacrificed
10	9.0–	56(P)	4	Died
11	8.5– 7.9	59(P)	30	Sacrificed
12	13.2–13.8	38(P)	23	Sacrificed
13	12.1–15.0	40(P)	30	Held for observation

[a] Letter in parentheses denotes whether the TEM was infused purified (P) or unpurified (U).

through a permanent jugular cannula (Cohn *et al.*, 1960) while the dog remained in his cage. Dogs were infused 5 days a week for a total of 30 infusions. At the end of this time they were sacrificed and autopsied. Dogs that died during the infusion period were also autopsied. Tissues were taken for microscopic examination and a duplicate set was sent to the U.S. Army Nutrition Laboratory[5] for analysis of the intravenous fat pigment. This pigment has been observed in the liver and spleen of dogs that have received multiple infusions of a cottonseed oil emulsion containing as one of its stabilizers, TEM (Hartwig *et al.*, 1961).

TEM is a synthetic product commercially prepared. In this investigation, suspensions were prepared with the TEM as received, or after the following purification procedures. TEM was dissolved in acetone, 1 part by weight to 2 volumes of acetone, and cooled to 2° C. The fraction which crystallized at this temperature was removed and discarded. The soluble portion was further cooled to —15° and filtered. The crystallized fraction, purified TEM, was stripped of solvent by means of nitrogen

[5] The authors wish to thank Major S. W. Thompson, II, U. S. Army Medical Nutrition Laboratory, Fitzsimons Army Hospital, Denver, Colorado, for staining and evaluating the many tissues sent him from this project.

Effusions (ml)			Hematology[b]			
Thorax		Pericardial	Hemoglobin (mg%)		WBC	
Right	Left	sac	Initial	Last	Initial	Last
0	0	0	15.7	13.0	25,100	46,600
0	0	0	10.7	6.9	5,000	29,000
0	0	0	11.0	8.5	25,000	16,000
400	400	100	15.7	12.0	17,400	15,300
400	400	20	13.4	10.7	27,000	21,000
20	32	0	13.0	12.3	16,100	11,600
0	0	0	11.6	10.7	15,400	16,050
0	0	0	11.6	10.7	14,800	17,300
2	5	3	8.5	8.8	32,000	23,600
25	25	5	10.2	8.8	28,100	13,700
0	0	0	16.6	11.6	12,800	10,800
800	800	20	11.6	11.6	29,300	13,300
0[c]	0	—	11.6	9.0	9,800	32,000

[b] Initial = preinfusion value; last = value at last infusion.
[c] As determined by thoracentesis.

and reduced pressure. Both the commercial and purified TEM products were analyzed by infrared spectroscopy, using a 1-mm cell at a concentration of 5 g/l of CCl_4. Four absorption bands were used as reference points, with absorption maxima at 2.8 mμ for hydroxyl, 3.5 mμ for CH_3CH_2 stretching, 5.8 mμ for ester group, and 8.4 mμ for carbon-oxygen. The fractionation procedure removed unreacted monoglyceride and fully esterified material from the commercial TEM, as determined by the spectrophotometric method. The acidity of TEM, as milliequivalents of KOH per gram, was 1.577.

Suspensions of TEM at a concentration of 0.5% in isotonic glucose were prepared by homogenization at 3500 psi and sterilized by autoclaving.

RESULTS

Daily infusion of a TEM suspension produced a bilateral pleural effusion in 46% of the dogs studied (Table 1). All but one of the dogs with a pleural effusion also had a pericardial effusion. The effusions were associated with both purified and unpurified preparations of TEM. In each instance of massive effusion the dogs showed no significant signs of

distress until 3 or 4 days prior to demise or sacrifice. Obvious signs at this time were dyspnea and downward displacement of the liver.

The accumulation of the fluid occurred rapidly. A thoracentesis was performed on a dog which had received 11 infusions of a TEM stabilized

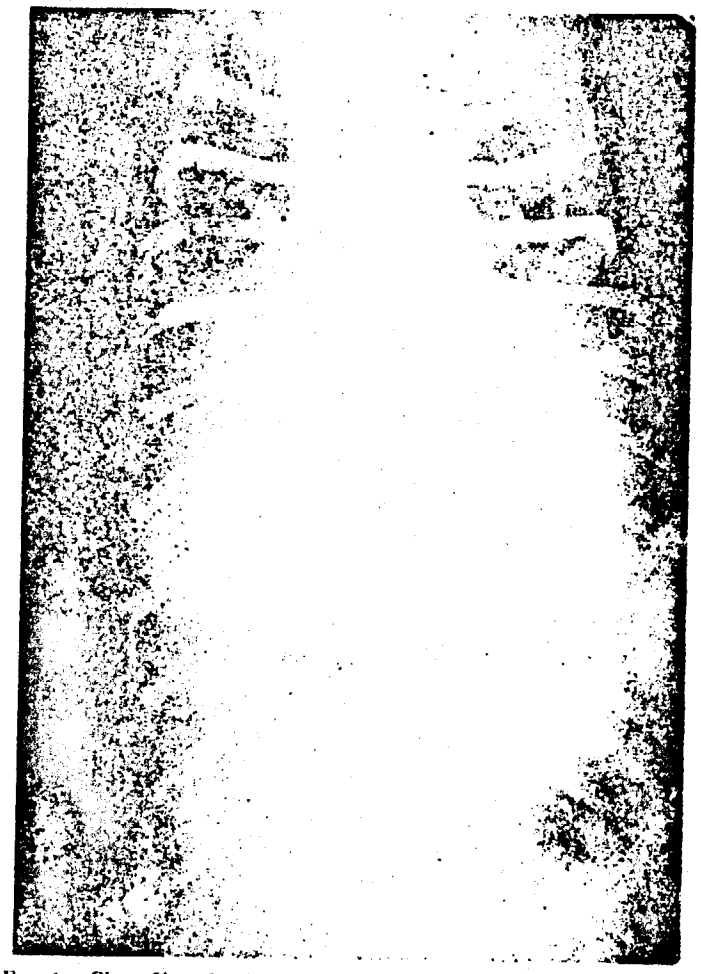

FIG. 1. Chest film of a dog with a massive pleural-pericardial effusion.

cottonseed oil emulsion as part of another experiment. With the dog in a supine position, 800 ml of serosanguineous fluid was removed from each thoracic cavity; at this time the animal was judged free of pleural effu

sions. The dog received no further infusions but expired 3 days later. At autopsy, 400 ml of fluid was present in each thoracic cavity.

Pleural effusion was readily seen on X-ray. Dog no. 12 became dyspneic following its 22nd infusion of TEM and thoracentesis verified

FIG. 2. Normal canine chest film.

a bilateral pleural effusion. A chest X-ray film (Fig. 1) of the dog showed opacification of the lower two-thirds of both lung fields and was in marked contrast to the clarity present in the normal canine chest X-ray (Fig. 2). At autopsy the dog had a pleural and pericardial effusion (Fig. 3).

No significant changes were noted in dogs that did not develop the effusions from TEM administration. Four of the six dogs that had pleural effusion, also had atelectasis. Two had pulmonary edema and two

FIG. 3. Pleural and pericardial effusion associated with daily intravenous infusion of TEM. Thorax of dog shown in Fig. 1.

congested livers. Microscopically the only findings that were generally consistent in both the effusion and noneffusion dogs were scattered areas of atelectasis. Six of the infused dogs exhibited a chronic hepatitis. The

intravenous fat pigment was demonstrated in the lung, liver, and spleen of dog nos. 1 and 7 and a similar type of pigment was found in the spleen of dog no. 3.

Hemoglobin concentration fell in eleven of the 13 dogs studied (Table 1). No particular trend was noted in the white blood cell count. The differential white cell count, bromosulfalein (BSP) test, and blood urea nitrogen (BUN) all remained within normal limits.

The pleural and pericardial effusion fluid was usually serous and rarely serosanguineous. The average ionic concentrations of sodium, potassium, and chloride in three of the effusions were: Na 122, K 4.4, and Cl 107 meq/1. The pleural fluid from two of the dogs had an average total protein concentration of 3.5% and albumin concentration of 1.08%.

DISCUSSION

TEM, a diacetyl tartaric acid ester of mono- and diglycerides, is prepared synthetically and the commercial grade contains not only the synthetic theoretical product, but also the unreacted monoglyceride and fully esterified triglycerides, as previously discussed under Methods.

The two major adverse effects which developed following daily intravenous administration of TEM were a drop in hemoglobin concentration and development of effusions. A fall in hemoglobin concentration occurred in 11 of 13 dogs. The relationship of hemoglobin levels to intravenous administration of TEM has been observed and discussed elsewhere (Hartwig, 1961). The severity of this drop, however, was never of a magnitude to warrant cessation of infusions of TEM. Pleural-pericardial effusions developed in 6 of 13 dogs. Effusion protein concentrations were about 50% lower than that found in normal dog sera (Singleton *et al.*, 1960). No serum protein determinations were made on the dogs. However, since there were no effusions into any other serous cavities nor any peripheral edema, it seems unlikely that a hypoproteinemia would be the sole mechanism responsible for the pleural and pericardial effusions. Findings in three dogs indicate that though infusions of TEM are discontinued, formation of the effusion may continue and rapidly refill drained pleural cavities with fluid.

Gross and microscopic examination presented no consistent clues as to the mechanism responsible for the effusions. Generally the lungs of the dogs with the effusion showed areas of atelectasis and/or consolidation. This, however, was probably the result rather than the cause of the

No adverse effects have been reported to follow oral ingestion of even large quantities of TEM. The chronic feeding tests on TEM did not affect the external appearance, behavior, or organ structure of rats and dogs when fed at 5, 10, and 20% levels of their entire food intake. Further, TEM did not appear to be toxic, either when added as such to the diet or baked in bread (Koppanyi and Dardin, 1950).

The development of pleural-pericardial effusions bears some similarity to the recently reported chick edema syndrome which develops after oral ingestion of specific lots of feed-grade animal fats (Brew *et al.*, 1959; Potter *et al.*, 1959). These fats were reported to contain the edema-producing factor as a trace impurity produced during certain fat-processing operations (Friedman *et al.*, 1959; Brew *et al.*, 1959). Further, Ames *et al.* (1960) reported that a number of samples of commercially prepared oleic acids and glycerol esters of oleic acids were shown to contain a material which produced pericardial edema in chicks. The active material was concentrated from either the oleic acid or the glycerol monoester by molecular distillation. If perchance, the chick edema factor is a glycerol monoester, then it may have some structural similarity to the pleural-pericardial principle in TEM, as TEM also contains glycerol monoesters.

The intravenous fat pigment was demonstrated in two dogs and possibly a third. The pigment was first described and characterized by Thompson *et al.* (1958) when they examined stained sections of liver and spleens obtained at autopsy from 10 patients who had received various amounts of an intravenous fat emulsion (Lipomul I. V., Upjohn). They found a brown granular pigment, invariably surrounded by lipoid substances, deposited in the reticuloendothelial cells of both organs. They then confirmed these results with experimental studies in rabbits. This same pigment has been demonstrated in dogs after multiple infusions of a fat emulsion, the SR emulsion (Hartwig *et al.*, 1961). TEM is a component of the SR emulsion, and the present studies show that the intravenous fat pigment after multiple infusions of the SR emulsion can be attributed in part to its TEM component. This may further mean that the major component of fat emulsions responsible for the intravenous fat pigments are the emulsifiers rather than the triglycerides.

Further experimental investigation of intravenously administered crude and purified TEM may provide a satisfactory method of studying the cardiopulmonary and other changes associated with pleural and pericardial

SUMMARY

Experimental pleural and pericardial effusions have been produced in dogs by the intravenous administration of TEM.

Thirteen dogs were infused daily with 0.5% TEM at a dose rate between 40 and 50 ml/kg. A fall in hemoglobin concentration occurred in 11 dogs, a pleural effusion in 6. BSP and BUN determinations remained within normal limits. The concentration of electrolytes in the effusion were slightly less than that of serum. Effusion protein concentrations were about half that of dog sera. The active principle of TEM or the mechanism responsible for the effusions are not known.

TEM has been demonstrated as a component of the intravenous fat emulsion. SR emulsion, capable of producing the intravenous fat pigment.

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Proc. Soc. Exp. Biol.
98: 347, 1958

Foreign-Body Type Reaction in Fat Cells.* (24039)

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During studies of the response of rats to diets containing very high amounts of saturated fats, a foreign-body type reaction was observed in fat cells of adipose tissue adjacent to sections of various organs prepared for histological examination. This report describes these altered fat cells and various dietary conditions under which they occur. Although a similar abnormality had been observed earlier with one of these fats by Cox and DeEds,† their data have not been published.

Methods and materials. Weanling male and female rats were obtained from Holtzman Rat Co. and fed one of the following diets. Each diet contained 20% vitamin-test casein, 4% B-vitaminized casein(1), 5% USP XIV salts, 4% corn oil, 17% glucose, and supplements of Vit. A, D, and E at levels of 40,000 I.U., 4,000 I.U., and 510 I.U./kg diet, respectively. The saturated fats incorporated at a level of 50% were fully-hydrogenated lard, palmitic acid, stearic acid, or a saturated partially acetylated monoglyceride(1). Ethyl stearate was fed at 45% with 5% cellulose added to provide body to the diet. A control group was given 50% lard. Rats were sacrificed at 8-week intervals and examined grossly. Portions of perigastric, perirenal, periadrenal, perigonadal, and subcutaneous fat were removed from each rat, fixed with Bouin's solution, sectioned, and stained with hematoxylin and eosin.

Results. Gross examination of the viscera and body cavities was not remarkable except for general fullness of the gastrointestinal tract with creamy colored food and an occasional mottled appearance of fat depots in animals fed the saturated fats.

Microscopic examination of sections showed an unusual foreign-body type reaction in fat from rats fed saturated fats (Fig. 1, top). The lesion consisted of a multinucleated cellular reaction usually located within the fat cell membrane with nuclei arranged evenly about periphery of the fat cell or in clumps. In many instances, the material which occupied the center of the fat cell, appeared to have been partially dissolved, leaving delicate slit-like spaces which were radially oriented and suggestive of fatty acid crystals (Fig. 1, bottom). The reactive process usually involved 2 or more adjacent fat cells and was separated from similar areas by uninvolved fat cells. No acute inflammatory reaction, evidence of recent hemorrhage, or birefringent material were associated with the fat reaction.

The phenomenon occurred within 8 weeks in rats fed diets containing palmitic acid, stearic acid, ethyl stearate, or saturated partially acetylated monoglycerides and within 16 weeks in rats fed the fully-hydrogenated lard diet, but was not observed in rats fed the control lard diet. The flecks of opaque white material observed grossly in the depot fat of some rats were associated with the severest occurrence of the foreign-body type reaction as seen microscopically.

* Communication No. 247 from Research Labs., Distillation Products Industries.

† Observed in rats fed saturated acetylated monoglycerides for long periods of time by A. J. Cox, Dept. of Pathology, Stanford Univ., Calif., and F. DeEds, Western Regional Research Laboratory, Albany, Calif., private communication. *Added in proof*: Recently published by Cox and DeEds, *Am. J. Path.*, 1958, v34, 263.

FIG. 1. *Top:* Periadrenal fat showing multiple islands of fat injury separated by normal fat. Hematoxylin and eosin, ×60. *Bottom:* Higher magnification showing variations of cellular reaction. Multinucleated giant cells are present at top of print. A fat cell containing radial slit-like spaces is seen near bottom of print. Hematoxylin and eosin, ×430.

This reaction in visceral and retroperitoneal fat of the rat bears some histologic resemblance to pathologic conditions of human fat such as fat necrosis of newborn(2), sclerema neonatorum(3), and traumatic fat necrosis of various types(4,5,6). Further studies are underway to determine the histochemical characteristics of the lesion.

Appearance of the foreign-body type reaction in all groups except that fed lard indicates that the reaction is a response to a relative or absolute excess of saturated long chain fatty acids. We are testing diets containing various proportions of saturated to unsaturated fatty acid to determine the critical ratio which produces this reaction.

Summary. A foreign-body type reaction has been observed in fat from rats fed diets containing high levels of saturated fat. The phenomenon and the dietary conditions under which it was produced, are described.

1. Herting, D. C., Ames, S. R., Koukides, M., Harris, P. L., *J. Nutrition*, 1955, v57, 369.

2. Flory, C. M., *Arch. Path.*, 1948, v45, 278.

3. Kohnstam, G. L. S., Herbert, F. K., *Arch. Dis. Childhood*, 1927, v2, 349.

4. Cameron, G. R., Basu Mallik, K. C., *J. Path. and Bact.*, 1954, v68, 525.

5. Collins, H. A., Stahlman, M., Scott, H. W., *Ann. Surg.*, 1953, v138, 880.

6. Farr, C. E., *ibid.*, 1923, v77, 513.

Received April 9, 1958. P.S.E.B.M., 1958, v98.

Arch. Indust. Hyg. and Occup. Med.
7(4): 282-291, 1953

COMPARATIVE TOXICITY OF SYNTHETIC AND NATURAL GLYCERIN

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THIS STUDY was undertaken in order to determine whether there are differences in toxicity between synthetic and natural glycerin and whether synthetic glycerin would be suitable and safe to use in cosmetic, pharmaceutical, and food preparations.

The acute toxicity was compared in rodents by intragastric administration to mice, guinea pigs, and rats and by application to the skin of rabbits. Chronic toxicity was evaluated by feeding rats for two years on diets containing 5, 10, or 20% of glycerin. The response of skin to chronic exposure was determined by applying glycerin to the skin of rabbits repeatedly over a period of 18 weeks. Guinea pigs were used for sensitization tests, and rabbits for determining ocular irritation.

The synthetic glycerin used was a commercial product manufactured by the Shell Chemical Corporation from propylene and chlorine by a number of different unit processes, including chlorination, chlorohydrination, and hydrolysis. These processes are depicted in the flow diagram. A series of purification treatments, including extraction and distillation, resulted in glycerin which averaged 99.5% by weight in purity. The nonglycerin content was mostly water, and there was no spectroscopic evidence of benzene or naphthalene nuclei or any other aromatic compounds. Apart from the impurities indicated by the U. S. P. specification tests, the only specific contaminants identified have been glycerin polymers and glyceraldehyde.

Samples of synthetic glycerin, typical of current production, were supplied monthly, or as requested, by the Houston plant of the Shell Chemical Corporation. Natural (U. S. P.) glycerin was selected at random from market stock. Each specimen was

This research was supported in part by a grant from the Shell Chemical Corporation, New York.

This paper was presented in part at the Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics, Omaha, Oct. 16, 1951.

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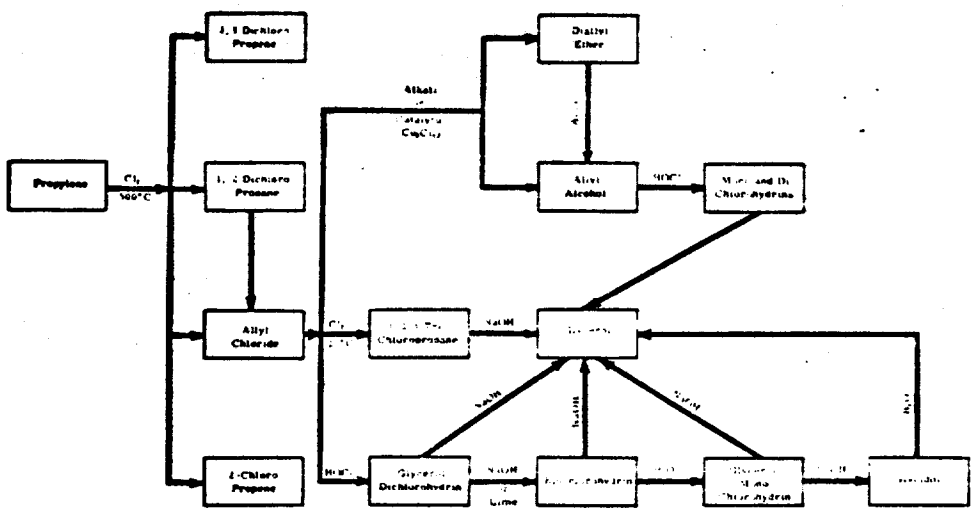
analyzed in accordance with the tests described in the "Pharmacopoeia of the United States" (13th and 14th editions), and chloride values were determined by the quartz tube combustion technique. All tests of purity were conducted under the supervision of the Shell Chemical Corporation and the Shell Development Company. There were minor differences between the natural and synthetic glycerin, namely, the residue on ignition was significantly greater ($p = < 0.001$) for natural glycerin, as was the content of fatty acids and esters. One lot of natural glycerin out of 25 failed the color test. Twenty-two out of 25 synthetic samples failed the test for readily carbonizable substances. The mean total chloride values were identical.

I. ACUTE TOXICITY

A number of investigators have studied the pharmacology, metabolism, and acute toxicity of glycerin, and two comprehensive reviews on its toxicity have been published, the first by Deichmann [1] and the second by von Oettingen.[2]



Flow diagram depicting possible different reactions in the manufacture of synthetic glycerin from propylene.

According to these reports, glycerin is absorbed readily from the intestines but not from the stomach. It may be metabolized to glucose and to a limited extent may replace carbohydrate in the diet. The feeding of 50% glycerin to starved rats increases the glycogen content of the liver, and liver tissue also forms glycogen from glycerin in vitro. The blood sugar of rabbits may double in an hour after peroral or intraperitoneal administration. There is considerable difference of opinion concerning the excretion of glycerin.

1. Deichmann, W.: Glycerol: Effects upon Rabbits and Rats. Indust. Med., Indust. Hyg. Sect. 2:5-6, 1941.

2. von Oettingen, W. F.: The Aliphatic Alcohols: Their Toxicity and Potential Dangers in Relation to Their Chemical Constitution and Their Fate in Metabolism. Public Health Bulletin 281, Federal Security Agency, U. S. Public Health Service, 1943.

Small doses of glycerin cause no rise in body temperature and no change in heart action or blood pressure in man. Intravenous administration may cause a fall in blood pressure.

Glycerin has a direct effect on renal function in both man and animals. Peroral administration is followed by diuresis. Hemoglobinuria may follow intravenous administration of glycerin, but premedication with ascorbic acid prevents this effect.

When glycerin is given intravenously to rabbits, there is a decided increase in the amplitude and frequency of respiration due to a specific action on the respiratory center.

Glycerin has a direct action on muscle tissue in vitro, and high concentrations cause severe dehydration. In vivo, it causes stiffness and clumsiness of movement.

Glycerin may cause irritation of mucous membranes presumably because of its dehydrating action. According to the Council on Dental Therapy of the American Dental Association,[3] however, there appear to be no clinical data to support a statement that glycerin is injurious to the tissues of the mouth. Distinct edema and hyperemia of the rabbit's eye have been reported to follow instillation of glycerin.

Glycerin has moderate narcotic properties, since rats are depressed to the same degree in their response to maze tests by 1.6 gm./kg. of glycerin as by 0.8 gm./kg. of alcohol (U. S. P.). Circus movements and convulsions may be elicited by auditory stimulation in frogs and mice treated with glycerin. Inactivity and tremors follow peroral administration to rabbits.

The fatal dose of glycerin by various routes of administration has been determined for a number of species of animals. The picture of acute poisoning is essentially similar for most species. Rapidity of pulse is followed by muscular weakness, tremors, twitchings, convulsions, diuresis, and diarrhea. Respiratory failure is the usual cause of death.

Anderson and others[4] compared the acute toxicity of natural (U. S. P.) glycerin with synthetic (Shell) glycerin by single peroral and intravenous administration. They found no difference in L. D_{50} values between the two glycerins.

Materials and Methods.—Test animals used for acute toxicity studies were male Swiss inbred white mice, female rats of the Long-Evans strain, male guinea pigs, and male albino rabbits. All were obtained from local suppliers and were quarantined for about two weeks prior to use. Metal cages bedded with sawdust housed them in approximately equal groups, which were separated according to species and sex. The temperature of the animal room varied between 15 and 17 C. The animals were fed commercial animal food pellets of known composition, supplemented weekly with greens.

a) Intragastric Administration: Undiluted synthetic or natural glycerin was given to 91 mice (20 ± 2 gm. after fasting six hours) in doses of 15 to 31.5 gm./kg., using a ball-point needle and syringe. Twelve rats (114 ± 8 gm. after fasting 22 to 24 hours) received 27.26 gm./kg., reported by von Oettingen[2] to be the $L.D._{50}$. A dose of 7.25 gm./kg., stated by von Oettingen to be the $L.D._{50}$ for guinea pigs, was used as the middle of three dose levels given to 29 guinea pigs (325 ± 50 gm.) by means of a catheter. Feed was offered two hours after the treatment, and water was available at all times.

Mortality, weight changes, and physiologic signs were recorded over a 10-day period. Examinations were made for gross lesions in all animals that died and in selected survivors that were killed for the purpose. Specimens of brain, heart, liver, spleen, stomach, intestine, and kidney

3. Glycerine in Tooth Paste, J. Am. Dent. A. **19**:500, 1932.

4. Anderson, R. C.; Harris, P. N., and Chen, K. K.: Toxicological Studies on Synthetic Glycerin, J. Am. Pharm. A. Scient. Ed. **39**:583-585, 1950.

were taken routinely for histologic study. Tissue specimens were fixed in 10% formalin, preserved in 80% alcohol, embedded in paraffin, sectioned, mounted, and stained with hematoxylin and eosin.

Results.—The chief pharmacologic effect was stimulation of the central nervous system. Body tremors, erection of the tail in the typical Straub manner, and generalized clonic convulsions preceded all observed deaths of mice. Deaths of rats were also preceded by muscle spasms and clonic convulsions; respiratory failure occurred 15 minutes to two and one-quarter hours after administration. Survivors appeared normal within two and one-half hours after administration. Tremors of the head and body, initiated by auditory stimuli, occurred in guinea pigs immediately after injection. Death was usually preceded by tremors, but guinea pigs in which tremors developed did not invariably die.

The approximate L. D_{50} values[5] for mice and rats were 23.0 and 27.2 gm./kg., respectively, for both glycerins. The values for guinea pigs were 10.0 ± 1.3 gm./kg. for natural glycerin and 11.5 ± 2.8 gm./kg. for synthetic glycerin.

Lesions.—Hyperemia of the small intestine and lungs occurred in some mice of both groups at the higher dosage levels. The only other lesion of interest was slight hyperemia of the kidneys and of the mucosa of the small intestine. In rats, there was invariably hyperemia of the pylorus and of the small intestine. The lungs were usually congested, and the spleen was pale. Three rats showed hyperemia of the cerebral meninges. Similar changes appeared in the guinea pigs with either glycerin.

(*b*) Cutaneous Application: Six rabbits (3.0 ± 0.5 kg.) whose trunks had been shaved 24 hours previously were immobilized in the multiple rabbit holder as described by Laug.[6] Glycerin in amounts of 6.25 to 18.7 gm./kg. was poured over the dorsal region and gently rubbed in with a glass rod. The glycerin was held in contact with the skin for eight hours by means of a rubber dam.

Results were negative; apparently neither glycerin was absorbed in quantities sufficient to produce a pharmacologic effect.

II. CHRONIC TOXICITY

A two-year feeding experiment with rats was designed to determine what changes might result from long-term ingestion of synthetic and natural glycerin by the rat. Several investigations of the feeding of natural glycerin have been reported. Whitlock, Guerrant, and Dutcher[7] found that levels up to 30% did not retard growth but that greater proportions did. Holck,[8] using smaller doses, did not find glycerin detrimental. Johnson and others[9] fed rats for 40 weeks a diet in which

5. Miller, L. C., and Tainter, M. L.: Estimation of the $E.D._{50}$ and Its Error by Means of Logarithmic-Probit Graph Paper, Proc. Soc. Exper. Biol. & Med. **57**:261-264, 1944.

6. Laug, E. P.: A Multiple Rabbit Holder, J. Lab. & Clin. Med. **29**:308-311, 1944.

7. Whitlock, G. P.; Guerrant, N. B., and Dutcher, R. A.: Response of Rats to Diets Containing Propylene Glycol and Glycerol, Proc. Soc. Exper. Biol. & Med. **57**:124-125, 1944.

8. Holck, H. G. O.: Glycerin, Ethylene Glycol, Propylene Glycol and Diethylene Glycol: Report on Feeding Experiments with Rats, J. A. M. A. **109**:1517-1520, 1937.

9. Johnson, V.; Carlson, A. J., and Johnson, A.: Studies on Physiological Action of Glycerol on Animal Organism, Am. J. Physiol. **103**:517-534, 1933.

41% of the starch had been replaced by glycerin without evidence of toxicity. Anderson and others [4] added 5% synthetic (Shell) and natural (U. S. P.) glycerin to the drinking water of rats for six months. They found no difference between the two glycerins when comparing growth rates, hemoglobin values, red and white blood cell counts, or gross and microscopic lesions.

In this experiment, glycerin was fed for a period of two years. The diet was prepared to contain 5, 10, or 20% of natural or synthetic glycerin. These levels were chosen because the side-products, which average less than 0.2% of the synthetic glycerin, would be ingested in significant amounts, while the highest level of glycerin fed, 20%, was not likely to disturb the metabolism.

The following criteria were selected for evaluating the results of the experiment: hemoglobin determination and urinalysis; lipid and glycogen content of the livers of the groups receiving 20% of glycerin in the diet; mean weekly food consumption in grams for each group for the first year of the experiment; mean organ/body weight ratios; growth curves; mortality; general appearance of the animals throughout the experiment, and gross and microscopic lesions.

Materials and Methods.—The test animals were young healthy rats of the Long-Evans strain, obtained from the inbred stock of the Institute of Experimental Biology of the University of California at Berkeley. There were 72 males, whose mean weight at the beginning of the experiment was 101.4 (96 to 109) gm., and 86 females, whose weight was 100.7 (92 to 108) gm.

Upon arrival at the laboratory, each rat was assigned to an individual numbered cage in a bank of cages, 8 units high and 22 units long. Distribution among the experimental groups was accomplished by reference to a standard random number table.[10] There were 22 animals of both sexes on each glycerin diet and 26 controls.

The diet consisted of a standard dog-food meal with which the glycerin was thoroughly mixed. The feed was prepared once a week and stored in stoneware crocks. The use of metabolism jars with mesh tops for feeding permitted free access to food as well as ease in weighing. The jars were weighed and refilled weekly, and the unconsumed feed was discarded. The mean weekly food consumption was calculated for the first year of the experiment. Water was allowed freely.

The animals were checked daily; at weekly intervals they were removed from their cages and carefully inspected, their health and activity noted, and their weight recorded. The mean per cent weight gain for each group was calculated at approximately four-week intervals.

At 3, 6, 12, 18, and 24 months after the start of the experiment, five rats from each group were placed in separate metabolism cages for from 24 to 48 hours, and urine specimens were collected. Those specimens which were satisfactory and uncontaminated were analyzed for albumin by the acetic acid heating method and for reducing substances by the qualitative Benedict's test. They were also examined microscopically for casts and red and white blood cells. Blood was withdrawn from the tail veins for hematologic studies. Erythrocytes and leucocytes were counted by standard methods. Hemoglobin values were determined by the Sahli method (13.8 gm. = 100%). Mean hemoglobin values were calculated for the different groups.

At the end of one year, all rats on the 20% diets were decapitated and exsanguinated. The glycogen content of the livers was determined according to the method of Good and others.[11] The livers were analyzed for total lipid content by the method of Jensen and others.[12] Organ/body weight ratios were determined both on these animals and on those which survived until

10. Fisher, R. A., and Yates, F.: Statistical Tables for Biological, Agricultural, and Medical Research, Edinburgh, Oliver & Boyd, Ltd., 1938, pp. 82-87.

11. Good, C. A.; Kramer, H., and Somogyi, M.: The Determination of Glycogen, J. Biol. Chem. **100**:485-491, 1933.

12. Jensen, D.; Chaikoff, I. L., and Tarver, H.: The Ethionine-Induced Fatty Liver: Dosage, Prevention, and Structural Specificity, J. Biol. Chem. **192**:395-403, 1951.

the termination of the experimental period of 105 weeks. The livers, kidneys, hearts, spleens, and lungs were removed, blotted on filter paper, and weighed. Statistical differences between the ratios of organ weight to body weight were determined for the different dosage levels.

Lesions.—Necropsies were performed on all animals that died or were killed during the experiment. Any gross abnormalities were noted. Sections of liver, spleen, adrenal gland, kidney, small intestine, bladder, and reproductive organs were prepared for microscopic study. Stains for lipid were made by the method of Rinehart and Abul-Haj [13] on the livers of all rats surviving the 105-week experimental period.

Statistical analyses were made in all cases in which they would be of value. The methods used were the standard chi-square, student *t*, and analysis of variance according to Fisher.[14]

TABLE 1.—*Mean Weekly Weight Gains of Rats on Natural (U. S. P.) or Synthetic (Shell) Glycerin*

Weeks on Diet	5%				10%				20%				Control	
	Natural		Synthetic		Natural		Synthetic		Natural		Synthetic			
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
4	124.3	60.4	119.1	72.0	112.4	66.6	117.8	77.8	112.9	77.2	98.9	73.4	134.3	79.2
8	188.2	104.0	175.2	100.9	174.3	97.1	181.5	114.6	183.3	108.9	174.0	107.3	187.0	119.6
12	236.7	114.5	229.8	130.1	221.6	121.1	233.7	142.0	240.1	245.6	224.4	141.8	245.0	154.4
20	255.3	120.3	266.7	139.9	260.3	133.8	265.8	152.6	275.0	152.9	253.6	157.3	289.5	170.2
28	281.6	136.8	288.9	155.2	287.3	154.2	289.7	171.0	290.9	160.4	282.3	170.2	324.4	183.1
32	295.9	146.4	296.4	156.3	303.1	153.1	290.9	172.7	303.4	168.8	277.5	172.7	335.1	190.4
36	298.8	145.9	298.7	163.6	306.9	156.5	302.6	178.7	292.0	173.8	279.3	174.1	331.2	193.7
40	304.5	150.9	310.5	166.9	311.4	162.7	258.6	181.7	294.3	178.5	275.3	181.2	331.9	198.9
44	298.9	151.6	315.1	171.9	319.7	167.2	304.8	184.1	302.5	178.9	280.5	191.2	336.0	202.4
48	298.2	151.7	321.2	172.1	319.9	165.3	318.8	187.8	308.7	176.7	299.0	191.8	337.1	203.0
52	307.9	153.3	330.5	171.3	324.9	164.7	328.1	190.8	320.6	178.9	318.4	205.3	345.7	206.3
56	319.3	156.6	343.7	180.1	336.0	171.1	338.3	196.0	....	.....	.....	.....	356.3	213.0
60	320.1	155.5	345.6	181.9	338.3	174.3	362.6	198.9	....	....	.....	.....	353.1	209.0
64	317.8	154.1	347.5	180.2	339.4	171.1	370.6	194.3	.....	.....	.....	....	363.7	206.4
68	304.1	161.3	351.7	179.7	340.0	174.2	372.8	100.0	....	.....	.....	.....	360.2	221.3
72	302.5	170.1	357.6	183.7	342.3	180.3	385.1	202.1	.....	.....	....	.....	366.2	227.7
78	327.2	162.6	355.6	182.4	352.0	175.7	380.1	201.5	.....	.....	.....	.....	357.9	223.8
82	322.0	159.9	356.3	186.5	351.4	172.0	383.6	203.1	.....	.....	.....	.....	394.5	223.7
86	328.8	167.7	360.5	181.2	358.7	180.9	397.8	208.6	.....	.....	.....	.....	394.6	229.4
90	316.4	167.1	364.0	179.3	348.6	174.0	390.5	206.3	.....	.....	....	.....	384.1	228.0
94	302.6	165.6	364.4	177.0	328.5	171.0	385.1	202.9	.....	.....	.....	.....	374.5	224.1
98	304.9	167.3	362.3	180.2	324.8	181.0	393.9	213.5	.....	.....	.....	.....	358.3	232.3
105	299.8	179.6	348.6	166.7	303.5	169.2	383.6	208.8	.....	.....	.....	.....	385.3	226.6

Results.—Table 1 presents the mean weight gains, expressed in per cent, of rats on natural (U. S. P.) or synthetic (Shell) glycerin. Examination of the growth curves and statistical analysis of mean values at 13, 52, and 105 weeks did not show any significant difference between the groups of males and the groups of females.

Hemoglobin determinations and urinalyses were carried out at 3, 6, 12, 18, and 24 months without demonstrating any differences between the blood and urine of the experimental groups and the blood and urine of the controls. There was no

13. Rinehart, J. F., and Abul-Haj, S.: Histological Demonstration of Lipids in Tissue After Dehydration and Embedding in a Polyethylene Glycol, A. M. A. Arch. Path. **51**:660-669, 1951.

14. Fisher, R. A.: Statistical Methods for Research Workers, Ed. 7, Edinburgh, Oliver & Boyd Ltd., 1938, pp. 128-177.

evidence of depression of hemoglobin in any group; the minimum concentration was 12.0 gm. per 100 cc. (87% on the Sahli scale). There was a high incidence of albuminuria, ranging from 30% in females on 5% natural glycerin to 92% in females on 20% natural glycerin. The incidence in male and female control groups was 64.0 and 53.8%, respectively. Albuminuria in the groups receiving the synthetic product ranged from 47.6% in females on 5% synthetic glycerin to 74% in males on 5% synthetic glycerin. The degree of glycosuria ranged from zero in male and female rats on 20% synthetic glycerin to 50% in male controls. However, due to the wide variation in degree of albuminuria and glycosuria and to their random distribution throughout the experimental groups, no significance could be attached to these findings.

No significant differences between natural and synthetic glycerin were demonstrable by the results of the specific biochemical analysis for liver glycogen and

TABLE 2.—*Mean Organ/Body Weight Ratios in per Cent for Each Group of Rats on Natural (U. S. P.) or Synthetic (Shell) Glycerin*

Diet	Sex	Liver	Lungs	Heart	Kidneys	Spleen
5%	M	3.97	0.74	0.26	0.89‡	0.43
Natural	F	3.93	0.47*	0.33	0.88	0.26
5%	M	3.41	0.45	0.28	0.75*	0.16
Synthetic	F	3.94	0.83	0.38§	0.89	0.26
10%	M	3.23	0.44	0.26	0.78	0.14
Natural	F	3.71	0.71	0.33	0.93	0.24
10%	M	3.60	0.55	0.25	0.71†	0.19
Synthetic	F	3.95	0.64	0.32	0.81*	0.23
20%	M	3.80	0.41	0.27	0.82	0.19
Natural	F	4.05	0.53	0.31*	0.91	0.21
20%	M	3.57†	0.37*	0.26	0.78	0.13*
Synthetic	F	4.20‖	0.61	0.29†	0.98	0.19
Control	M	3.98	0.50	0.29	0.90	0.18
	F	3.78	0.66	0.35	0.92	0.27

* Control group heavier, $p = 0.05$.
† Control group heavier, $p = 0.01$.
‡ Synthetic group lighter, $p = 0.01$.
§ Natural group lighter, $p = 0.05$.
‖ Control group lighter, $p = 0.05$.

lipid. These tests were carried out on rats fed 20% glycerin in the diet and killed at the end of one year. The mean glycogen values in rats on natural glycerin were 4.2% for males and 4.3% for females. Corresponding values for synthetic glycerin were 4.2% for males and 3.7% for females. The mean values for total lipid were 7.5% for males and 6.9% for females on natural glycerin and 7.0% for males and 6.7% for females on synthetic glycerin.

The mean organ/body weight ratios were tabulated and compared statistically (Table 2). Of the 180 comparisons, 12 were found to be significant, 8 being at the 0.05% level and 4 at the 0.01% level. In only two groups were the organs of animals fed synthetic glycerin significantly heavier than those of the controls or of those fed natural glycerin.

Females receiving 20% synthetic glycerin had liver/body weight ratios significantly higher than those of the controls; females receiving 5% synthetic glycerin

had heart/body weight ratios significantly greater than those on 5% natural glycerin.

From comparison of these data we concluded that there was no evidence of stress due to the ingestion of either glycerin, as would have been indicated by increased organ/body weight ratios.

Pathology.—In none of the rats were there lesions attributable to either natural or synthetic glycerin or to theoretical contaminants (aromatic, heterocyclic, or halogenated hydrocarbons). In 89 of the 157 rats there were no discernible natural diseases.

A variety of spontaneous diseases was present in the remaining 68 rats. The lowest incidence was in the groups of rats fed 20% natural or synthetic glycerin and those fed 5% synthetic glycerin. No obvious significance could be attached to this observation.

Only inflammatory diseases occurred in 27 rats. Bronchiectasis, pneumonia, and pulmonary abscesses were the most frequent abnormalities. Taenia infestation of the liver occurred rarely. Hydronephrosis and pyelonephritis were observed in several animals.

TABLE 3.—*Summary of Pathologic Lesions in Rats*

Group		Number of Rats	Normal	Inflammatory Lesions	Tumors *		Miscellaneous †
					Malignant	Benign	
Natural	5%	12	11	6	1	2	2
	10%	12	10	4	5	1	2
	20%	12	19	2	0	0	1
Synthetic	5%	21	14	3	0	4	0
	10%	13	7	5	5	4	1
	20%	12	14	2	0	1	5
Control		26	14	5	5	0	2
Total		157	89	27	16	12	13

* Rats with both neoplastic and inflammatory lesions are included in this group and are not included in the group with inflammatory lesions. Rats with multiple tumors are included in the table once only.
† In this category are rats showing lymphoid hyperplasia, epidermoid cysts, advanced postmortem autolysis, etc.

Twenty-eight of the rats had tumors; in one rat there were two benign lesions. Several rats had not only neoplasms but also unrelated inflammatory lesions.

Malignant neoplasms occurred in 16 rats. There were five each in the groups on 10% natural glycerin and on 10% synthetic glycerin as well as five each in the control group. The other malignant neoplasm was in the group on 5% natural glycerin.

Benign tumors were present in 12 rats. The most unusual neoplasms were pheochromocytomas, occurring in three rats, and granulosa cell tumors, occurring in two rats. Nine of these 12 tumors occurred in the rats on synthetic glycerin; there were none in the controls.

A summary of the pathologic changes in these animals is given in Table 3.

III. IRRITATION AND SENSITIZATION TESTS

In determining whether synthetic glycerin would be suitable and safe to use in cosmetic, pharmaceutical, and food preparations, the following tests were made.

Skin Irritation.—Twenty-four female albino rabbits (2.0 to 3.8 kg.) were used for the determination of skin irritating properties. The method of application was adapted from the procedure of Draize and others.[15] The animals were clipped as frequently as necessary with an electric hair clipper. They were divided into two groups, and each group was subdivided into four sections. The daily applications of 0.5 to 4.0 ml./kg. of synthetic or natural glycerin covered a dorsal area which was approximately 30% of the body surface. Animals at the two lower dose levels were not confined in the usual holders,[6] since the glycerin could be completely worked into the skin. The other groups were confined for an eight-hour period, covered with a rubber dam to hold the glycerin in intimate contact with the skin. The skin of each rabbit was examined before and after exposure for edema, erythema, or other sign of irritation. At the end of the period unabsorbed glycerin, which rarely was present, was removed by gentle blotting with absorbent cotton.

The urine of the rabbits on the two higher dosages was examined microscopically and analyzed for pH, sugar, and albumin at 30 and 60 days after the start of the experiment. The blood was examined for total hemoglobin and red and white blood cell counts at 45 and 90 days.

Glycerin was applied five times weekly until 90 applications had been made. The animals were then killed with thiopental sodium for autopsy. After gross examination, specimens of thyroid, heart, lung, stomach, spleen, liver, adrenal gland, kidney, small intestine, ovary, uterus, bladder, and treated skin were taken for microscopic study. Tissues were fixed in formalin, dehydrated, and stained with hematoxylin and eosin.

Results.—All rabbits remained in satisfactory health throughout the experiment, except one whose back was fractured accidentally. This rabbit, which was receiving applications of 4 ml./kg. of synthetic glycerin, had to be killed after only 53 applications. Its skin was perfectly normal, and there was no gross or microscopic evidence of tissue change. Mild and transient infections of the foot pads were noticed in seven animals receiving synthetic glycerin and in six animals receiving natural glycerin. This infection involved the hind feet primarily and began with a falling out of hair above the pads. A mild inflammatory process led to scab formation, followed by healing. Methylrosaniline chloride was applied topically.

Eight of the 12 animals treated with natural glycerin showed weight gains of 20 to 1,300 gm.; 3 lost 160 to 350 gm., and the weight of 1 remained stationary. Eight of the 12 animals treated with synthetic glycerin gained 401 to 1,230 gm., and 4 lost 90 to 250 gm. None of the losses are considered relevant.

The results of the urinalysis were within normal limits. Blood counts also showed no significant deviation from normal. Animals on natural glycerin had 5,010,000 to 8,380,000 erythrocytes, 7,200 to 14,250 leucocytes, and 75 to 94% hemoglobin (Sahli scale). Animals receiving synthetic glycerin had 5,300,000 to 7,780,000 erythrocytes, 7,500 to 11,500 leucocytes, and 78 to 95% hemoglobin.

Neither glycerin damaged the skin of any animal. Indeed, the skin appeared exceptionally soft and pliable. Occasional accidental abrasions made during clipping healed rapidly without infection.

A variety of lesions were found in the tissues of both groups on autopsy. These were abnormalities customarily found on autopsy of mature animals, and no consistent lesion indicative of a specific degeneration was found on gross or microscopic examination.

Eye Irritation.—Four normal untreated eyes of albino rabbits were selected on the basis of absence of grossly visible staining by 5% aqueous solution of fluorescein sodium, flushed with

15. Draize, J. H.; Woodard, G., and Calvery, H. O.: Methods for the Study of Irritation and Toxicity of Substances Applied Topically to the Skin and Mucous Membranes. J. Pharmacol. & Exper. Therap. **82**:377-390, 1944.

distilled water 20 seconds after instillation. After a two-hour interval to permit the eyes to return to normal, 0.1 ml. of glycerin was applied to the center of the cornea. The eyes were examined 1, 24, and 48 hours later in strong diffuse daylight. They were stained with fluorescein sodium, and the reactions were evaluated on the basis of the scale of weighted scores suggested by Draize and others.[15]

The eye irritation score averaged two for the natural glycerin and five for the synthetic glycerin at the end of one hour. The two subsequent readings were negative for both glycerins.

Sensitization.—Adult white male guinea pigs (350 to 429 gm.) were divided into two groups of 12 each and given 0.1 ml. injections of 0.1% solution of synthetic or natural glycerin in isotonic sodium chloride solution.[16] Ten injections were made on alternate days. Two weeks after the last injection, test injections of 0.05 ml. were made in the flank below the sensitizing area, using freshly prepared solutions. Twenty-four hours later, the diameter, height, and color of the reactions were noted and compared with readings made after the first sensitizing injection. There was no response to the challenging injection of either glycerin, except a barely perceptible papule at the site. This would be classified as both colorless and negative on Landsteiner's scale.

SUMMARY

The acute toxicity of natural and synthetic glycerin, based on intragastric administration, is presented as follows in tabular form in grams per kilogram:

	Natural	Synthetic
Mouse	23.0 ± 1.3	23.0 ± 1.9
Rat	27.2	27.2
Guinea pig	10.0 ± 1.3	11.5 ± 2.8

The chief pharmacologic effect of either glycerin was stimulation of the central nervous system. The chief alteration in anatomic structure following intragastric administration of either glycerin was congestion of the pylorus, small intestine, lungs, and kidneys.

Chronic toxicity studies showed that after rats had been fed 20% natural or synthetic glycerin for one year there were no significant differences between experimental and control subjects. After rats had been fed 5 or 10% natural or synthetic glycerin for a period of two years, there were no significant differences as regards per cent of weight gain, results of urinalysis and hemoglobin determinations, or percentage of mortality. There was no evidence of organ stress at any level of either glycerin, and there were no gross or microscopic lesions attributable to the glycerin feeding. Statistical comparison of the mean weekly food consumption of the various groups indicated a slightly greater intake by male rats receiving 5 and 10% natural glycerin, in comparison with males receiving the same levels of synthetic glycerin.

Sensitization tests on guinea pigs and skin and eye irritation studies on albino rabbits showed that no sensitization occurred and that no skin irritation appeared after 90 days of application. There was no measurable eye irritation.

Conclusion.—There were no toxicologic differences between the natural and the synthetic glycerin, so far as our studies could detect.[17] Neither gave evidence of toxic effects.

16. Draize and others.[15] Landsteiner, K., and Jacobs, J.: Studies on the Sensitization of Animals with Simple Chemical Compounds, J. Exper. Med. **61**:943-955, 1935.

17. A feeding experiment with dogs has indicated that there are no differences between the two glycerins. These data will be published in a later report.

EXPERIMENTS ON THE ABSORPTION OF ORGANIC SOLUTES IN THE SMALL INTESTINE OF RATS

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For many years, it has been generally accepted that intestinal absorption of dissolved substances is more than a mere diffusion. This belief has been established mainly on the basis of the statement of Nagano ('02) in Roehmann's Laboratory, that hexoses like glucose, galactose, fructose, mannose, though very nearly related to each other in regard to their chemical and physico-chemical properties, are very strikingly different in their rates of absorption, the more physiological sugars showing the stronger absorption. Another old statement, likewise at variance with diffusion being assumed as the ruling factor of absorption, is the following: Katzenellenbogen ('06) (see also Höber, '26) in the laboratory of one of us, has pointed out that Cl ions, in presence of organic compounds of low diffusion rate, like mannitol, move from the intestinal content into the body against the concentration gradient. Clearer evidence of the same effect has been presented by Goldschmidt and Dayton ('19) applying, instead of mannitol or another non-electrolyte, the slowly diffusing Na_2SO_4, and more recently by Burns and Visscher ('34) and Ingraham and Visscher ('36) emphasizing again the influence on the Cl transport of ions with lower mobility, like SO_4, phosphate, citrate in comparison with those of higher mobility, NO_3, SCN. Now, considering that the mechanism of entrance and transport of dissolved substances in the living beings is one of the central problems of general physi-

[1] A preliminary report has been published in the Proceedings of the Society for Experimental Biology and Medicine, 1936, vol. 34, pp. 486–488.

ology, it seems to be of great value to obtain more knowledge of the factors involved in the higher rate of penetration of some solutes in comparison with others. In this respect, it is conceivable that the finding of Magee and Reid ('31) that phosphate augments the intestinal absorption of glucose, but not of xylose, has stimulated the interest in the problems of absorption remarkably, although the interpretation of this effect is still ambiguous. All the more, it seems profitable to extend the fairly small experimental basis, providing support for the idea that diffusion is an essential factor of absorption and, by doing so, to trace a sharper line between merely physico-chemical and—what may be called—physiological factors, where processes in the interior of the cells are involved. This plan appeared to be promising also for the reason that knowledge concerning the physico-chemical properties of organic membranes has largely increased since the time when physical chemistry was considered to be more extensively serviceable in this field of physiology.

To arrive at such a distinction between physico-chemical and physiological factors, we decided to study absorption 1) of some more unphysiological, but indifferent compounds, i.e., substances which are chemically fairly inactive in the body, not poisonous, not metabolized, and 2) of some more physiological substances, turning our interest chiefly to the amino acids, the behavior of which has been investigated only occasionally.

METHODS

The experiments have been carried out under nembutal anaesthesia with rats of 150 to 200 gm. body weight, starved for 24 hours. Five hundredths cubic centimeter per 100 gm. body weight were injected subcutaneously. The greatest part of the small intestine was isolated by ligatures. Small glass canulae were fixed on both ends of the loop. The loop was carefully washed with warm Ringer's solution and emptied by soft pressing. In each experimennt, the loop was refilled several times with equal volumes (4 cc.) of different solutions, each of these being equimolar to the others. After identical intervals, the loop was emptied by rinsing with Ringer and pressing again softly. In this way, four to six different solutions could be administered in one experiment. The total

residues of solute contained in the loop were analytically determined. We have resigned to measure the volumes emptied as well as the final osmotic pressure. Though apparently rather rough, de facto the method turned out to give generally satisfactory results.

The fluid gained in each period of an experiment was precipitated with tungstic acid. Insofar as amino acids or acid amides were concerned, the material contained in the loop was determined by the Kjeldahl method applying for titration n 20 sulphuric acid. Sugars were titrated following Benedict's prescription, taking into a small test tube not more than 1 to 3 cc. of the copper reagent. For N-free substances we applied Bang's method using n 10 chromic acid and n 10 thiosulphate. Blank determinations have to be made not with distilled water, but with NaCl solution containing the same percentage of Cl as the solutions collected from the intestinal loop.

The results calculated from the analyses are expressed either as per cent absorbed of the total amount introduced into the loop or as absolute amount absorbed, given in millimols.

THE ABSORPTION OF UNPHYSIOLOGICAL SUBSTANCES

We have investigated the behavior of a number of polyhydric alcohols and of aliphatic acid amides. Provided these substances would penetrate the intestinal wall as they would pass an artificial diffusion membrane, we could expect an influence chiefly 1) of the molecular volume (MV)[2] in case the structure of the membrane would be sieve-like, resembling that of an inert filter, and 2) an influence of partition coefficients, if the character of the membrane would be that of a lipoid-like solvent allowing the solute present to be distributed between both phases.[3] As a matter of fact, such behavior is fairly generally accepted to characterize the surface membrane of living cells. However, concerning total living organs, like the intestinal wall, our knowledge is at present rather poor in this respect, although, obviously, it is desirable to be well informed for each organ, how far substances are enabled to pass as they would through an inert diaphragm and how far they have to suffer some kind of intermediate alterations

[2] The MV values recorded in this paper are those calculated from Kopp's formula.

[3] The partition coefficients concerned are in general those of ether: water and of olive oil: water taken from the paper of Collander and Bärlund ('33).

on their pathway. To some extent this question has been attacked by Amberson and Höber ('32) on the cat's salivary gland and by Schmengler and Höber ('33) on the kidney tubules of the frog. In both cases the isolated and artificially perfused glands, tested with indifferent lipoid-insoluble nonelectrolytes, appeared to be permeable in the direction blood vessel-lumen like a filter only to such molecules, the volume of which does not exceed a certain limiting value. In addition, the experiments on the salivary gland furnished evidence that the permeating power of distinctly lipoid-soluble compounds surpasses that of lipoid-insoluble ones, even though the former

TABLE 1

Absorption of polyhydric alcohols. Solutions: 0.1 molar in one-third Ringer. Each period of absorption: 25 minutes

	SUBSTANCE	MV	PART. COEFF. ETHER : WATER	PART. COEFF. OLIVE OIL : WATER	PER CENT ABSORPTION
Ia	Mannitol	189.2	0.00001	.....	0
b	Glycerol	87.8	0.00066	0.00007	88.6
c	Erythritol	130.2	0.00011	0.00003	41.4
d	Mannitol	189.2	0.00001	.....	0
IIa	Mannitol	189.2	0.00001	.....	0
b	Erythritol	130.2	0.00011	0.00003	23.6
c	Glycerol	87.8	0.00066	0.00007	69.8
d	Glycerol	87.8	0.00066	0.00007	75.7
e	Erythritol	130.2	0.00011	0.00003	30.2

ones have the larger volume (i.e., butyramide in comparison with acetamide). Now, we have carried on studies upon the question of passive permeability of total organs taking as another subject the intestine which provides, for this purpose, especially suitable conditions.

1. The absorption of polyhydric alcohols

The rates of absorption of the following substances have been compared: ethylene glycol, glycerol, erythritol, mannitol. Table 1 shows the outcome of two experiments with the same substances to give an idea as to the consistency of the result under our experimental conditions.

The result is obvious: the absorption rates of the polyhydric alcohols rise with decreasing MV. In both experiments the absorption rate of mannitol is 0.

2. *The absorption of aliphatic acid amides*

The same rule is valid with acid amides. This has been proved by comparison of acetamide, lactamide, urea, malonamide and succinamide, all of them being fairly lipoid insoluble. Table 2 gives an example.

Now, having suggested that the evidence of such a conspicuous connection between absorption rate and MV, as demonstrated in these experiments, supports the concept that absorption is like the diffusion across a porous membrane,

TABLE 2

Absorption of acid amides. Solutions: 0.66 molar in two-thirds Ringer. Each period of absorption: 25 minutes

	SUBSTANCE	MV	PART. COEFF. ETHER : WATER	PART. COEFF. OLIVE OIL : WATER	PER CENT ABSORPTION
a	Succinamide	126.4	.....	.....	3.4
b	Lactamide	98.5	0.0018	0.00058	42.5
c	Acetamide	68.7	0.0025	0.00083	78.2
d	Lactamide	98.5	0.0018	0.00058	36.7
e	Succinamide	126.4	.....	.....	6.3
f	Acetamide	68.7	0.0025	0.00083	68.4

the results obtained still need some further comments. Obviously, the differences of absorption rates of the chemicals applied in our experiments are far greater than the differences in the rates of free diffusion, as determined usually with membranes provided with wide pores, f.i., with membranes of Jena sintered glass, diffusion-coefficient ratios as large as 1 : 2 being considered very large. However, we know mainly from investigations of Michaelis and of Collander that with sieve-like membranes, where the pore diameter approaches more or less the diameter of the molecules of the solutes, the diffusion rates differ to a much greater extent from each other (Höber, '36). We conclude, therefore, that the intestinal membrane resembles such a molecular sieve.

This conclusion will be supported later by another observation (p. 410).

Another point needy of discussion is the following: it might be concluded from tables 1 and 2 that the differences of absorption rates are dependent as well upon the MV as upon the lipoid solubilities, the smaller volumes being correlated with the higher partition coefficients. As a matter of fact, it seems hardly possible to decide at present whether minute partition coefficients like those indicated in tables 1 and 2, though comparatively different from each other, have any significance. For instance, supposing that the plasma membrane of the cells is like a monomolecular film of lipoid, such a film would behave like a porous structure with its intermolecular spaces involving porosity and, due to the existence of thermal agitation, involving the alternative formation of wide and narrow pores. In this regard, such a film would resemble a rigid molecular sieve, like a collodion membrane, the various pore diameters fluctuating around an average value. However, the same film could resemble a solvent layer, provided the lipoid molecules would show a distinct affinity toward solute molecules approaching them from a bordering solution. Following the action of such an adhesion force, 'lipoid-soluble' molecules with volumes not surpassing a limiting value would be allowed to pass some intermolecular spaces, where 'lipoid-insoluble' ones of the same size would not be allowed, solely the axial, not the total column of solvent in such a space being available for their passage. Consequently, the strength of those adhesion forces would be the main factor determining whether such a lipoid film would disclose the properties of a sieve or those of a layer of oil.

3. *Comparison between polyhydric alcohols and acid amides*

From the foregoing, it could be expected that indifferent 'lipoid-insoluble' non-electrolytes of equal MV will be absorbed with the same speed. However, they fail to do so. We have found that, under comparable conditions, the amides are more slowly absorbed than the polyhydric alcohols and that

an amide with two —$CONH_2$-groups moves more slowly across the intestinal wall than one with one such group. This is shown by table 3.

Actually, the differences in the absorption rates are even larger in favor of the alcohols than it appears from this table.

TABLE 3

Absorption of polyhydric alcohols and of acid amides compared

SUBSTANCES COMPARED		PER CENT ABSORPTION			
		alc.	am.	alc.	am.
Erythritol (130.2) (0.00011)	Malonamide (104.4) (0.00030)	18.2	12.6	23.2	5.8
Glycerol (87.8) (0.00066)	Lactamide (98.5) (0.0018)	56.6	41.8	57.4	44.7
Erythritol (130.2) (0.00011)	Succinamide (126.4)	51.5	12.2	36.3	16.4
Ethylenglycol (65.5) (0.0068)	Urea (59.2) (0.00047)	71.3	34.6	76.4	39.4
Erythritol (130.2) (0.00011)	Malonamide (104.4) (0.00030)	89.2	25.9	87.1	12.8
		mono-am.	di-am.	mono-am.	di-am.
Acetamide (68.7) (0.0025)	Urea (59.2) (0.00047)	83.7	49.9	80.2	48.1
Lactamide (98.5) (0.0018)	Malonamide (104.4) (0.0003)	69	27	66	

Numbers in parentheses mean MV and partition coefficients ether: water. Numbers headed by alc. and am. (di-am., mono-am.) mean per cent absorption of alcohols and amides alternatively attempted.

since the amounts of alcohols have been determined by the Bang method, and its titration values mostly appear to be fallacious to some small extent by the reducing effect of some additional amounts of organic substances leaving the intestinal tissue during the experiment.

What is the reason for this remarkable difference in the absorption rates? It will be shown later (p. 412) that the preferential absorption of the polyhydric alcohols is by no means indicative of a preferential absorption as brought about by some special cellular activity, further that such a preferential absorption is by no means conflicting with the view that the absorption of the alcohols is a diffusion process as well as that of the amides. On the other side, it seems impossible at present to give a definite explanation of this behavior. But, at least, this phenomenon resembles a number of other observations well known from studies on permeability of singular cells. Jacobs ('31) was the first to turn the attention to the striking fact that the red cells of mammals and reptiles are more permeable to urea than to ethylene glycol, whereas for those of fishes and birds the inverse relation holds true. Wilbrandt ('31) generalized these statements, as, studying permeability to a number of non-electrolytes with various plant species, substances containing in their molecule acid amide groups turned out to enter the cells either more rapidly or more slowly than those containing alcoholic OH-groups, according to either one or another species being concerned. This generalization has been proved to be justified by many other observations (Höber and Ørskov, '33; Collander and Bärlund, '33; Höfler, '32, '34; Hofmeister, '35; Wahry, '36). The following interpretation has been proposed by one of us (Höber, '30, '31). The cell surfaces differ from each other by the composition of their building material. Among others, their lipoids, due to a more or less acidic character, could display a greater or smaller affinity to amides. As a matter of fact, such a behavior can be imitated by a mixture of oil and oleic acid which is a better solvent for acid amides than is neutral oil (Watzadse, '29; Collander and Bärlund, '33). In a corresponding manner, a lack of affinity to amides could be granted by a more basic character. But it seems possible also to find lipoid solvents exhibiting special affinities to polyhydric alcohols (Fleischmann, '28). Finally, instead of some real dissolving power, the structure of the cell surface might

exhibit particular adsorption affinities toward different substances (Höber, '36).

4. *The absorption of lipoid-soluble compounds*

Lipoid solubility, in the following experiments, means a more definite affinity to a lipoid-like solvent than in the previously described experiments with polyhydric alcohols and with acid amides. The results of three experiments appear in table 4.

TABLE 4

Influence of lipoid solubility on the absorption of acid amides

	SUBSTANCE	MV	PART. COEFF. OIL: WATER	PER CENT ABSORPTION	REMARKS
Ia	Lactamide	98.5	0.00058	65	Solution: 0.06 mol. in one-half Ringer. Each period 25 minutes
b	Valeramide	134.7	0.023	81	
c	Lactamide	98.5	0.00058	69	
d	Malonamide	104.4	0.00008	27	
e	Valeramide	134.7	0.023	89	
f	Lactamide	98.5	0.00058	66	
IIa	Acetamide	68.7	0.00083	81.3	Solution: 0.18 mol. in one-third Ringer. Each period 10 minutes
b	Butyramide	112.7	0.0095	84	
c	Acetamide	68.7	0.00083	76.3	
d	Butyramide	112.7	0.0095	80	
IIIa	Malonamide	104.4	0.00008	13.3	Solution: 0.1 mol. in one-third Ringer. Each period 25 minutes
b	Succinimide	102.9	0.0049	84.3	
c	Malonamide	104.4	0.00008	13.2	
d	Succinimide	102.9	0.0049	79.3	

The first experiment in table 4 indicates the influence of lipoid solubility insofar as valeramide, with its partition coefficient (oil: water) being about forty times greater than that of lactamide, is more rapidly absorbed than the latter one, though its MV is one-third greater. In the second experiment acetamide and butyramide have been compared, the MV of the second surpassing by far that of the first. However, the absorption rate of butyramide is as great as that of acetamide or a little greater, apparently on account of its higher lipoid solubility. The third experiment displays an enormous difference in the absorption rates of malonamide and succinimide, the MV values being very near to each other, but

the oil: water coefficient being 60 times higher with succinimide, than with malonamide. This difference may partially be due to the two acid amide groups contained in the malonamide molecule.

5. *Absorption as limited by a certain molecular size*

The two experiments reported in table 1 seem to prove that, during an absorption period of 25 minutes, mannitol failed entirely to be absorbed. The question arises whether this behavior would be indicative of the existence of a limiting cross section of the pores of a sieve-like membrane. If so, we could expect 1) that extending the absorption period over more than 25 minutes would have no differential effect on

TABLE 5

Experiments concerning the limiting size of MV. Solutions: 0.1 mol. in one-third Ringer

MANNITOL C_6 189.2		MANNITOL C_6 189.2		SORBOSE C_6 183.2		INOSITOL C_6 178.8		ADONITOL C_5 160.0		ERYTHRITOL C_4 136.2	
After min.	P.c. unabs.	After min.	P.c. unabs.	After min.	P.c. unabs.	After min.	P.c. unabs.	After min.	P.c. unabs.	After min.	P.c. unabs.
10	104	20	100	15	92	15	105.5	15	97.7	15	94.7
20	108	40	100	30	92	30	103	25	82.9	30	71.2
30	113.7	60	101.8	45	89	47	103	35	80.0	45	60.3
40	113.5	110	97.7	60	79	60	107.6	45	75.1	60	43.3

the absorption rate, and 2) that other substances resembling mannitol in their physico-chemical properties likewise would fail to be absorbed. Experiments where conditions have been varied in these directions are comprised in table 5.

In these and in a number of other experiments the results have been secured by Bang titration of the intestinal contents. This is the reason why, with mannitol and inositol, the absorption rates are partially negative, in other words, some substances reacting with chromic acid have escaped from the gut wall into the content of the loop (p. 407). On the other hand, the experiments with sorbose, adonitol and erythritol show a distinct increase of absorption with time, although presumably partially veiled by a corresponding exit of material

from the gut wall. As a matter of fact, we may be permitted to interpret the results of this series of experiments as indicating a certain limit of molecular size, the size of mannitol (C_6) and inositol (C_6) being above, that of adonitol (C_5) and erythritol (C_4) being below this limit. This concept being accepted, sorbose could be expected to be unable to pass the intestinal wall like mannitol or inositol, unless the hexoses would display a preferential absorption different from diffusion. In this case sorbose would display a lesser degree of preferential absorption than for instance glucose or galactose.

However, we may have to raise the question whether the behavior of mannitol and of inositol can be looked upon only as caused by a threshold porosity of the intestinal membrane, or whether it might be due to low lipoid solubility. We have already faced this problem without arriving at a definite answer (p. 408). The partition coefficients ether: water are <0.00001 for mannitol and 0.00011 for erythritol, respectively. Interpreting the greater absorption rate of erythritol as inconsistent with its MV, even such a low partition coefficient 0.00011 would have to be considered as an effective lipoid-solubility (Marklund, '36). But, taking into account a number of other observations, it seems to be more reasonable to adhere to the concept of a sieve-like architecture. For instance, comparing the permeating power of the polyhydric alcohols and sugars from glycerol (C_3) to raffinose (C_{18}) in regard to various cells and tissues, one fails to meet the distinct difference in the rate of penetration always lying between C_5 and C_6 as in the intestine. For the sulfur alga Beggiatoa (Ruhland and Hoffmann, '25; Schönfelder, '30) and for the marine alga Chaetomorpha (Hoffmann, '37) the border line is drawn between C_{12} (disaccharide) and C_{18} (trisaccharide), for the placenta of mammals (Schlossmann, '32) between disaccharide C_{12} and hexose C_6, whereas the fresh water plant Chara, like the gut wall, is enabled to distinguish between hexose and pentose (Collander and Bärlund, '33). Furthermore, mentioning still larger molecules, the glomerular membrane of

the kidney has been found to be freely permeable to inulin (MW = 5000) (Richards et al., '34; Shannon, '34), and to permit even ovalbumin (MW = 36,000) to pass, but not serum globulin (MW = 4 × 36,000) (Bayliss, Kerridge and Russell, '33). In all these cases, it seems to be appropriate to account for the smallest permeability by only a small number of comparatively wide pores being accessible to the largest molecules.

6. *Absorption at different concentrations*

So far, the absorption of unphysiological substances seems to be like a diffusion across an inert membrane. This concept is in agreement with further experiments, where the

TABLE 6

Absorption of urea, erythritol and malonamide at different concentrations

UREA			ERYTHRITOL			MALONAMIDE		
	After 15 minutes			After 25 minutes			After 35 minutes	
Molar	P.c. abs.	Mmol. abs.	Molar	P.c. abs.	Mmol. abs.	Molar	P.c. abs.	Mmol. abs.
0.06	20.9	0.050	0.06	54.1	0.130	0.066	16.9	0.045
0.09	19.0	0.068	0.09	65.0	0.235	0.132	16.8	0.089
0.12	17.1	0.082	0.12	62.2	0.297	0.198	16.5	0.131
0.18	20.0	0.144	0.18	54.4	0.393	0.264	18.4	0.194
0.24	17.8	0.170	0.24	55.5	0.535			

influence of concentration upon absorption rates has been studied (table 6). Under those conditions, considering that the solution in touch with the outside of the membrane, the circulating blood plasma, has a fairly constant composition and that, particularly, the concentration of the substance submitted to the absorption is there equal to 0, we have to expect from Fick's law of diffusion that, with equal amounts of solution and with equal lengths of time, the percentage of absorption will be approximately constant. For singular cells, evidence of a corresponding behavior is chiefly available from the more recent experiments by Jacobs ('33, '34, '36) and by Collander and Bärlund ('33). With the intestine the theoretical requirements have been satisfied at first by experiments of

Verzár ('35), showing that the absolute amount absorbed of the 'unphysiological' sugars, mannose, sorbose, xylose rises more or less in proportion to the concentration applied, in other words, that the relative amounts absorbed are practically constant. The same relation, within a certain range of deviations, holds true in our experiments with compounds of smaller molecular size.

It is of particular interest to learn from these experiments that polyhydric alcohols as well as acid amides are equally absorbed according to Fick's law. This means, that the preferential absorption of the polyhydric alcohols in comparison with the acid amides is by no means a sign of cellular activity, as taken into consideration before (p. 408).

THE ABSORPTION OF AMINO ACIDS

The experiments so far referred to have provided evidence that, after applying several criteria, the influence upon the rate of absorption of molecular size, of lipoid solubility, of time, of concentration, of characteristic features of the molecular structure, we may be allowed to conclude that absorption of a number of unphysiological chemically fairly inert organic non-electrolytes is essentially like a diffusion process. Now, we shall apply some of these criteria to see, whether substances of great physiological importance, the amino acids, behave otherwise. At first we have studied the influence of MV.

1. The absorption of amino acids compared with that of other substances with regard to their molecular volume

In studying this connection, we have to take into account that the amino acids present a particular behavior in their diffusion rates. It is conspicuous already from old observations of Overton ('02) concerning the osmotic properties of frog muscles that amino acids like glycin or alanin enter, if at all, with very great slowness, although one could expect them to exceed, f.i., erythritol—which enters—because of their smaller molecular size. Still more evident is their inertia in penetrating the cell surface in the experiments of Ruhland and

Hoffmann ('25) and of Schönfelder ('30) on Beggiatoa, which is remarkable for its fairly incomparable behavior as a molecular sieve, and where the permeation rates of glycin, alanin, asparagin, leucin have been found to be abnormally low. The reason for this slowness is not of a physiological nature, since, according to Schmengler ('33), the slowness becomes evident also in diffusion experiments with collodion membranes, when the amino acids are compared with other substances of corresponding MV. Obviously, for several reasons, this slowness

TABLE 7

Amino acids compared with acid amides, polyhydric alcohols and sugar. Indicating, whether the absorption from equimolar solutions of amino acids is faster or slower

AMINO ACID	MV_{calc}	MV_{eff}	ABSORPTION RATIO	SUBSTANCE COMPARED WITH AMINO ACID	MV
Glycin	78	~ 110	<	Acetamide	69
Glycin			>	Lactamide	98
Glycin			>	Malonamide	104
Glycin			>	Succinamide	126
dl, α-alanin	98.5	~ 150	=	Glycerol	87.8
dl, α-alanin			>	Succinamide	126
dl, α-alanin			>	Erythritol	130
dl, α-aminobutyric acid	120	....	>	Malonamide	104
dl, α-aminobutyric acid			>	Succinamide	126
dl, asparagin	130	~ 170	>	Malonamide	104
dl, asparagin			>	Succinamide	126
dl, asparagin			>	Erythritol	130
dl, asparagin			>	Xylose	153
dl, α-aminovaleric acid	142	....	>	Erythritol	130
dl, α-leucin	164	~ 190	=	Malonamide	104

is based upon the ampholyte character of the amino acids, according to which in aqueous solution they are practically entirely present as zwitterions, i.e., as strong dipoles enlarging their MV, between others, by the formation of a shell of water molecules. Consequently, a porous membrane such as the intestinal wall could be expected to be passed also comparatively slowly. But the contrary is true. This will be learned from table 7, which comprises briefly our main results, and from table 8, which shows more in detail the general

course of these experiments. The columns headed in the tables by MV show the values calculated from Kopp's formula and the approximate effective values as derived from the diffusion experiments of Schmengler.

It follows from the fourth column in table 7 that, in nearly all experiments reported, the absorption of amino acids exceeds that of the substance correlated. Only, comparing glycin with acetamide, the latter turned out to be faster, obviously, because in this case the effective molecular size of

TABLE 8

Three experiments concerning amino acids as compared with acid amide, polyhydric alcohol and sugar

	SUBSTANCES	MV		PER CENT ABSORBED		REMARKS
Ia	Malonamide, xylose	104	153	11.3	13.1	Solutions: 0.09 mol. in one-half Ringer. Each period 20 minutes
b	Asparagin, xylose	—170	153	65.7	10.5	
c	Malonamide, xylose	104	153	11.1	6.9	
d	Asparagin, xylose	—170	153	72.7	18.7	
IIa	Malonamide	104		11.3		Solutions: 0.05 mol. in two-thirds Ringer. Each period 25 minutes
b	Aminobutyric acid	(120)		66.2		
c	Malonamide	104		9.1		
d	Aminobutyric acid	(120)		66.1		
IIIa	Erythritol	130		7		Solutions: 0.1 mol. in one-third Ringer. Each period 25 minutes
b	Aminovaleric acid	(142)		28.4		
c	Erythritol	130		12.6		
d	Aminovaleric acid	(142)		29.5		

glycin is overbalancing. For the same reason, alanin is assumed not to surpass glycerol in speed, leucin not malonamide. For the rest it appears that, even with the MV of the amino acid rather largely exceeding that of the compared substance, like in the experiments with aminobutyric acid and malonamide, with aminovaleric acid and erythritol and particularly with asparagin and malonamide, the amino acid is the faster one.

In table 8 the numbers in brackets mean the MV_{calc} of aminobutyric and aminovaleric acid, the MV_{eff} not being available. In the first experiment, mixtures of two substances have been introduced into the intestinal loop, both components being

analyzed one beside the other. The far greater absorption of the amino acid is evident in this as well as in the other two experiments.

2. *Absorption at different concentrations*

Considering the results so far described, the question arises whether the preferential absorption of the amino acids resembles that of the polyhydric alcohols in respect to the acid amides (p. 407), in other words, whether the diffusion of the amino acids is merely speeded up by some adsorption affinities exerted by the pore walls of the sieve-like structure of the surface membrane, so that the concentration gradient inside

TABLE 9

Absorption of glycin, alanin, asparagin and valin at different concentrations

GLYCIN			ALANIN			ASPARAGIN			VALIN		
	After 20 min.			After 20 min.			After 20 min.			After 20 min.	
Mol.	P.c. abs.	Mmol. abs.	Mol.	P.c. abs.	Mmol. abs.	Mol.	P.c. abs.	Mmol. abs.	Mol.	P.c. abs.	Mmol. abs.
0.071	71.0	0.201	0.055	48.5	0.107	0.026	64.3	0.067	0.056	62.0	0.139
0.152	55.2	0.313	0.110	45.9	0.202	0.052	53.4	0.112	0.084	37.0	0.124
0.212	52.3	0.445	0.165	40.7	0.268	0.078	41.8	0.132	0.115	27.8	0.128
0.283	45.5	0.515	0.220	26.8	0.235	0.104	28.4	0.118	0.167	15.0	0.100
									0.223	10.6	0.094

the pores would be raised. In order to answer this question, we have studied the influence of varying concentrations upon the rate of absorption, in the same manner as done with other non-electrolytes. Provided that absorption would be like a diffusion, the percentage absorption would turn out to be constant, the absolute absorption to be increasing. Some of our experimental results are recorded in table 9.

Evidently, the behavior of amino acids is different from that of the other non-electrolytes as described above. Whereas, according to table 6, the percentage of absorption of urea, of erythritol and of malonamide turned out to be fairly constant, whatever concentration was introduced for absorption, the percentage absorption of amino acids falls off with rising

molarity. This is just what has been observed in earlier experiments of Auchinachie, Macleod and Magee ('30), of Donhoffer ('35) and chiefly of Verzár ('35) with the 'physiological' sugars, particularly with glucose and galactose, in contrast to the 'unphysiological' sugars, like mannose, xylose, arabinose. Apparently it means that, beside the concentration gradient, there is still another factor involved as a driving force in the absorption of special solutes through the intestinal wall. This factor displays its effect most vigorously with low molarities, where the concentration gradient is relatively ineffective. Therefore, the percentage absorption is highest at those low concentrations. Whenever diffusion would fail to work, the special factor alone would be responsible, in other words, the absolute amount absorbed would appear to be independent from the concentration present. This conclusion seems to be justified by the data in table 9 especially in regard to the amino acid with the highest MV, valin, whereas, with glycin, allowing a larger diffusion because of its low MV, an increase of concentration is more or less paralleled by an increase in the absolute amount absorbed. However, the experiments carried out so far may not be numerous enough to permit a definite conclusion.

How to explain this special behavior of some sugars and that of the amino acids? Since Wilbrandt and Laszt ('33) have published their well-known observations upon the influence of iodoacetate on the absorption of hexoses and pentoses, there has been much discussion of the possibility, that, by an intermediary phosphorylation and dephosphorylation of the sugars, variable in their rates from one sugar to the other, the absorption of some of these might be speeded up. The main objection to this concept is resting on the evidence, that, wherever cellular transport is associated with cellular metabolism, iodoacetate seems to work as an inhibitor. Regarding our own experiences, this follows from observations concerning the absorption of Cl ion and the secretion of phenol red by the tubular wall of the frog kidney, both moving from a lower to a higher level of concentration (Ferrari and

Höber, '33), further from observations concerning the inhibitory effect of iodoacetate on the accumulation and secretion of dyestuffs in the liver (see also Koll-Schröder, '34). Consequently, phosphorylation cannot be taken as mediating primarily the higher rate of absorption of sugars. More specific appears to be the influence of another poison, phlorizin, which displays a strong inhibitory effect on the absorption of the 'physiological' sugars not only in the kidney, but also in the intestine, whereas the absorption of amino acids in these organs remains unaffected by phlorizin (Lundsgaard, '33; Wertheimer, '33). In this case as well as in others, where we are dealing with specific transporting cellular properties, obviously useful for the total organism—as f.i., in the frog kidney the secretory transport of metabolic end products, like urea, uric acid, thiocyanate, the absorbing transport of Cl, glucose, aminoacids (Laug and Höber, '36)—we have to demand a special metabolic reaction as initiating the specific transport, which might be detected by a systematic investigation of the local influence of a great variety of poisons upon the isolated transporting structure. We are continuing our studies in this direction.

SUMMARY

1. In experiments on isolated loops of the small intestine of rats, the absorption of a number of organic substances has been followed. The absorption rates of these substances have been compared by filling alternatively into the loop equal amounts of the equimolar solutions for equal time periods. Further the influence of varying concentrations upon the rate of absorption has been investigated.

2. In the series of polyhydric alcohols (ethylenglycol—mannitol), the absorption rate falls off with rising molecular volume. The absorption rate of mannitol is approximately equal zero. Varying the concentration of a substance shows the percentage of absorption to be rather constant. It is concluded, that absorption of these substances is like a diffusion through a sieve-like membrane, the maximal pore diameter of

which controls the limiting molecular volume allowing permeation.

3. The absorption of aliphatic acid amides is analogous to that of the polyhydric alcohols except the fact that the amides are absorbed more slowly than the alcohols of corresponding molecular volume. A similar behavior has been encountered in studies upon the permeability of single cells of animals and plants. The differences are assumed to be based on special affinities between the pore walls of the membranes and the solutes.

4. Substances of distinct lipoid solubility are absorbed at a higher rate than lipoid insoluble substances of very low lipoid solubility.

5. Amino acids are absorbed at a higher rate than could be expected from their calculated molecular volume and even at a higher rate considering the volume of the zwitterions of the ampholytes being enlarged by a shell of water dipoles. Varying the concentration, it appears that the percentage of absorption is not constant, but falls off with rising concentration. Evidently, these substances of physiological importance resemble the 'physiological' sugars (glucose, galactose). Their absorption is not like a diffusion, but is a process complicated by the presence of an accelerating factor, the effect of which becomes more visible with lower than with higher concentrations.

ACKNOWLEDGMENT

One of the authors (R. H.) wishes to express his appreciation to the Faculty Research Committee of the Medical School, University of Pennsylvania, for a grant in support of this work.

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J. A. M. A.
109: 1517-1520, 1937

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GLYCERIN, ETHYLENE GLYCOL, PROPYLENE GLYCOL AND DIETHYLENE GLYCOL

REPORT ON FEEDING EXPERIMENTS WITH RATS

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The substitution of diethylene glycol for glycerin as a hygroscopic agent in industry makes it desirable to compare the toxicity of these two chemicals. Both have high boiling points, 245 and 290 C., respectively. Hence, small amounts may possibly pass unchanged with smoke from a cigaret into the body in addition to anything absorbed from the moist end of the cigaret in the mouth.

In view of the rather high toxicity of the related ethylene glycol and because of the use of propylene glycol as a solvent for iodobismitol, it was thought desirable also to include a rough comparison between commercial samples of diethylene glycol and these two related chemicals.[1]

Recent exhaustive studies [2] have shown that glycerin may constitute a large proportion of the food of rats over long periods and that smaller amounts may be added to adequate diets of growing dogs and of man without apparent harm. In fact, the rats showed normal growth and reproduction on such a diet and glycerin could be substituted for part of the food. Only with very large quantities was there diuretic action in dogs and rats. In spite of this favorable report my associates and I decided to include glycerin in our feeding experiments so as to make certain that the rats had the usual tolerance to this compound.

The recent literature dealing with the properties of the glycols is fairly extensive. A comprehensive paper by von Oettingen and Jirouch [3] deals with seven glycols, including ethylene glycol and diethylene glycol. Both caused hyaline shrinking of muscle fiber; ethylene glycol caused a slight and diethylene glycol a questionable hemolysis; methemoglobin formation was absent with ethylene glycol and uncertain with diethylene glycol. The minimal fatal dose in mice was twice as high for diethylene glycol as for ethylene glycol. Both had a slight action on the central nervous system, caused slight hyperemia and muscle rigor and had a slight antiseptic action toward yeast. Diethylene glycol was more depressing to striated muscle than ethylene glycol but had an uncertain depressive action on the intestine as compared to ethylene glycol. Neither caused edema, sensory nerve ending depression or motor nerve fiber depression. The authors concluded:

> Local irritation is comparatively small. Their application to the skin seems to be without risk. Given orally in larger doses they may produce severe gastro-enteritis and systemic symptoms. For intramuscular injection only small doses should be used, else systemic effects may arise. Subcutaneous injections should be avoided on account of local irritation. The intravenous administration would appear to be dangerous, because of the hemolytic action and of the fall of blood-pressure observed with these compounds.

Ethylene glycol was also studied extensively by Hanzlik, Seidenfeld and Johnson,[4] who used both intra-

From the Department of Physiology of the University of Chicago.
Profs. A. J. Carlson and A. B. Luckhardt gave helpful suggestions regarding this problem.

1. These two glycols were supplied by the Carbide and Carbon Chemical Corporation, South Charleston, W. Va.
2. Johnson, Victor; Carlson, A. J., and Johnson, Adelaide: Am. J. Physiol. 103: 517 (March) 1933.
3. von Oettingen, W. F., and Jirouch, E. A.: J. Pharmacol. & Exper. Therap. 42: 355 (Aug.) 1931.
4. Hanzlik, P. J.; Seidenfeld, M. A., and Johnson, C. C.: J. Pharmacol. & Exper. Therap. 41: 387 (April) 1931.

muscular injections and drinking experiments. In chronic feeding experiments on rats, smaller daily doses (0.7 Gm. per kilogram) stunted growth but did not injure the kidney, whereas the higher daily dose of 2.2 Gm. per kilogram caused variable degrees of oxaluria and renal calculi, verifying previous reports. As a solvent for medicinal products with intramuscular injections it was considered "comparatively innocuous." Hunt [5] reported kidney injury by oxalic acid formation when ethylene glycol was administered in the drinking water of different species of animals and cautioned against continued administration of small doses to man. In this and a subsequent paper he [6] also warned strongly against the promiscuous use of ethylene glycol in food products or as a substitute for glycerin. As to propylene glycol he found that, with a 5 per cent solution as the only liquid for drinking, rats showed normal growth and also good tolerance to it when their livers or kidneys had been injured by various poisons; Hunt suggested that propylene glycol may have a "true food value in the sense that ethyl alcohol does, and without the drug action of the latter" Wiley, Hueper and von Oettingen [7] concluded that low concentrations of ethylene glycol vapor in the air (300 mg. per thousand liters) is not toxic when intermittently given to mice or rats as judged by weight curves and histopathologic examinations, although one of ten rats and three of twenty mice died during the tests with 398 mg. per thousand liters for eight hours a day, five days a week, sixteen weeks, with no controls to show that similar mortality would occur in untreated rats and mice.

In their extensive study on bismuth excretion in rabbits and patients, Hanzlik, Mehrtens and Spaulding [8] used ethylene glycol as the solvent for iodobismitol without any untoward effects by this glycol. Seidenfeld and Hanzlik [9] found propylene glycol to be much less toxic than ethylene glycol in injection and feeding experiments as judged by animal growth and tissue appearance by microscopic examination. No demonstrable accumulation effects were seen with concentrations of from 1 to 10 per cent in the drinking water. However, propylene glycol did cause somewhat greater local irritation than ethylene glycol. Finally, Hanzlik, Mehrtens and Spaulding,[10] in a comparison of propylene glycol and diethylene glycol, concluded that iodobismitol prepared with propylene glycol is less toxic and prepared with diethylene glycol is more toxic systemically than ordinary iodobismitol prepared with ethylene glycol.

Haag and Ambrose [11] report that concentrations of 3 and 10 per cent, respectively, of diethylene glycol in the drinking water of rats proved rapidly fatal.

TOXICITY OF DIETHYLENE GLYCOL [12] IN FOOD AND WATER AND THAT OF GLYCERIN IN FOOD, AND ETHYLENE GLYCOL AND PROPYLENE GLYCOL IN WATER

The results of an eleven weeks experiment show that female rats 4½ months old on a control diet of fox-chow (Purina Mills) and water gained in weight about to the same extent as did those who had eaten fox-chow containing 20 per cent glycerin or 20 per cent water.

However, the fox-chow containing 20 per cent diethylene glycol killed five of six rats within four weeks. One kidney (found to be normal) was removed from the sixth sick rat after two weeks; on a normal diet this rat promptly recovered. When the fox-chow contained 10 per cent diethylene glycol, three of five rats died within eight weeks, but one showed normal gain in weight. From the fifth rat, which was losing weight, one kidney (found to be normal) was removed after the eighth week. On our control diet this rat recovered promptly. With fox-chow containing 5 per cent diethylene glycol, one out of six rats died after seven weeks and another after ten weeks. Of the remainder, one showed but a slight increase in body weight and the other three had made approximately normal gains.

When the drinking water contained 5 per cent diethylene glycol, the toxicity was markedly greater. All five rats died within eleven days (average duration of life, eight days). However, an equimolecular solution of commercial ethylene glycol (2.92 per cent) was even more toxic, killing all six rats within six days (average duration of life, four days). On the other hand, an equimolecular solution of commercial propylene glycol (3.58 per cent) was much less harmful than that containing the diethylene glycol. One of the six rats died after the tenth week and one had lost considerable weight at the end of the eleven weeks. The remaining rats were beginning to lose weight at the end of the experiment. Thus, the commercial propylene glycol appears to be more toxic than the highly purified substance administered to rats by Hanzlik and his associates [9] or the propylene glycol used by Hunt.[13]

Further analysis of the food and water intake showed that with fox-chow containing 20 per cent glycerin the rats ate 23 per cent less fox-chow per day than the ones fed pure fox-chow; but they ate only 11 per cent less than those who had had fox-chow with 20 per cent water as their food. The average daily consumption of glycerin was 2.7 Gm. As to water intake, no conclusion seems warranted because the rats who ate fox-chow containing 20 per cent water drank considerably more water than did the controls, indicating that there may be considerable variation in drinking habits even with animals of nearly the same size.

Considering fox-chow consumption per average hundred grams of gain in body weight during the eleven weeks, 11 per cent less of actual fox-chow was needed when the fox-chow contained 20 per cent of glycerin, indicating that glycerin was used as a food substitute.

The average daily fox-chow consumption was 6 per cent less than in the controls when the fox-chow contained 5 per cent of diethylene glycol. When it contained 10 per cent, the fox-chow consumption was 10 per cent less, or about the same as when the fox-chow had 20 per cent of glycerin. Considering again the small number of animals, these differences are not significant. However, the large increase in the average daily water intake (75 per cent) when the fox-chow contained 10 per cent of diethylene glycol may indicate an attempt to wash down the ill tasting food with water.

When the diethylene glycol proportion in the fox-chow reached 20 per cent, the average daily fox-chow consumption declined sharply to 24 per cent of the control value in these quickly dying rats. The aver-

5. Hunt: Indust. & Engin. Chem. 24: 361, 1932.
6. Hunt: Indust. & Engin. Chem. 24: 836, 1932.
7. Wiley, F. H.; Hueper, W. C., and von Oettingen, W. F.: J. Indust. Hyg. & Toxicol. 18: 123 (Feb.) 1936.
8. Hanzlik, P. J.; Mehrtens, H. G., and Spaulding, J. B.: Iodobismitol, Arch. Dermat. & Syph. 29: 298 (Feb.) 1934.
9. Seidenfeld, M. A., and Hanzlik, P. J.: J. Pharmacol. & Exper. Therap. 44: 109 (Jan.) 1932.
10. Hanzlik, P. J.; Mehrtens, H. G., and Spaulding, J. B.: J. Pharmacol. & Exper. Therap. 49: 300 (Nov.) 1933.
11. Haag, H. B., and Ambrose, A. M.: J. Pharmacol. & Exper. Therap. 59: 93 (Jan.) 1937.
12. [illegible]

sharper reduction in the average daily water intake to 18 per cent of normal indicates that these rats became too sick even to drink.

When the water contained 5 per cent of diethylene glycol, the reduction in average daily eating of fox-chow was even more severe than when the fox-chow had contained 20 per cent of diethylene glycol; the intake went down to 17 per cent of normal. The average daily water intake was also markedly diminished to 31 per cent of normal.

When the water contained an amount of ethylene glycol equimolecular with the 5 per cent diethylene glycol, the average daily consumption of fox-chow was nearly twice and the average daily water intake nearly three times that of the rats that had been drinking 5 per cent diethylene glycol; thus the water intake was nearly normal. Therefore, on an average, the rats took in 2.7 times more ethylene glycol molecules daily than those of diethylene glycol, which may partly explain why they were dying twice as rapidly.

When an amount of propylene glycol equimolecular with the 5 per cent diethylene glycol was present in the water, the rats ate and drank approximately as much as the control rats; thus, on an average they took in three times as many propylene glycol molecules daily as when diethylene glycol had been added to the water. The latter fact further emphasizes the much greater toxicity of diethylene glycol as compared with propylene glycol.

A COMPARISON OF THE EFFECTS OF GIVING VARYING CONCENTRATIONS OF PURE DIETHYLENE GLYCOL (EASTMAN) IN THE DRINKING WATER OF YOUNG RATS

Because the toxic effects following administration of commercial diethylene glycol may possibly have been due to impurities, we added a nine weeks experiment in which pure diethylene glycol was added in varying amounts to the drinking water of rats which were from 7 to 8 weeks old; concentrations started at a level 20 per cent lower than the one that had killed all rats within eleven days. This new concentration of pure diethylene glycol was thus 4 per cent; it killed three of the five rats within nine days, but the other two survived.

On the lower concentrations of 2, 1, 0.5, 0.25 and 0.125 per cent, respectively, of diethylene glycol, no animals died. However, the average actual and percentage gains in body weight during the nine weeks was less than half of that of the controls. Not until the concentration had become as low as 0.125 per cent were these growth averages exactly up to the control values.

Looking next on the average daily amounts of fox-chow eaten, the values are 24 per cent lower than the control intake when the water contained 2 per cent of diethylene glycol. In case of the two highest concentrations (2 and 1 per cent, respectively), there was also a noticeable diminution in actual water intake to 59 and 67 per cent of normal, respectively.

With the higher concentrations of pure diethylene glycol in the water there were dark discolorations around the genitalia; these were not blood; this indicates that diethylene glycol or some darker decomposition product was being passed in the urine. Thus, some diethylene glycol was being absorbed.

In the case of the rats which died acutely from diethylene glycol poisoning, the stomach and intestine showed signs of marked local irritation and in some

Comparing the liver, kidney, lung and myocardium of the rats that had been given commercial ethylene glycol, propylene glycol, diethylene glycol or glycerin in the food or water with those of the control rats, no certain damage was demonstrable to these vital organs as judged by examinations of sections stained with scarlet red or with hematoxylin and eosin. These examinations were done by courtesy of Dr. Paul R. Cannon.

Considering the same four organs of the rats which had been given varying concentrations of the pure diethylene glycol in the drinking water, no certain damage could be seen in the liver, kidney or lung. Dr. Eleanor Humphreys, who carried out these examinations, reported an indication of a possible slight change in the myocardium with the three highest concentrations as judged by the hematoxylin and eosin staining technic, but it was slight and not present in all cases.

COMMENT

The results of these experiments place diethylene glycol between ethylene glycol and propylene glycol in toxicity. A high concentration of glycerin in the solid food (20 per cent) seemed harmless. Diethylene glycol in one fourth of this concentration in the fox-chow and in much smaller concentrations in the drinking water was distinctly harmful as judged by cases of mortality, stunted growth in the young, and impaired reproductive capacity.

Even after prolonged administration of diethylene glycol in the concentration employed, however, visible microscopic changes in the liver, kidney, lung, heart and testis were absent. Both the frozen section method and the staining with hematoxylin and eosin were used in the first four of these tissues. Although diarrhea was not observed, I believe that the absence of pathologic changes in these vital organs may indicate that local irritation probably is an important factor in the toxicity.

Because the rat in some cases is much more tolerant to poisons than man, for example, to atropine, and in other cases shows peculiar susceptibilities, for example, delayed death after slightly hypnotic doses of nostal, we do not know with certainty what the relation is with diethylene glycol. However, the report by Hunt[6] of greater susceptibility of man to ethylene glycol than the rat—comparing the per kilogram dosage—probably means that man is also more susceptible to diethylene glycol.

SUMMARY

1. In an eleven weeks experiment, younger adult female rats gained about the same in body weight as control animals when a high concentration of glycerin (20 per cent) was mixed with their solid food

2. Similar concentrations of commercial diethylene glycol killed all rats within about two weeks, and even 10 and 5 per cent proved fatal to some of the rats. Diethylene glycol is even more toxic when added to the drinking water. When the rats drank from a 5 per cent solution, the average duration of life was only eight days.

3. With the equimolecular concentration of commercial ethylene glycol (2.92 per cent) in the drinking water, the rats lived only an average of four days but actually had consumed 2.7 times as many molecules of ethylene glycol per rat daily.

4. With an equimolecular concentration of commercial propylene glycol in the water (3.58 per cent), one rat died at the tenth week, one was sick and others were

intake was three times as many molecules of propylene glycol as of diethylene glycol. This places diethylene glycol between ethylene glycol and propylene glycol in toxicity, but nearer the first one.

5. When pure diethylene glycol was added to the drinking water of 2 months old female rats, some died with the highest concentration of 4 per cent and growth was markedly stunted at this level. Even in the concentration of 0.25 per cent, some indication of impaired growth was detectable.

6. That diethylene glycol was being absorbed with the higher concentrations was indicated by discolorations around the genitalia. With high concentrations, local injury and bleeding of the stomach and intestine were frequently seen in the fatally poisoned rats.

7. In microscopic examinations of the liver, kidney, lung and myocardium the only possible change seemed to be a slight one in the myocardium with 1, 2 and 4 per cent of pure diethylene glycol, respectively, in the drinking water; but even this change was not uniformly present and hence may not be significant. Both the frozen section method and the hematoxylin and eosin staining method were employed.

8. Pregnancy did not occur when males and females were mixed and both received 0.5 per cent of pure diethylene glycol in the drinking water. There was some indication of smaller and fewer litters in females receiving 0.25 and 0.5 per cent in the drinking water when mated with frequently changed fresh stock males.

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Acta Physiol. Scand.
7: 69-79, 1944

From the Institute of Medical Physiology, University of Copenhagen.

Glycerol Oxydation in the Animal Organism.

By

ERIK J. HOLST.

Received 12 November 1943.

There seems to have been little research into the question of the metabolism of glycerol, probably because of the lack of suitable methods of analysis. As I have devised a method of glycerol analysis for another purpose (1943), a method capable of application to series determinations on blood, it was so to say the obvious course to make use of it in an investigation into glycerol metabolism.

The method is based on an extremely sensitive colour reaction, which is specific for glycerol and is brought about by heating it with anthron and sulphuric acid. This reaction has been employed for the colorimetric determination of glycerol in aqueous extracts of parchment paper (F. SCHÜTZ, 1938).

Technique of Method.

By means of a pipette 0.2 ml whole blood (or 0.2 ml oxalate plasma) is transferred to 2.5 ml distilled water in a centrifuge tube. Add 0.1 ml basic lead-acetate solution (GOULARD's extract prepared according to PETERS and VAN SLYKE 1932), 0.4 ml of a 5 % solution of zinc sulphate and 0.5 ml of 0.85 n. sodium hydroxyde. Shake after adding each component. Centrifuge after standing at least five minutes. Decant the clear, colourless fluid into a conical centrifuge tube. At this stage the samples may be stored in the refrigerator for 24 hours without affecting the result of the analysis. The excess lead is removed by adding a small drop of saturated carbonate of soda and renewed centrifuging. Of the clear fluid transfer 2 ml by pipette into a Pyrex test tube, and add 4 ml of a fresh solution of anthron[1] in concentrated sulphuric acid (0.1 mg per ml). Add the same quantity to 2 ml standard solution (0.5—2.0 or 5.0 mg%) and to 2 ml water. After thorough mixing cover the tubes with tinfoil and place for 10 minutes in an oil bath maintained at 170—175°. This will produce a beautiful red-orange colour with green fluorescence when glycerol is present. Compare colorimetrically according to BÜRKER's principle. The precipitation fluids have a low blank value (0.1—0.2 mg%), which is determined separately and subtracted.

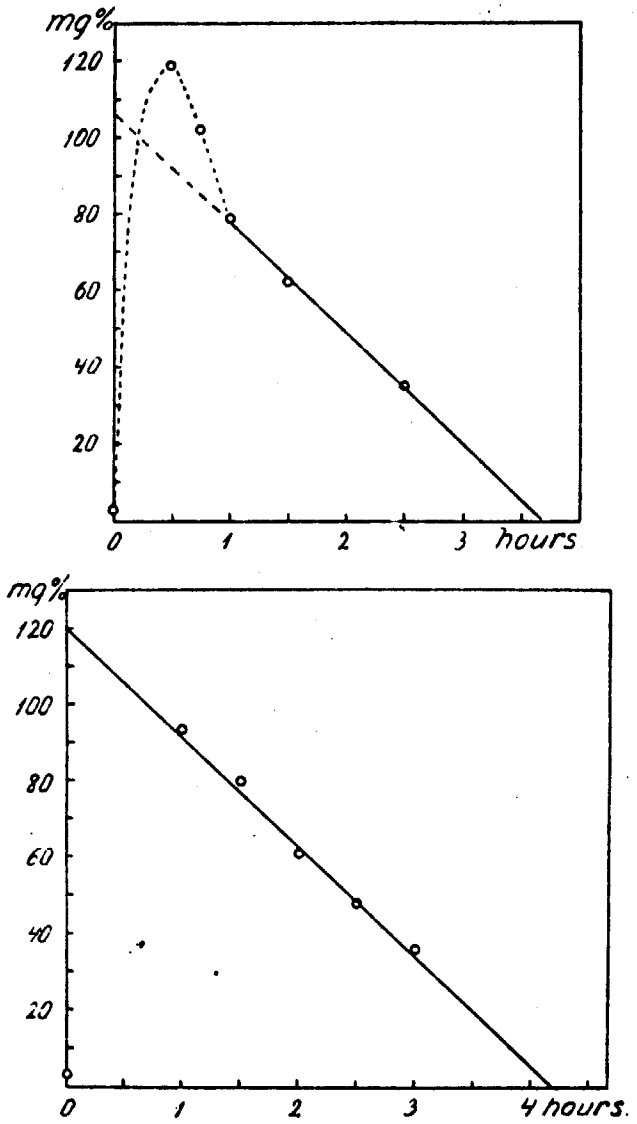
In experiments to find known quantities of glycerol added to blood the mean error of a single analysis is found to lie at about 3 %.

There being good reasons for believing that the liver plays a role in glycerol metabolism, I first experimented with a series of glycerol administrations both to patients with a healthy liver and to liver patients, for the purpose of determing whether the rate of decrease in blood glycerol after glycerol administration if of any value as a liver-function test.

The technique was as follows: To the fasting patient I administered 0.54 g glycerine per kilogramme. (In practice I gave the patients ½ ml glycerinum bidestilatum "Merck" pro kg body weight). It was administered in ten times the volume of water. A blood sample was taken from the ear just before and five times in the course of 2½—3 hours after the glycerol administration.

The blood glycerol curve (figs. 1 and 2) describes a course that is reminiscent of a blood alcohol curve after alcohol administration, as in all cases the concentration in the blood after one hour decreases quite rectilinearly. Consequently it must be justifiable — as with alcohol — to assume that absorption and distribution in the organism are complete after one hour, so that the first part of the curve may be regarded as the resultant of absorption, distribution and elimination, whereas the remaining part is purely an elimination curve. This can be characterized by its rate of descent and its point of intersection with the ordinate. The first expressed as the fall in concentration in per mille per minute corresponds to WIDMARK's factor β (WIDMARK 1932). The second indicates the concentration that would have been obtained immediately after the glycerol ingestion if absorption

[1] Anthron is prepared by means of a weak reduction of anthrachinon in acetic acid solution with granulated tin and fuming nitric acid after LIEBERMANN and GIMBEL (1887).



Figs. 1 and 2. Glycerol Concentration of the Blood in two Patients after Oral Ingestion of Glycerol.

and distribution had proceeded instantaneously. From this figure it is possible to calculate what fraction of the total mass of the organism acts a a solvent for glycerol like the blood. This frac-

tion, which after WIDMARK is denoted by r, is calculated according to the equation

$$G = p \cdot r \cdot c_0$$

Where G = the quantity of glycerol in g,
p = the body weight in kg,
c_0 = the concentration in ‰ at 0 hours.

Table 1.

Glycerol Ingestion 0.54 g/kg Except Patient No. 6, who received 0.45 g/kg.

No.	Weight kg	C_0 ‰	r	β ‰	r β g/kg/M	Diagnosis	Age	Sex
1	68.5	1.18	0.46	0.0049	0.0023	Sciatica	41	♀
2	70.8	1.08	0.50	0.0048	0.0024	Sciatica	36	♀
3	63.2	1.10	0.49	0.0052	0.0025	Sciatica	37	♀
4	57.0	1.14	0.48	0.0057	0.0027	Myalgia	48	♀
5	65.1	1.20	0.45	0.0049	0.0022	Mb. Basedowii	29	♀
6	58.0	(0.80)	0.56	0.0051	0.0029	Mb. Basedowii	65	♀
7	60.0	0.94	0.58	0.0056	0.0032	Diabetes mellitus	30	♀
8	74.0	1.21	0.45	0.0054	0.0024	Obstructive jaundice	53	♀
9	54.0	1.04	0.52	0.0048	0.0025	Sub-icterus	44	♀
10	63.4	0.96	0.56	0.0048	0.0027	Acute hepatitis	43	♀
11	58.1	0.94	0.58	0.0045	0.0026	Cirrhosis hepatis	55	♂
12	54.3	0.88	0.62	0.0035	0.0022	Cirrhosis hepatis	58	♂

In addition to C_0, r and β Table 1 contains the velocity of elimination r · β expressed in g/kg/min. Taking all the tests together it will be seen that the mean of r is 0.52 with a dispersion that is at any rate no greater than for alcohol. For alcohol the average for both sexes is 0.61. The average values for β are 0.0049 and 0.0025 respectively, so that the velocity of metabolism in g/kg/min. is 0.0026 for glycerol and 0.0015 for alcohol. No account is taken here of the excretion, which for alcohol is insignificant as the alcohol will be found in the same concentration in the urine as in the plasma, and the loss of alcohol through the expiration air will be of importance only in cases of excessive ventilation. For glycerol the loss is somewhat larger and amounts to about 10 % of the elimination, as found in experiments 1, 4 and 12, where the urine excretion in the period from 60 to 180 minutes was determined at 0.00025, 0.00024 and 0.00026 g/kg/min. respectively.

It is also to be seen from these experiments that there is no indication that the glycerol curve would be clinically useful as a

liver function test (cp. Tests 1—4 with 8—12). Of the only two cases of hyperthyreoidism one has a high, the other a low velocity of metabolism. The highest velocity observed was that of patient No. 7, who has diabetes. This calls for further experiments on diabetics.

These results also call for a further comparison between glycerol and alcohol metabolism. LUNDSGAARD (1937), partly by means of artificially perfused isolated livers and hind-leg preparations, partly with the aid of exviscerated animals, has shown that the alcohol oxydation takes place almost exclusively in the liver and that the oxydation of alcohol does not take place in the muscles. He has also shown that the alcohol metabolism in the liver is a partial oxydation which leads to the formation of an organic acid, probably acetic acid, which presumably is further oxydated in the tissues.

The following experiments are a partial repetition of LUNDSGAARD's experiments with glycerol instead of alcohol. Cats narcotized with chloralose (5 cg/kg) were used in these experiments.

Table 2.

Glycerol Experiments on Cats.

No.	Weight kg	r	β	Elimination g/kg/min.	Elimination mg/min.
1	3.4	0.54	0.0059	0.0032	10.7
2	3.8	0.45	0.0081	0.0036	13.9
3	2.7	0.57	0	0	0
4	2.9	0.50	0	0	0
5	2.5	0.52	0.0008	0.0004	1.0

Cats Nos. 1 and 2 were nephrectomized, partly to eliminate any loss of glycerol through the urine, partly that they might serve as a better basis for comparison with Cats Nos. 3, 4 and 5, which were abdominally exviscerated. All the animals received 1.2 g glycerol intravenously at 0 hours except No. 6, which was exviscerated only and served for control purposes.

It will be seen from experiments 1 and 2 that the rate of metabolism was again constant and independent of the blood concentration. In one test (No. 2) it can be seen that even after 10 minutes there was no even distribution in the animal; it can also be seen that at very low concentrations the rate of decrease does not follow the straight line curve but becomes slower. The

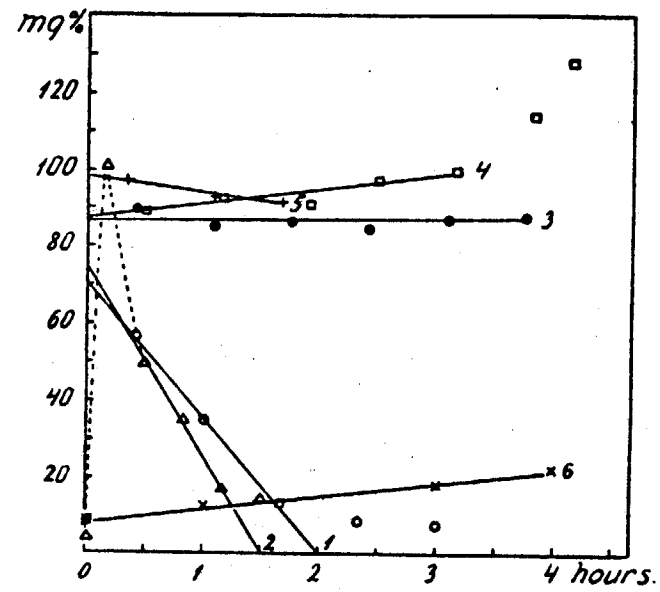


Fig. 3. Elimination Curves from the Experiments in Table 2.

quantity of glycerol remaining, which was thus catabolized more slowly is so small in proportion to the administered quantity that it is of no practical importance to the calculation of the velocity of metabolism, and therefore it is disregarded. The mean r value is the same as for human beings, whereas the rate of metabolism in g/kg/min., i. e. $r \cdot \beta$, is about 30 % higher than for man.

The exviscerated animals do not metabolize glycerol, the blood concentration being unchanged in one case (No. 3), slightly falling in another (No. 5) and slightly rising in a third (No. 4). During the experiment three of the animals (Nos. 3, 4 and 6) received a constant infusion of glucose (10 mg/min.) in order to prevent the fall in blood sugar which otherwise would occur in the hepatectomized animals. These three had increasing blood sugar. However, the increase in the glycerol concentration in the one case cannot be due to the rise of the blood sugar, as I have ascertained by glycerol analyses on blood with various quantities of glucose added, but must be due to an accumulation in the blood of other products as a consequence of the abnormal metabolism. The control experiment (6) also supports this

Accordingly, these experiments show that the glycerol metabolism must, in any case primarily, be dependent on the function of the excised organs, and thus it is natural to suppose the liver as the seat of that metabolism. As I am not master of the technique of liver perfusion experiments, Professor LUNDSGAARD made them for me, and I would here express my thanks to him. The technique was described by LUNDSGAARD, NIELSEN and ØRSKOV (1936). Moreover, the oxygen consumption was determined by a photoelectric measurement of the oxygen in the venous blood, as recently described by LUNDSGAARD (1942). By this means a considerable drop in the oxygen consumption of the liver is observed in the first 30—60 minutes after perfusion begins, whereafter it remains almost constant.

Table 3.

Liver Perfusion Experiment.

No.	1	2	3	4
Rate of glycerol metabolism mg min. .	3.00	3.19	6.25	7.90
Computed oxygen consumption resuming complete oxydation of glycerol metabolized ml min.	2.56	2.97	5.33	6.73
Oxygen consumption found ml min. .	2.00	2.25	3.25	3.35
Liver weight, g	70	78	100	86

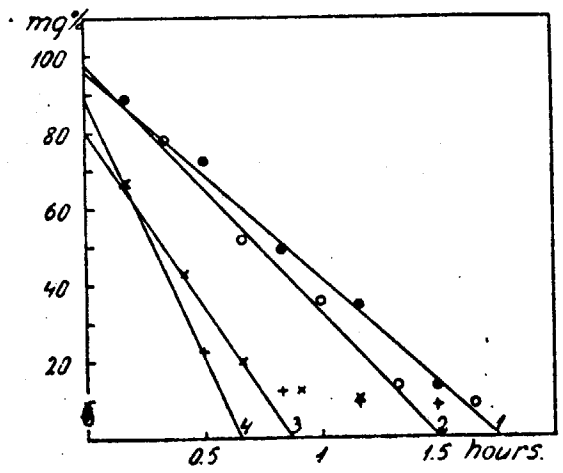


Fig. 4. Elimination Curves for the Experiments in Table 3.

In each test 300 mg glycerol were added to the perfusion blood. In experiments 1 and 2 the addition was not made until after the oxygen consumption had become constant. Here again the curves have a rectilinear course, and the rate of metabolism averages 3.25 mg/min., which is about 25 % of the metabolism in an intact animal. Under corresponding experimental conditions LUNDSGAARD found that the alcohol metabolism in the isolated liver was about 60 % of that of an intact animal and he ascribed this to the initial fall in the oxygen consumption. I therefore added the glycerol shortly after perfusion began, i. e. under decreasing oxygen consumption, the result being that these experiments (Nos. 3 and 4) gave a much higher rate of metabolism for glycerol, about 60 % of that of an intact animal.

The addition of glycerol causes no great increase in the oxygen consumption of the liver. If the catabolized quantity of glycerol were completely oxydized, the oxygen consumed by this alone would clearly exceed the liver's real oxygen consumption, as will appear from Table 3. The oxydation in the liver must, therefore, be incomplete.

A partial oxydation would cause a fall in the respiratory quotient. The most likely partial oxydation would be a conversion to glucose, which requires a much lower consumption of oxygen than for total oxydation (14 %). Provided that the whole of the glycerol is converted into carbo-hydrate without an increase in the total oxygen consumption, everything else being equal, the RQ will be reduced, e. g. from 0.70 to 0.59. In a single test, in which the RQ was determined before and after the administration of the glycerol, I was unable to demonstrate any change in RQ, whereas in LUNDSGAARD's alcohol experiments it fell from 0.69 to 0.37. It should be observed, however, that the fall in the RQ which under the same circumstances one would expect to find when alcohol is oxydated to acetic acid is about four times as great as when glycerol is converted into carbo-hydrate. When it is considered that part of the glycerol is undoubtedly oxydized completely with a respiratory quotient (0.86) which lies above the normal of the isolated liver, it is no wonder that the addition of glycerol results in no distinct change in the quotient. No production of acid can be demonstrated during the glycerol metabolism, the alkali reserve being constant.

Owing to the disagreement between the rate of metabolism in the isolated liver and in the intact animal, the possibility cannot

be excluded that glycerol is also broken down in the intestine. For the purpose of approaching this problem a little nearer an experiment was made by perfusing an isolated cat intestine with blood to which glycerol was added. No definite change was observed in the glycerol concentration, but the intestinal function undoubtedly suffers under perfusion, as oedema sets in rapidly with sanguineous exudation into the lumen. I therefore tried leaving out the liver without compromising the circulation of the intestine. With a G-shaped glass cannula I connected the portal vein and the right renal vein, thus making a kind of Eck's fistula. At the same time the hepatic artery and the right renal artery were ligatured and the left kidney was removed. The animal received heparin intravenously just prior to the setting up of the anastomosis, and glucose was infused constantly as in the case of the exciscerated animals. After the experiment there was no stasis in the portal region, and the intestine had a perfectly natural appearance with no exudation into the lumen.

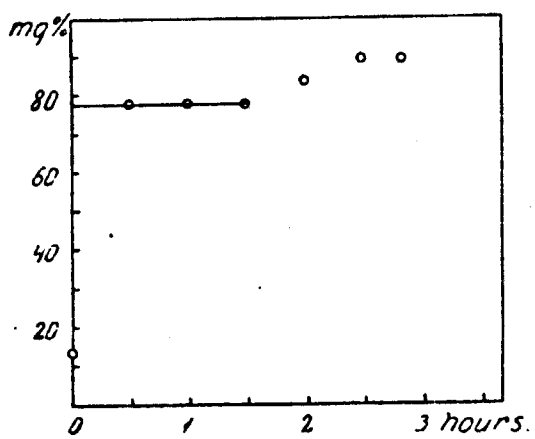


Fig. 5. Elimination Curve of Hepatectomized Cat.

Fig. 5 shows the result of an experiment of this kind. Here again the curve is horizontal at first, whereupon the values rise as in one of the experiments with exviscerated animals. It would seem that in hepatectomized animals a substance accumulates in the blood which has a colour reaction like that of glycerol.

For comparison brief mention may be made of earlier experiments by LEDERER (1936). The glycerol method he used is based

on a reduction of bichromate in sulphuric acid. The so-called chrome index of the plasma is determined by means of titration of the excess bichromate; by subtracting from this the value corresponding to the glucose content the so-called residue index is reached. The increase of the glycerol content is then calculated from the difference between the "residue index" before and after the administration of the glycerol.

Employing this somewhat uncertain method LEDERER studied three intravenous injections of glycerol in dogs, 1 g glycerol per kg; four blood samples were taken during the first 90 minutes. By plotting the results into a coordinate system it is found that two of the curves are rectilinear and have the same inclination, the third one being more irregular. I have calculated the r values at 0.65, 0.44 and 0.33 and the velocity of metabolism at 11.1, 13.7 and 9.4 mg/kg/min., that is to say they are three or four times as high as in my own experiments on man. Perfusion of dog livers by LEDERER gave metabolic values of 7.4 and 29.3 mg/min., but in a control test without glycerol there was a marked rise in the "residue chrome index", so that in reality the metabolism must be assumed to have been much greater. Thus the results of LEDERERS experiments, through carried out with a primitive technique for glycerol determinations correspond in some respects to mine.

Summary.

There is a distinct similarity between the metabolism of glycerol and that of ethylalcohol. This similarity is recognizable by the fact that:

1) The elimination curve is rectilinear, i. e. the rate of glycerol metabolism is constant, and independent of the blood concentration.

2) The glycerol metabolism is localized exclusively, or almost exclusively in the liver.

3) At most a fraction of the glycerol can be completely oxydized in the liver, while at least the larger fraction must be incompletely oxydized.

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Boll. Soc. Ital. Sper.
47: 595-596, 1971

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THE EFFECT OF ORALLY TAKEN GLYCEROL ON THE THICKNESS OF THE NORMAL HUMAN CORNEA

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Section of Naples -- Meeting of July 31, 1971

As is well known, glycerol causes a rapid and significant ocular impotence through a dehydration of the ocular tissues, both in subjects affected by various forms of glaucoma and in normal subjects. The possible dehydrating effects of glycerol on the thickness of the cornea has not yet been studied.

The scope of this note is to relate the results of our study on the effects of glycerol--administered orally--on the central and peripheral thickness of the normal human cornea.

Materials and methods: Our experiments were conducted on twenty healthy eyes belonging to ten normal subjects, ages 15 to 48 years old (the median age was 30).

After the base value of the corneal thickness, whether central or peripheral, and of the intraocular pressure was established, the subject was given orally a solution of glycerol at 50% (Luxoral Tubi, Lux, Naples) in a dose of 1 ml/kg of bodily weight. After one hour and thirty minutes, we again determined the corneal thickness, central and peripheral, and the intraocular pressure. If we found that an eye had lost more than 10 mm Hg in pressure, that eye was excluded. [We chose the figure 10 mm Hg as the maximum limit to avoid a marked reduction in pressure producing by itself a variation in the corneal thickness (1)].

The corneal thickness was measured with the "apparatus I for measuring in depth" (haag--Streit, Bern) mounted on a fissure lamp (Haag--Streit, model 900).

The central thickness was determined at the center of the cornea; the peripheral thickness was determined at six o'clock immediately adjacent to the limbar vessels. The technique used for measuring the corneal thickness has already been described by us (2). As soon as we obtained the value of the central and peripheral corneal thickness, we measured the intraocular pressure with the tonometer of Goldmann. Local anesthesia was effective with Novesine 0.4% (Wander S.A., Milan). Each subject was always examined at the same time of day (long after he awakened).

The variability of the apparatus that we used was determined on a given eye. We made ten measurements, either on the central corneal thickness or on the peripheral corneal thickness, and so were able to calculate the respective standard deviations.

Results: The standard deviation of ten measurements of the central corneal thickness was $\pm$ 0.013 mm; hence we considered significant a variation of the said thickness equal to or greater than 0.039 mm. The standard deviation of ten measurements of the peripheral corneal thickness was $\pm$ 0.015 mm; hence we considered significant a variation of this thickness equal to or greater than 0.045 mm.

TABLE: Mean values of central and peripheral corneal thickness (mm) and of the intraocular pressure (mm Hg) before and after an orally administered dose of a solution of glycerol at 50%.

	N. occhi	0 h	1 h 30 min	Variazioni
Spessore corneale centrale	20	0,501	0,481	—0,020
Spessore corneale periferico	20	0,721	0,705	—0,016
Pressione intraoculare	20	12,25	10,33	—1,92

As can be seen from the table, the mean corneal thickness, both central and peripheral, of the normal human eyeball--under our experimental conditions--shows a slight reduction after the oral administration of glycerol at 50%, but such a reduction is not statistically significant.

The behavior of the central or peripheral corneal thickness of each individual's eyes, when the glycerol was orally administered, was, in general, uniform.

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OSMOTIC PROPERTIES OF THE ERYTHROCYTE

VII. THE TEMPERATURE COEFFICIENTS OF CERTAIN HEMOLYTIC PROCESSES

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ONE FIGURE

I

It is well known that the rate of penetration of cells by water and by dissolved substances is usually greater at high than at low temperatures. Quantitative data on this subject, however, are still somewhat scanty, particularly data which permit comparisons to be made between the behavior of different penetrating substances in the presence of single kinds of cells or of single substances in the presence of different kinds of cells. This paper, having to do with the temperature coefficients of hemolysis for eleven species of mammals in solutions of certain typical penetrating non-electrolytes is designed to fill some of the gaps in the existing literature in this field and to bring out certain facts of possible significance from the standpoint of the comparative physiology of cell permeability.

The advantages of the erythrocyte for such investigations are obvious. Material representing a large number of species of different degrees of zoölogical relationship can be obtained in abundance at all times, and can be studied under readily standardized and controlled conditions. Of the various methods for the quantitative estimation of the permeability of the erythrocyte, the hemolysis method is by far the simplest, and if used with judgment and with due regard for

its limitations, yields very satisfactory results. The details of this method as here employed have been described in earlier papers of the present series. The times measured have in all cases been those required for the attainment of 75 per cent hemolysis in a mixture of 1 part of blood to approximately 500 parts of solution. The blood, defibrinated but otherwise untreated, has been kept, up to the time of its mixture with the hemolytic solutions, within a few degrees of 0°C. Rapid mixing has been facilitated by mechanical stirring, which in short experiments has been continued to the end of the experiment and in those of considerable length has been applied at intervals.

Subject to the validity of certain assumptions, which have been discussed more fully elsewhere (Jacobs, '34), the rate of hemolysis in water and in hypotonic solutions of non-penetrating substances may be used as a quantitative measure of the permeability of the erythrocyte to water. The most important of these assumptions are, 1) that Fick's law of diffusion is followed, but that the rates of diffusion within the cell and in the external medium are so rapid compared with that across the cell membrane that only the latter process need be dealt with, 2) that the hemolysis of each cell takes place at what for it is a definite 'hemolytic volume,' V_h, and 3) that during the swelling of the cell no processes occur, other than the entrance of water from without, which alter either its internal osmotic pressure, its permeability, or the readiness with which it gives up its hemoglobin.

Of these assumptions, 1) has been shown by Collander and Bärlund ('33) by direct chemical methods to be substantially valid for the cells of Chara and by less direct methods by Lucké, Hartline and McCutcheon ('31) probably to be applicable to the eggs of Arbacia. Earlier work on the erythrocyte, for example, Jacobs and Parpart ('31), has also yielded results which as far as can be judged from the available data seem to be consistent with it. Assumption 2) has been shown experimentally by Ponder and Saslow ('31) probably to be sufficiently accurate for the purposes of the present study.

As contrasted with the first two assumptions, the third is frequently far from true. One important disturbing factor in all temperature studies on the erythrocyte has been pointed out in earlier papers (Jacobs, '28, '31 a). It arises from the fact that the base-binding powers of hemoglobin as compared with those of carbonic acid increase fairly rapidly with rising temperature (Stadie and Martin, '24). It follows from principles discussed at length by Warburg ('22) and by Van Slyke, Wu and McLean ('23) that other conditions remaining equal, the internal osmotic pressure of the erythrocyte must necessarily decrease with rising and increase with falling temperature. To disregard the significance of this factor, as has been done in several recent papers on osmotic hemolysis, is tacitly to deny the truth of the careful physico-chemical studies mentioned above, and many other similar ones to be found in the literature, on the sharing of base between hemoglobin and carbonic acid.

Since in spite of its frequent emphasis by one of the present authors this effect of temperature on the osmotic behavior of the erythrocyte is still neglected by many workers, it may be useful at this point to discuss a concrete experiment, performed several years ago with the assistance of Dr. A. K. Parpart but hitherto unpublished, which illustrates very clearly the fallacious conclusions that might be drawn from uncritical comparisons of rates of hemolysis obtained at different temperatures. In figure 1 is represented the course of hemolysis of ox erythrocytes at 0°, 20° and 40°C. in solutions of NaCl of the concentrations 0.095 M, 0.100 M and 0.105 M, respectively. The ordinates as plotted represent scale readings of the instrument previously described (Jacobs, '30), but a number of horizontal lines have been drawn to convert these readings into percentages of hemolysis.

An inspection of the figure immediately shows the futility of attempting to use temperature coefficients in cases such as this, in which obviously two sorts of effects are involved. The first of these effects has to do with the degree of hemolysis ultimately attained; it increases progressively with decreasing temperature, and vice versa. The second is a true rate

effect, which for any given system determines the rapidity with which the position of final equilibrium is approached; it increases with rising temperature. Since the two effects, in general, have opposing influences on the observed times of hemolysis, it is evident that the latter, if determined merely for certain arbitrarily selected sets of conditions might vary

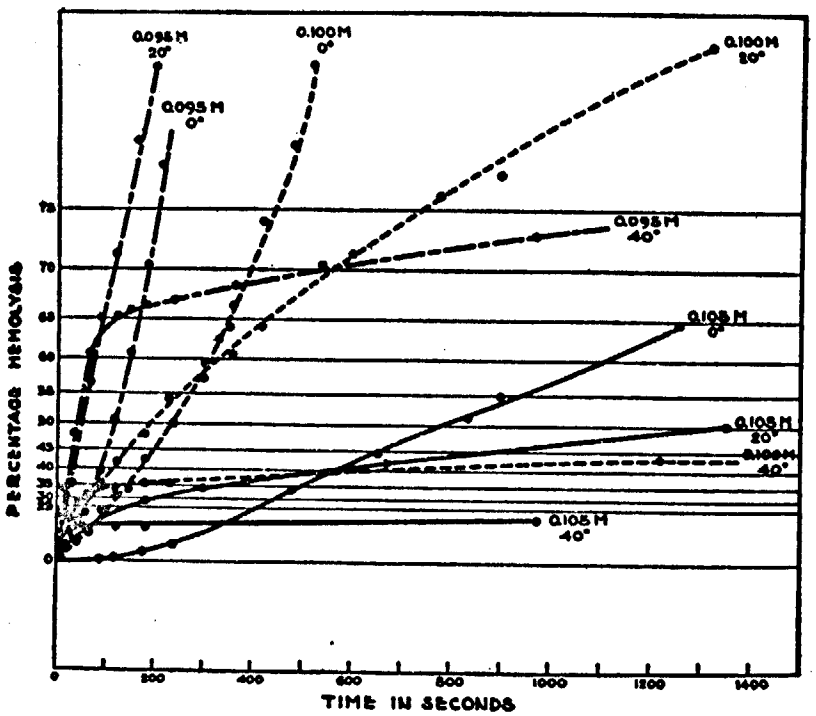

Fig. 1 The course of hemolysis of ox blood at 0°, 20° and 40°C. in 0.095 M, 0.100 M and 0.105 M NaCl.

in what at first sight would appear to be an entirely inexplicable manner. For example, if in the case of 0.100 M NaCl, the time required to reach 25 per cent hemolysis were selected as a criterion for estimating the rate of penetration of water, it would with entire correctness be inferred that permeability increases with increasing temperature, though the calculated Q_{10} values might be considerably in error. If, on the other

hand, 32 per cent hemolysis were similarly selected and observations were made only at 20° and 40°, the inference might be drawn that temperature has no appreciable effect on permeability. (Many statements in the literature concerning cell permeability are based on evidence no better than this.) Furthermore, if the end-point chosen had by chance been 65 per cent hemolysis, it would appear by the same reasoning as before that the entrance of water is slower at 20° than at 0°, and slowest of all at 40°C.

Though when the experimental data are fully presented in this way, a confusion of rate and equilibrium factors might seem to be unlikely, the fact remains that such factors have very frequently been confused in the past, not only in the study of hemolytic processes but in other fields of physiology as well, and they will doubtless continue to be in the future in spite of warnings such as this. The question naturally arises whether this source of error can ever be avoided. Strictly speaking it cannot, though for practical purposes the equilibrium factor usually becomes of negligible importance when swelling of the cells is allowed to take place in water or in extremely dilute solutions in which the volume curve crosses the line representing the hemolytic volume steeply, i.e., in which hemolysis occurs at a volume far removed from the theoretical equilibrium volume. By working under such conditions, consistent and reproducible values of Q_{10} for the entrance of water may be obtained. Numerically, these usually lie between about 1.25 and 1.40, and are, perhaps significantly, of the order of magnitude of the temperature coefficients of diffusion processes. Such values were previously reported by one of the authors (Jacobs, '28, '31 a) and have been confirmed many times since (see for example the figures for water in table 4).

A second factor that may possibly cause difficulties in the determination of temperature coefficients for osmotic processes in the erythrocyte is that of leakage from the cell, during the swelling process, of significant quantities of osmotically active solutes. Ponder and his co-workers have strongly emphasized the importance of this factor, attributing to it not

merely certain observed departures of the behavior of cells from that of perfect osmometers, when exposed to a single solution at a single temperature, but even explaining in the same way the greater osmotic resistance of the erythrocyte in solutions of non-electrolytes than in those of electrolytes (Ponder and Saslow, '31, p. 290) and at high than at low temperatures (Ponder, '34, p. 114).

In the case of the last-mentioned temperature effect, which is of particular importance in connection with the present work, Jacobs and Glassman ('34) have recently found that cells which fail to hemolyze at high temperatures may readily do so when the temperature is subsequently lowered, a fact which would be difficult to account for by a leakage hypothesis. Jacobs and Parpart have also shown ('33 and further work soon to be published) that leakage is probably by no means the most important factor in determining osmotic differences between electrolyte and non-electrolyte solutions. While it is almost certain, therefore, that the significance of this factor has been over-emphasized in certain cases, it is by no means impossible that it may introduce troublesome complications into experiments of the type here reported. In the absence of a complete knowledge of the full significance and magnitude of leakage in any given case, however, it seems best for the present merely to recognize its possible existence and to present the data on osmotic hemolysis exactly as they were obtained, without attempts to apply to them corrections which would, in the present state of our knowledge, be highly uncertain.

II

It is possible to apply the hemolysis method to the measurement of the permeability of the erythrocyte to dissolved substances, as well as to water, by taking advantage of the fact that the entrance of the solvent into the cell from a given solution tends constantly to equalize any differences of osmotic pressure that might otherwise be set up by the penetration of the solute. In theory at least, definite mathematical relations can be deduced between the movements of water and those of the solute, and the rate of penetration of the latter

can be inferred from the rapidity with which observed volume changes of the cell occur (Jacobs, '34). When the rate of penetration of the dissolved substance is relatively slow as compared with that of water (see for example the case dealt with by Schiødt, '33), the relation is very simple, the permeability of the cell to the solute, subject to the qualifications already discussed, being approximately inversely proportional to the time of hemolysis (Jacobs, '34). When, on the other hand, the penetration of the solute is rapid, as in the case of urea, ethyl alcohol, etc., the relation becomes much more complex, and the times of hemolysis may give rise to very

TABLE 1

Theoretical temperature coefficients for osmotic hemolysis corresponding to assumed coefficients of 1.3 for permeability to water and 2.6 for permeability to a solute

20°C. $k_2 = 1$ $t_{20} = 1$		30°C. $k_2 = 1.3$ $t_{30} = 0.77$			(Q_{10}) HEMOLYSIS
k_1	F_{20}	k_1	k_1/k_2	F_{30}	
50	1.04	130	100	1.02	1.33
10	1.20	26	20	1.10	1.42
2	1.88	5.2	4	1.48	1.65
1	2.57	2.6	2	1.88	1.78
0.5	3.77	1.3	1	2.57	1.91
0.1	12.0	0.26	0.2	6.93	2.25
0.05	22.0	0.13	0.1	12.0	2.38
0.01	102.0	0.026	0.02	52.0	2.55

misleading ideas as to the true permeability of the cell at different temperatures.

This important point may be illustrated by a concrete example. Suppose for simplicity that with a permeability constant for water, k_2, equal to unity when expressed in the manner previously described (Jacobs, '34), hemolysis occurs at 20°C. in some given time which may also be called unity; suppose further that at 30°C. hemolysis takes place in 0.77 of the same units of time, i.e., that Q_{10} for water is equal to 1.3, which is an entirely possible value. Now let the permeability constant, k_1, for the solute at 20°C. assume in different cases the values represented in column 1 of table 1, and let the

value of Q_{10} for the change of k_1 with temperature have the convenient and not improbable value of 2.6.

In a previous paper (Jacobs, '34, p. 178) a table has been given which shows for different k_1/k_2 ratios the values assumed by a factor, here designed by F, which indicates how many times longer is theoretically required for the attainment of a given hemolytic volume, V_h, in an isosmotic solution of a penetrating solvent than in water under the same conditions. In the present case, for purposes of illustration, we may chose 1.7 as a possible value for V_h. Multiplying the times required to reach this volume in water at 20°C. and 30°C., which are 1.0 and 0.77, respectively, by the appropriate values of F given in the table, we obtain the times in which hemolysis should occur at the two temperatures in question in isosmotic solutions of the penetrating substances. From these times the theoretical temperature coefficients of hemolysis given in the last column may then readily be calculated.

Comparison of these values with the assumed value of 2.6 for the temperature coefficient of permeability to the solute shows in a striking manner the errors that would result from the attempt, in this particular instance, to treat the two entirely dissimilar temperature coefficients as if they were identical. It will be noted, however, that these errors become less the larger the value of F, i.e., the smaller the value k_1 as compared with k_2. When the permeability of the cell to the solute is sufficiently low so that the time of hemolysis in an isosmotic solution exceeds that in water fifty or 100 times, or even somewhat less, comparatively small errors are caused by using the hemolysis figures directly as a measure of permeability. With increasingly rapid rates of penetration of the diffusing substance, however, the situation becomes more and more dominated by the effect of temperature on the movements of the solvent, and the lower limiting value of Q_{10} for hemolysis is simply that of Q_{10} for the entrance of water alone.

Of greater practical usefulness than the calculation of theoretical temperature coefficients for hemolysis from hypothetical assumptions as to the behavior of k_1 and k_2 is the

reverse process of obtaining from direct measurements of the rate of hemolysis at different temperatures true temperature coefficients for permeability. Subject to the various simplifying assumptions that have already been made, the theoretical treatment of this problem is simple. Let the observed times of hemolysis in water at two temperatures separated by 10°C., say 20° and 30°C., be represented by t_{20} and t_{30}, respectively. Let the corresponding times for hemolysis in an isosmotic solution of a penetrating substance be similarly represented by t_{20}' and t_{30}' and the ratios of the two observed times of hemolysis at a single temperature by F. For hemolysis by water alone, the product k_2t is theoretically constant (Jacobs, '34), hence,

$$(Q_{10})\ \text{water} = \frac{(k_2)_{20}}{(k_2)_{30}} = \frac{t_{30}}{t_{20}} \qquad (1)$$

From the table already mentioned there may be found for experimentally determined values of F and v_h corresponding values of the ratio k_1/k_2; for convenience this ratio may be designated by R. Having found in this way the appropriate values of R_{20} and R_{30} we may write

$$\frac{(k_1)_{20}}{(k_2)_{20}} = R_{20}\ (2)\ \text{and}\ \frac{(k_1)_{30}}{(k_2)_{30}} = R_{30} \qquad (3)$$

Dividing (3) by (2) and introducing (1), we have

$$(Q_{10})\ \text{solute} = \frac{(k_1)_{20}}{(k_1)_{30}} = \frac{R_{20}}{R_{30}} \cdot \frac{t_{30}}{t_{20}} \qquad (4)$$

The corresponding equation for the hypothetical case discussed above, is derived in a very similar and entirely obvious manner. It is

$$(Q_{10})\ \text{hemolysis} = \frac{t_{20}'}{t_{30}'} = \frac{F_{20}}{F_{30}} \cdot \frac{t_{30}}{t_{20}} \qquad (5)$$

It would doubtless be advantageous, at least in the case of rapidly penetrating substances, if equation (4) could be employed to convert temperature coefficients for hemolysis into the corresponding temperature coefficients for permeability to the solute. This treatment of the available data, unfortunately, is as yet scarcely practicable in most cases. In the first place, there is no certain information available about the

value of V_b for most species of animals. Furthermore, in view of uncertainties as to the importance of the various complicating factors discussed above, it is questionable how far a simplified mathematical treatment of this sort would add to the exactness of our knowledge. Finally, there is the practical difficulty that in cases of hemolysis in water and in solutions of very rapidly penetrating solutes, where such methods would be most useful, measurements of times of hemolysis are more difficult, and unavoidable errors are relatively much more serious than in the slower hemolytic processes where corrections of the crude data are scarcely necessary.

TABLE 2

Calculation of Q_{10} for the permeability of erythrocytes of the dog to urea, from times of hemolysis in water and in 0.3 M urea at four different temperatures. Each time represents the average of five determinations

TEMPERATURE IN DEGREES C.	WATER		0.3 M UREA		R	(Q_{10}) UREA
	Time of hemolysis in seconds	Q_{10}	Time of hemolysis in seconds	Q_{10}		
0	2.62		4.08		3.43	
		1.38		1.46		1.65
10	1.90		2.80		4.11	
		1.42		1.41		1.38
20	1.34		1.98		4.00	
		1.31		1.37		1.54
30	1.02		1.44		4.70	

For these reasons, no attempt has been made to apply further mathematical treatment to the experimental data here presented except by way of illustration (table 2) in a single case in which calculations of Q_{10} for the entrance of urea into the erythrocytes of the dog have been attempted from the observed times of hemolysis in water and in 0.3 M urea at several temperatures. The results of these calculations, doubtless because of the large mathematical effects of small errors in the observed times of hemolysis, are not very satisfactory, and they are presented more as a demonstration of the use of the method than as quantitative data of much scientific value. By the employment of more refined methods for measuring very rapid rates of hemolysis, however, better results could probably be obtained.

In view of the numerous difficulties that have so far been mentioned, it might seem that information derived from studies of hemolysis could contribute comparatively little to an exact understanding of the effects of temperature on cell permeability. Such a view, however, is unduly pessimistic. In the first place, it may be noted that while accurate absolute values of permeability coefficients for a given erythrocyte to different substances at different temperatures cannot at present be obtained with great certainty, definite conclusions may nevertheless be drawn from such data both as to the relative order in which a series of substances penetrate a cell at any given temperature and of the general nature of the temperature effect on each substance of the series. Furthermore, it is possible by means of data on hemolysis at different temperatures to demonstrate certain very characteristic and constant specific differences among erythrocytes which conceivably may, and in the opinion of the authors probably will, throw important light upon the various current theories of cell permeability, and may even ultimately be instrumental in introducing new points of view into this highly controversial field.

III

The experimental data presented in this paper involve four variables, namely, species, hemolytic solution, temperature and time of hemolysis. In summarizing these data in table 4, it has been thought best to adopt a method of grouping which facilitates primarily comparisons of the behavior of the same species with different substances, though in the accompanying discussion the comparative behavior of different species with the same substances also receives consideration. In addition to the experiments here reported, most of which represent determinations of the times of hemolysis for a complete series of six temperatures beginning at 0°C. and separated by 10° intervals, a considerable number of earlier ones have been omitted, either because the temperatures at which hemolysis was observed could not readily be included in the table, or because slight differences in technique were involved.

These unreported experiments are, on the whole, in excellent agreement with those chosen for publication, and considerably strengthen the conclusions drawn from the latter.

Unfortunately, considerations of space have prevented the separate presentation of the data obtained for each individual animal, and it has therefore been necessary to average for each species all the figures pertaining to a single temperature, the numbers in parentheses in the tables being those of the individual observations on which the averages are based. This method of averaging data is open to certain objections, and if uncritically employed might lead to false conclusions, but in the present instance the precaution has been taken of constantly comparing average temperature coefficients with those

TABLE 3

Effect of temperature on the time of hemolysis in seconds, in 0.3 M glycerol, of the blood of seven individual rabbits

DATE	0°	10°	20°	30°	40°	50°	CALCULATED MEAN Q_{10} (0°–40°C.)
8/7/35	103	74	55.9	37.5	27.7	16.0	1.39
8/9/35	86	61	45.0	27.4	19.8	14.2	1.44
8/11/35	144	97	56.2	35.9	24.9	17.2	1.55
8/12/35	139	96	50.2	43.6	34.2	20.2	1.42
8/19/35	99	66	39.0	28.6	24.4	17.0	1.42
8/20/35	105	65		30.0	23.4	16.6	1.46
8/21/35	125	82	57.0	37.2	27.4	18.0	1.46

for single individuals, and the differences so found have usually proved to be of negligible importance. By way of illustration of this point, the full data for the times of hemolysis in 0.3 M glycerol at six temperatures of the blood of seven rabbits are given in table 3. The agreement here is fairly typical of that obtained with mammalian blood in general. In only a few instances will attention need to be called to relatively large discrepancies in individual data.

The choice of a method for representing quantitatively the effect of temperature on the various hemolytic processes has presented certain difficulties. On the whole, it has seemed best to include in the tables 3 and 4 only a single temperature coefficient for each series, that employed usually being the

fourth root of the ratio of the observed time of hemolysis at 0°C. to that at 40°C. or, in a few cases, in which parentheses are used, the appropriate root of the ratio for a smaller interval. Though figures for 50° were available in most cases, it seemed wisest not to include them in the calculations because of the possibility of incipient heat hemolysis or other unknown changes of the cell at such high temperatures. It should be strongly emphasized that no great quantitative significance is to be attached to a calculated mean Q_{10} value of the sort here employed. As a matter of fact, such a value is sometimes misleading in obscuring the considerable, and occasionally highly characteristic, differences between the Q_{10} values for actual 10° intervals. But to avoid an undue complexity of the table and to provide a ready, even though somewhat crude, method of comparing the extensive data for different substances and different species, this 'short cut' has appeared to be justified. A more accurate analysis of the data may, of course, readily be made at any time by those interested. The general nature of the temperature coefficients observed will now be brought out more clearly by a consideration of the results obtained with various individual penetrating substances.

1. Glycerol. This substance, which has been much more extensively studied than any of the others, is a particularly favorable one for investigations of specific differences in the permeability of erythrocytes. In itself, apart from the osmotic processes with which it may be associated, it is relatively harmless, and the recent chemical studies of Parpart and Shull ('35) indicate, for several species at least, that the time of hemolysis in its solutions is a fairly accurate indication of its rate of penetration into the cell. Furthermore, the specific differences in the observed times of hemolysis in 0.3 M solutions at 20°C. are particularly striking, these times ranging from 10 or 15 seconds for the rat to over 30 minutes for the ox, with the other species occupying fairly definite intermediate positions. Finally, the permeability of the erythrocyte to glycerol can be modified more easily and profoundly

TABLE 4

Effect of temperature on the time of hemolysis of mammalian blood in 0.3 M solutions of penetrating non-electrolytes. The letters in parentheses are used to designate particular series, which are more fully described in the text

SPECIES	SUBSTANCE	TIME OF HEMOLYSIS IN SECONDS						MEAN CALCULATED Q_{10} (0°–40° EXCEPT WHEN ENCLOSED IN PARENTHESES)
		0°	10°	20°	30°	40°	50°	
Rat (a)	Glycerol	19.8(8)	18.7(8)	18.2(8)	16.2(8)	16.5(8)	21.0(8)	1.05
Rat (b_1)	Glycerol	2666 (10)	1135 (10)	224 (10)	114 (10)	49 (10)	29.2(10)	2.72
Rat (b_2)	Same + $NaHCO_3$	21.2(10)	14.8(10)	10.5(10)	7.8(10)	6.0(10)	4.8(10)	1.37
Rat (b_3)	Same boiled	21.0(5)	17.9(5)	14.6(5)	17.4(5)	17.4(5)	21.7(5)	1.05
Rat (c_1)	Glycerol	25.0(1)	25.4(1)	12.2(1)	21.4(1)	19.2(1)	24.9(1)	1.07
Rat (c_2)	Same + acetic acid	7440 (1)	1925 (1)	403 (1)	112 (1)	46 (1)	16.8(1)	3.57
Rat	Ethylene glycol	11.5(5)	6.7(3)	3.6(4)	1.9(4)	1.5(5)	1.3(5)	1.66
Rat	Erythritol			25500 (1)	10920 (1)	4692 (1)	1417 (1)	(2.62)
Rat	Urea	2.8(1)	2.4(1)	1.9(1)	1.3(1)	1.2(1)	1.0(1)	1.24
Rat	Thiourea	163 (5)	103 (2)	44.3(5)	25.9(5)	17.2(4)	9.6(5)	1.75
Rat	Water	3.0(1)	2.1(1)	1.5(1)	1.1(1)			(1.40)
Man	Glycerol	66 (2)	43.4(2)	32.2(2)	25.7(3)	24.9(3)	25.3(3)	1.28
Man (d_1)	Glycerol	61 (1)	41.7(1)	29.5(1)	31.2(1)	34.7(1)	33.3(1)	1.15
Man (d_2)	Same + CO_2	7080 (1)	1654 (1)	244 (1)	161 (1)	78 (1)	43 (1)	3.09
Man (d_3)	Same + $NaHCO_3$	47.7(1)	35.7(1)	27.2(1)	22.6(1)	17.9(1)	13.8(1)	1.28
Man	Ethylene glycol	11.8(2)	6.2(2)	4.2(2)	2.8(2)	1.9(3)	1.0(3)	1.58
Man	Erythritol			25500 (1)	7200 (1)	3512 (1)	1670 (1)	(2.48)
Man	Urea	7.3(1)	4.5(1)	2.6(1)	1.7(1)	1.4(1)	1.2(1)	1.51
Man	Thiourea	470 (2)	229 (2)	104 (2)	53.5(2)	30.2(2)	16.0(2)	1.99
Man	Water	3.9(1)	2.7(1)	1.9(1)	1.6(1)	1.3(1)	1.0(1)	1.32
Mouse	Glycerol	127 (3)	78 (3)	44.9(4)	29.2(3)	18.1(3)	8.9(3)	1.63
Mouse	Ethylene glycol	27.7(3)	11.3(3)	5.7(3)	2.7(3)	1.5(3)	1.1(3)	1.58
Mouse	Erythritol	1736 (3)	711 (3)	363 (3)	188 (3)	128 (3)	73 (3)	1.92
Rabbit	Glycerol	119 (8)	77 (7)	51.4(7)	34.3(7)	26.0(7)	16.9(8)	1.46
Rabbit (e_1)	Glycerol	86 (1)	61 (1)	45.0(1)	27.4(1)	19.8(1)	14.2(1)	1.44
Rabbit (e_2)	Same + acetic acid	4560 (1)	663 (1)	125 (1)	52.8(1)	31.0(1)	23.0(1)	3.14
Rabbit	Ethylene glycol	14.7(2)	7.4(2)	4.8(3)	2.6(2)	1.6(2)	1.2(3)	1.74
Rabbit	Urea	3.3(3)	2.1(1)	1.4(3)	1.0(3)			(1.49)
Rabbit	Thiourea	365 (3)	208 (1)	93 (3)	53.5(3)	30.5(3)	15.5(3)	1.86
Rabbit (f_1)	Ethylene glycol	12.0(2)	6.6(2)	4.2(2)	2.4(2)	1.6(2)	1.2(2)	1.65
Rabbit (f_2)	Di-ethylene glycol	231 (2)	85 (2)	26.8(2)	10.8(2)	4.4(2)	2.5(2)	2.69
Rabbit (f_3)	Tri-ethylene glycol	1304 (2)	452 (2)	82 (2)	22.7(2)	8.2(2)	4.1(2)	3.55
Rabbit (f_4)	α, γ dioxypropane	150 (2)	51.6(2)	16.5(2)	5.8(2)	2.9(2)	1.9(2)	2.68
Rabbit (f_5)	α, β dioxypropane	49.2(2)	17.2(2)	7.0(2)	2.8(2)	1.4(2)	1.2(2)	2.41
Rabbit (f_6)	n-propyl alcohol	2.2(2)	1.75(2)	1.35(2)				(1.28)
Rabbit (f_7)	Water	2.1(2)	1.75(2)	1.2(2)				1.32
Guinea pig	Glycerol	450 (1)	251 (1)	188 (2)	98 (2)	63 (2)	38.2(2)	1.63
Guinea pig	Ethylene glycol			4.9(1)	3.7(1)	3.3(1)	2.9(1)	....
Guinea pig	Urea	4.8(3)	3.6(3)	2.6(3)	1.8(3)	1.5(3)	1.2(3)	1.34
Guinea pig	Thiourea	660 (3)	336 (2)	131 (3)	57.8(3)	33.0(3)	19.0(3)	2.66
Guinea pig	Water	3.0(3)	2.3(2)	1.7(3)	1.2(3)	1.0(2)		1.33
Cat	Glycerol	17160 (2)	4415 (3)	1135 (3)	329 (3)	117 (3)	52.5(3)	3.48
Cat	Ethylene glycol	82.2(2)	40.0(3)	13.2(3)	5.1(3)	2.6(3)	1.1(3)	2.37
Cat	Urea	3.4(2)	2.5(1)	1.7(2)	1.4(2)	1.1(2)		1.33
Cat	Thiourea	146 (2)	83 (1)	44.8(2)	24.2(2)	16.2(2)	7.9(2)	1.73
Dog	Glycerol	19350 (2)	5340 (2)	1272 (3)	337 (3)	123 (3)	63 (3)	3.54
Dog	Ethylene glycol	94 (2)	36.9(2)	12.9(3)	5.1(3)	2.8(3)	1.5(3)	2.41
Dog	Urea	4.1(1)	2.8(1)	2.0(1)	1.4(1)			(1.42)
Dog	Water	2.6(1)	1.9(1)	1.3(1)	1.0(1)			(1.37)
Horse	Glycerol	18000 (1)	4080 (1)	1105 (1)	323 (1)	132 (1)	33 (1)	3.42
Horse	Ethylene glycol	120 (1)	40 (1)	14.3(1)	5.8(1)	2.8(1)	1.2(1)	2.56
Pig	Glycerol	22260 (1)	4650 (2)	1316 (2)	391 (2)	119 (2)	48 (2)	3.70
Pig	Ethylene glycol	123 (1)	38 (2)	15.6(2)	6.0(2)	2.0(2)	1.2(2)	2.80
Sheep	Glycerol	30000 (1)	6840 (2)	1397 (3)	397 (2)	140 (3)	51 (3)	3.83
Sheep	Ethylene glycol	161 (2)	67 (1)	24.5(3)	7.2(3)	3.5(2)	1.7(2)	2.60
Ox	Ethylene glycol	45870 (4)	9420 (5)	2169 (7)	617 (5)	192 (5)	73 (6)	3.93
Ox (g_1)	Glycerol	46980 (1)	8760 (1)	2264 (1)	681 (1)	232 (1)	88 (1)	3.77
Ox (g_2)	Same + acetic acid	24900 (1)	4255 (1)	1291 (1)	416 (1)	146 (1)	60 (1)	3.61
Ox	Ethylene glycol	238 (2)	85 (1)	27.4(2)	10.4(3)	4.4(2)	2.2(2)	2.71
Ox (h_1)	Ethylene glycol	247 (2)	61 (2)	31.0(2)	8.3(2)	3.8(2)	2.2(2)	2.84
Ox (h_2)	Di-ethylene glycol	449 (2)	155 (2)	51.8(2)	16.6(2)	7.0(2)	3.0(2)	2.83
Ox (h_3)	Tri-ethylene glycol	1384 (2)	413 (2)	114 (2)	36.4(2)	13.0(2)	5.8(2)	3.21
Ox (h_4)	α, γ dioxypropane	400 (2)	137 (2)	37.9(2)	12.4(2)	4.8(2)	2.3(2)	3.02
Ox (h_5)	α, β dioxypropane	160 (2)	42 (2)	13.7(2)	4.4(2)	2.2(2)	1.2(2)	2.92
Ox (h_6)	Propyl alcohol	3.1(1)	2.1(1)	1.9(1)				(1.28)
Ox (h_7)	Water	2.0(1)	1.7(1)	1.3(1)				(1.24)
Ox	Urea	4.2(2)	3.2(2)	2.2(2)	1.6(2)			(1.38)
Ox	Thiourea	1082 (2)	492 (2)	183 (2)	86 (2)	48.3(2)	23.0(2)	2.18

than that to any other known solute. This possibility should render it a substance of peculiar usefulness for testing experimentally various hypotheses as to the fundamental nature of cell permeability.

One striking modification of the permeability of the erythrocyte to glycerol may in some species be produced by traces of copper or of copper salts in the water used for making up the solutions (Jacobs and Corson, '34). This effect, which is not obtained at all with the blood of some animals (cat, dog, sheep, ox) and only slightly with that of others (rabbit, guinea pig), may be so pronounced with the blood of man, for example, that the use of water distilled in an ordinary copper still may change the time of hemolysis in 0.3 M glycerol at 20°C. from 30 seconds to 15 minutes or more. Equally striking effects may be obtained with the blood of the rat. For reasons that will be discussed more fully elsewhere, this effect is believed to involve a true rather than merely an apparent change in the permeability of the cell.

Another interesting modification of the time of hemolysis in glycerol solutions, which is also shown most clearly by the erythrocytes of man and the rat, is associated with the pH of the medium. This effect, which has recently been independently observed in a somewhat less striking form by Ørskov ('35) in the case of the blood of the rabbit, seems likewise to be associated with changes in permeability to glycerol and is highly specific in its nature both as to the species of animal and as to the penetrating substance. It is perhaps most easily demonstrated by saturating an isosmotic glycerol solution with alveolar air, though much lower tensions of carbon dioxide are also very effective. In such solutions the times of hemolysis may be prolonged to fifty or more times their normal values, the effect being readily reversible by the removal of CO_2 or by the addition of $NaHCO_3$ to the solution. In the case of rabbit and guinea pig blood similar effects are also obtained, but they are less pronounced and cease at higher tensions of CO_2. In striking contrast to the blood of these animals is that of the dog, cat, sheep and ox in which the same

treatment accelerates instead of retarding hemolysis. These highly characteristic specific effects, which can also be produced by other acids than CO_2, and the equally specific effects of $NaHCO_3$ and other alkalies have recently been investigated with the assistance of Mr. S. A. Corson and Dr. E. P. Laug and will be described more fully elsewhere.

A third factor which may influence the rate of glycerol hemolysis, but which, unlike the two just mentioned, is believed not to be caused primarily by a change in cell permeability, and which is in the same direction for all the species so far studied, is an acceleration of the hemolytic process by low concentrations of neutral electrolytes. This effect has been explained by Jacobs, Parpart and Corson ('34) as being due to an increase in the internal osmotic pressure of the cell resulting from a fairly complex shift in the ionic equilibrium. Further evidence of the correctness of this view will be published elsewhere; for present purposes it is necessary merely to know of the existence of the effect in order to be able to guard against errors resulting from it.

Earlier published data of one of the authors (Jacobs, '31 a, b) as well as the figures for 20°C. in table 4 indicate that at room temperature the eleven species of mammals studied fall into two well-marked groups with respect to their permeability to glycerol at room temperature. The first group includes the rat,[1] the mouse,[2] the rabbit and man, and also, though standing somewhat apart from the other members of the group, the guinea pig. In the first four species mentioned, under the conditions of these experiments and at 20°C., hemolysis usually occurs in from 10 to 60 seconds; in the guinea pig, gen-

[1] Through the kindness of Dr. H. H. Donaldson, which it is a pleasure for us to acknowledge, it was possible not only considerably to extend our rat series but to compare the blood of albino rats with that of three Norway rats from The Wistar Institute colony. Though slight differences in the times of glycerol hemolysis appeared to be present in the two types of animals, further work will be needed to determine whether these differences are really significant. In the meantime the data for the Norway rats are included in series (b) in table 4.

[2] In the case of the mouse alone, of all the animals used, was it necessary to mix blood obtained from several individuals to obtain enough for an entire series. The mouse experiments therefore differ in this respect from those with the other species.

erally within 2 to 5 minutes. As contrasted with these five forms, the cat, the dog, the horse, the pig, the sheep and the ox show average times of hemolysis under the same conditions of from 15 to over 30 minutes. No species has as yet been found which falls within the rather wide interval separating the two groups.

It is of considerable interest to note that the groups in question are essentially the same as those already mentioned above in describing the effects of copper and of pH on glycerol hemolysis. It will be further observed by an examination of table 4 that the temperature coefficients for glycerol hemolysis also show very characteristic differences between the two groups. Those for species belonging to the second group have extremely high values, ranging from 3.48 for the cat to 3.93 for the ox. The guinea pig, the rabbit and the albino mouse show intermediate values of 1.63, 1.46 and 1.63, respectively, while in the case of man and the rat whose bloods are most similar with respect to the other characters discussed above, the temperature coefficients under properly standardized conditions are extremely low, tending in the rat to approach unity throughout most of the temperature range and in man to do so above 20°C. This general correlation between the time of hemolysis at 20° the magnitude of the temperature coefficient and effect of pH changes and of traces of Cu seems to be too striking and too complete to be a mere coincidence. What its meaning may be is not yet certain, but it is likely that a further study of data of this sort may throw important light upon the fundamental nature of the permeability of the erythrocyte.

It might perhaps be thought that in the case of glycerol the association of low temperature coefficients with rapid penetration is due to the increased importance of the rate of entrance into the cell of water, which, as has been pointed out above, has a very low temperature coefficient. This explanation can scarcely be valid in the case of glycerol, however, though it conceivably might be in the case of some other substances. It will be noted, in the first place, that the times of

glycerol hemolysis for the guinea pig, rabbit and mouse all so greatly exceed the corresponding ones for water that the influence of the temperature coefficient for the latter substance, though probably not inappreciable, could not be very great (see in this connection page 204 and in particular table 1). It is certainly inconceivable, that the large differences in the temperature coefficients for the guinea pig and the ox, to take an extreme case, could be accounted for in this way.

In the second place, in the two species, man and the rat, in which glycerol hemolysis is most rapid, the temperature coefficient for hemolysis does not tend to approach that for water but rather to behave in such a peculiar manner as to indicate the probable interaction of several factors. At the same time in these two species, ethylene glycol, which penetrates even more rapidly than glycerol shows fairly high and typical temperature coefficients which are obviously not greatly influenced by that for the penetration of water. The conclusion therefore seems inescapable that there are actual characteristic specific differences in the temperature coefficients for glycerol hemolysis which for some reason are correlated with the absolute rates of penetration of the solute. Further discussion of this rather striking fact may be reserved until another important point has first received attention.

The enormous sensitiveness of the erythrocytes of the rat to slight changes in the pH of glycerol solutions has already been mentioned. It proved to be responsible in the work here reported for what at first appeared to be an unusual and very troublesome individual variability of this particular species. Even when the precaution was taken of using copper-free distilled water for making up the glycerol solutions, it was found that sometimes hemolysis of rat erythrocytes at 20°C. occurred consistently in 15 to 20 seconds, while at other times for no obvious reason it required several minutes. Worse than this was the fact that sometimes in two successive experiments with the same blood and the same solution sudden changes of the same magnitude might occur. An unpredicta-

bility of results of this sort had not hitherto been encountered with other forms of mammalian blood.

After many annoying experiences of this sort, appropriate experiments finally established the fact that even in some lots of so-called 'good' distilled water the amounts of CO_2 present are sufficient to cause marked retardation of glycerol hemolysis of rat erythrocytes. It was also found that when such amounts are not originally present, they may be introduced by an incautious breadth by the experimenter. An additional complication proved to be caused by the fact that the addition of as little as 1 part of blood to 500 of solution introduces into the latter what for rat blood are significant traces of bicarbonates. In some cases these bicarbonates are sufficient in amount to raise the pH of the solution above the point of retardation; in others they are insufficient, depending upon the carbon dioxide content of the liquid and the size of the drop of blood. As a matter of fact, it was easy to show that under certain conditions where 1 drop of blood required several minutes for hemolysis, 4 drops of the same blood in the same amount of solution might be hemolyzed within 10 or 15 seconds.

As an illustration of the various kinds of results that may be obtained with rat erythrocytes, the series designated by (a) in table 4 may be compared with series (b_1). In the former, the distilled water used was sufficiently free from CO_2 to give over a period of 18 days with blood from eight different animals times of hemolysis which were both very short and in excellent agreement with each other. In series (b_1), however, a different lot of distilled water, copper-free and supposedly of good quality, was used; the times of hemolysis obtained in this case were of an entirely different order of magnitude. On adding to some of this latter solution $NaHCO_3$ to the concentration 0.0002 M, the results represented in series (b_2) were obtained. Finally on heating another lot of the same solution to the boiling point to drive off the CO_2 and then cooling it, the results obtained were those given under (b_3), which are seen to be very similar to those

of series (a). If space permitted the reproduction of the data for the blood of each individual rat separately, these experiments would appear even more striking than they do, since series (a) was based upon eight and series (b) upon ten separate animals among which the most surprising constancy of behavior was obtained, without the occurrence at any temperature a single aberrent result. It seems, therefore, that even in the case of the rat, by sufficiently careful attention to experimental technique, results of a highly satisfactory degree of reproducibility can be secured; without a knowledge of the necessary precautions, however, the results obtained with this species may be both unpredictable and inexplicable.

The relatively enormous changes that can be produced in the rate of glycerol hemolysis of rat blood by very slight changes in the character of the hemolytic solution provide a means of investigating further the relation between the magnitude of the temperature coefficient and the absolute rate of hemolysis. In every case where with the blood of the rat a retardation of hemolysis was produced, whether by CO_2, by acetic acid or by copper, a high temperature coefficient of the order of magnitude of >2.0 was obtained. In the absence of retardation, however, the temperature effect in the rat was always found to be very slight and apparently unique in that above a certain point the rate of hemolysis actually tended to decrease slightly with increasing temperature. That this condition is apparently a constant one is indicated by the fact that it was shown more or less clearly by each of the individual rats composing the two series (a) and (b_3). It will be noted on the other hand that while the bicarbonate series (b_2) was likewise characterized by very short times of hemolysis, there was present in it a slow but regular increase in the time of hemolysis from the lowest to the highest temperatures.

As might be expected from the other facts mentioned above, human blood has been found to resemble rat blood in the general character of the changes in the temperature coefficients produced by varying the pH of glyceral solutions. This peculiarity is made clear by a comparison of the figures designated (d_1), (d_2) and (d_3), respectively, in table 4. Series (d_1)

represents blood exposed to a 0.3 M glycerol solution made up with relatively CO_2—free distilled water. The time of hemolysis obtained with it were short and the temperature coefficients low, approaching unity above 20°C. In series (d_3) in which the glycerol solution contained 0.0002 M $NaHCO_3$, hemolysis was likewise rapid and the temperature coefficients low, but they were more uniform than those in the preceding series. Finally in series (d_2), in which a low but not accurately measured concentration of carbon dioxide was introduced into the solution by passing into it a small amount of expired air, slow hemolysis and high temperature coefficients were observed. A somewhat similar experiment with rabbit blood in which 0.0002 M acetic acid was added to the glycerol solution is summarized under (e). With beef blood on the other hand, the addition of various amounts of carbon dioxide and low concentrations of other acids somewhat accelerates hemolysis but does not greatly change the observed temperature coefficients as is shown in the series designated (g).

In concluding the discussion of the relation between the absolute rates and the temperature coefficients of hemolysis in glycerol solutions, reference should be made to the interesting suggestion of Danielli and Davson ('35) that in the case of the permeability of certain types of membranes a relation such as that described can be predicted from the kinetic theory of solutions. A further point of interest about the theory of Danielli and Davson is that it provides a possible physical explanation for the high temperature coefficients so frequently found in permeability processes that seems to be much more plausible than the chemical one usually advanced. The fairly extensive data here presented on the differences in temperature coefficients from species to species, and more particularly the very striking differences that can be produced in the same species by slight changes in external conditions would be very difficult to explain by the rather naive view that high temperature coefficients for a given process necessarily indicate a chemical, and low ones a physical mechanism. It is sufficiently unlikely that the various specific differences that have been found should be of this

nature, but far more unlikely that mere traces of CO_2 or of bicarbonate could, in the same species, reversibly convert a physical into a chemical mechanism and vice versa. On the other hand, the kinetic theory of Danielli and Davson, perhaps appropriately modified by taking into account changes in solubility, in the character of the membrane, etc., produced by temperature changes, not only explains but demands differences of the sort here reported. It should be emphasized, however, that while with a single substance a negative correlation between the absolute rate of penetration and the temperature coefficient of hemolysis is very commonly found (further examples will be given below), this generalization is not necessarily true where different substances are involved. A striking example of its somewhat limited applicability will appear when the results obtained with glycerol are compared with the similar ones for the next substance studied, namely, ethylene glycol.

2. *Ethylene glycol.* Like glycerol, ethylene glycol is a substance which apart from its indirect osmotic effects is harmless to the erythrocyte, and its penetration may readily be studied by the hemolysis method. As would be expected from its smaller molecular volume and its behavior with many other cells, its absolute rate of entrance of any given mammalian erythrocyte seems to be considerably more rapid than that of glycerol, though the reverse may be true in the case of several species of birds which will receive consideration in another place. The general correlation, already noted under glycerol, between absolute times of hemolysis and temperature coefficients for a series of different species again appears with ethylene glycol, though with more exceptions and otherwise in a less striking form than with glycerol. Thus, the members of the first group mentioned above all show lower absolute times of hemolysis at 20° and lower temperature coefficients (3.6 to 8.7 seconds and 1.58 to 2.07) than those of the second group (12.9 to 27.4 seconds and 2.37 to 2.80). How far the irregularities that appear within the two larger groups are really significant can be determined only by a study of more

individuals than have as yet been available; in any case the considerations mentioned above render it unlikely that in the case of a substance which penetrates as rapidly as ethylene glycol the relation between permeability and time of hemolysis should be as simple as that in the case of glycerol.

Perhaps the most interesting fact to be brought out by a comparison of the figures for glycerol and ethylene glycol is that in all the animals belonging to the first group (with the possible exception of the guinea pig for which data are not yet available) the temperature coefficients are lower rather than higher for the more slowly penetrating of the two substances. This striking exception to the principle proposed by Danielli and Davson ('35) does not necessarily invalidate the latter, but may merely indicate in the case of the penetration either of glycerol or of ethylene glycol, or of both, the presence of additional complicating factors. That such factors are probably associated chiefly with the behavior of the glycerol is indicated, first, by the fact that the rate of entrance of this substance into the erythrocytes in question is incomparably greater, in comparison with that of other chemical compounds of nearly the same molecular volume, than in the case of any other known cells; perhaps some specific solubility of the glycerol in the materials composing the surface of these erythrocytes may be involved. In the second place, as has already been pointed out, the peculiar character of the temperature effect found with rat and human erythrocytes indicates that the situation in these cells must be a complex one. The facts in question, therefore, while suggesting caution in the use of the generalization that the absolute rates and the temperature coefficients of hemolytic processes are definitely related, by no means destroys its value as first approximation to the truth in many cases.

3. Erythritol. This substance enters most erythrocytes so slowly that very few experiments were made with it except in the case of those of the mouse, which show to it an apparently specific permeability (Jacobs, '31 a, b; Höber and Ørskov, '33; Ulrich, '34). As was perhaps to be expected from the behavior of glycerol in the somewhat similar cases just discussed, the temperature coefficient for hemolysis of mouse erythrocytes by erythritol proved to be relatively low, namely 1.92. The corresponding values of 2.48 and 2.62 for man and the rat, the only other species with which experiments were practicable, are scarcely comparable with that for the mouse, since they cover only the upper part of the temperature range, for which the coefficients are, in general, relatively smaller than at lower temperatures.

4. Substances related to glycerol and ethylene glycol. It is of interest to compare for a given kind of blood the behavior of a series of substances which are more or less closely related to one another chemically, and in this way to determine the general effect of changes in its structure on the ability of a molecule to enter the erythrocyte. A favorable series of this sort consists of a number of compounds derived from propane by the introduction of –OH groups in different numbers and positions. Experiments with such a series were carried out with the blood of the ox and the rabbit, two individuals of each species being used. The results are given under (f) and (h) in table 4; it may be mentioned that the individual figures in all cases were satisfactorily close to the averages given in the table.

Considering first the results obtained with ox blood, it will be observed that the first member of the series, propyl alcohol, lengthens only very slightly the time required for hemolysis in water alone. Evidently it enters the cell so rapidly that, for the reasons discussed above, definite conclusions cannot be drawn as to the effect of temperature on its rate of penetration. As would be expected from the known behavior of other cells, the introduction of a second –OH group into the molecule considerably slows its rate of penetration. That the position of this second hydroxyl group is by no means immaterial is shown by the figures in the table which indicate a much slower rate for α, γ dioxypropane where both ends of the molecule are occupied by polar groups than in its isomer where one end is of a hydrocarbon nature. Corresponding

to this difference in the absolute rates of hemolysis, there appears the one that might be expected in the temperature coefficient of the process. The introduction of the third hydroxyl group in glycerol leads to conditions which have already been discussed, namely, to very high values both of the absolute time of hemolysis and of the temperature coefficient. The blood of the rabbit behaves similarly in principle to that of ox except for the anomalous position of glycerol, which has already been discussed.

Another instructive series is that derived from ethylene glycol by the condensation of two or of three molecules of the substance, with the loss in each case of one less molecule of water. With this series, both in the case of the ox and of the rabbit, the permeability of the erythrocyte may be seen in table 4 to decrease with the size of the molecule; and again the temperature coefficients of hemolysis change in the expected direction.

5. *Urea and thiourea.* The peculiar behavior of the mammalian erythrocyte with respect to urea has previously been emphasized by one of the authors (Jacobs, '31 a, b). Indeed, the rate of entrance of this substance is so rapid that only very doubtful estimates of the effect upon it of temperature changes can be made (see p. 206 above). It is of interest, however, to note that in the case of the erythrocytes of fishes, in which the rate of penetration of urea is slow, the temperature coefficients are high. On the other hand, Höber and Ørskov ('33) have pointed out that thiourea enters the erythrocytes of mammals far more slowly than does urea, but that in the case of the single fish which they studied this relation is reversed. In the course of the present work, this last observation has been confirmed for many other species of fishes, and further details of the study will be presented elsewhere. It is sufficient at this point merely to mention that while in the mammals the temperature coefficients for thiourea are consistently high, and indeed fairly accurately parallel the times of hemolysis from species to species, in the fishes they are generally somewhat lower than those for urea. In a

few species of fish (menhaden, alewife) in which, apparently as a specific character, the permeability of the erythrocytes to urea is very great, it is interesting to note that the relative positions assumed by the two substances are the same as in the mammals, and this is true not only of their absolute rates of penetration but of the temperature coefficients for hemolysis as well.

SUMMARY

1. The limitations of the hemolysis method as a means of studying the permeability of the erythrocyte to water and to solutes at different temperatures is discussed, and a method is suggested for the quantitative treatment, in favorable cases, of hemolysis data for rapidly penetrating solutes.

2. With respect to their behavior in isosmotic glycerol solutions, two sharply separated groups of mammals have been found. The first, which includes man, the rat, the mouse, the rabbit and the guinea pig is characterized by rapid hemolysis, by a low temperature coefficient and, particularly in man and the rat, by a strong retardation of the process by CO_2 and other acids, as well as by traces of copper. The second group, which contains the ox, the sheep, the pig, the horse, the dog and the cat, shows slow hemolysis, high temperature coefficients and no retardation, but even frequently an acceleration, by the substances mentioned. Retardation in the first group is accompanied by striking increases in the magnitudes of the temperature coefficients.

3. In the case of at least several penetrating substances (glycerol, ethylene glycol and thiourea), the relative times required for hemolysis of the erythrocytes of different species by the same substance at the same temperature tend to run parallel with the temperature coefficients for the hemolytic processes in question. A similar correlation, to which, however, several important exceptions are noted, is found in comparisons of the times and temperature coefficients of hemolysis of the same erythrocytes by different substances.

4. The observations here reported are, on the whole, in good agreement with the physical theory of the effect of tempera-

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J. Nutr.
101 (3): 299-306, 1971

Utilization of Fat and Glycerol for Glycogenesis by the Neonatal Rat [1]

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ABSTRACT Hepatic glycogenesis from fat and glycerol has been studied in the fasted rat during development. Administration of either olive oil or glycerol resulted in marked hepatic glycogenesis in 5- to 10-day-old animals; this response was diminished in weaned rats and was almost absent in animals less than 48 hours old. The incorporation of ^{14}C-glycerol into hepatic glycogen was greater in 9-day-old neonatal rats than in weaned animals. Although glycerol kinase activity was low in liver of animals less than 48 hours of age, these young animals readily converted ^{14}C-glycerol into $^{14}CO_2$. Our results indicate that glycerol is utilized via different pathways depending on the age of the animal. Hepatic glycogenesis, following fat administration, in the 5- to 10-day-old rat can be explained on the basis of increased utilization of triglyceride-glycerol for glyconeogenesis during this period of neonatal life. J. Nutr. *101:* 299–306, 1971.

In the transition from the suckling to the weaning period, there is a marked qualitative change in the composition of food ingested by the rat. During the suckling period more than 60% of the calories are derived from fat (1), but after weaning only about 30% of the calories in the diet is in the form of fat (2). Numerous biochemical and physiological differences between suckling and weaned rats have been described and correlated with this major change in diet. These include: a greater utilization of fat relative to protein as an energy source during starvation in suckling animals (2); impaired hepatic lipogenesis during the newborn period (3); increased production of ketone bodies by the liver of infant rats (4); and a decreased rate of intestinal absorption of glucose and its incorporation into liver glycogen during the suckling period (5). Thus, absorption, transport and metabolism of fat and carbohydrate are different in neonatal rats than in adult animals; there is greater metabolic dependency on dietary fat as a source of energy for the neonatal rat.

A striking difference between neonatal and adult rats was reported by Hahn et al. (6). These workers found that when olive oil was fed to fasted rats 5 days of age there was a large increase in content of hepatic glycogen, whereas no increase was noted in adult animals when they were fed the same diet. The explanation for this difference between the neonatal and adult animals was not evident since neither of the individual components of olive oil, glycerol and oleic acid, administered separately or in combination, resulted in an increase in concentration of hepatic glycogen in the neonatal rats.

We have studied hepatic glycogenesis following administration of fat and glycerol to rats during development. Our results indicate: that glycerol and triglyceride-glycerol are major sources for glycogenesis in the suckling animal; and that there is accelerated glycogenesis during the neonatal period in the rat.

METHODS AND MATERIALS

Rats of the Wistar strain were used in all these experiments and were fed a standard laboratory diet.[2] They were fasted for various periods of time, according to age. After preliminary experiments, fast-

Received for publication September 18, 1970.
[1] This research was supported by National Institutes of Health Grants HD-0391, HD-0241, and Training Grant HD-0049.
[2] Purina Laboratory Chow, Ralston Purina Co., St. Louis, Mo.

ing of the newborn and suckling rats and all experimental manipulations were performed in a constant temperature room maintained at 30 to 32°. This procedure significantly reduced the variability in results, probably by prevention of hypothermia. Postweanling rats were fasted at room temperature (22°). All solutions were administered to the animals by intubation with polyethylene catheters of different sizes. Control animals received a volume of water equivalent to the volume of test substance administered.

Animals were killed by decapitation at various periods of time after administration of the test material. All tissue preparations were carried out in a cold room at a temperature of 0 to 4°. Livers were immediately removed and placed in preweighed tubes containing 60% KOH. The tubes were reweighed and placed in a boiling water bath for 3 hours. Enough water was added to dilute the mixture to 45% KOH; glycogen was precipitated by adding absolute ethanol to a final concentration of 65%. The samples were kept overnight at 0 to 4° and the precipitated glycogen was collected by centrifugation, then washed three times with 60% ethanol. After dissolution in distilled water and recentrifugation, glycogen was determined in the supernatant fluid by the anthrone procedure (7). In experiments where radioactivity of glycogen was determined, samples were treated in the above fashion except that two additional washings with 60% ethanol were employed. The aqueous solution of glycogen was added to hyamine hydroxide and radioactivity determined in a dioxane counting fluid (containing 0.5% PPO and 0.01% POPOP)[3] with a scintillation spectrometer[4] at 50% efficiency. These small amounts of glycogen did not require corrections for quenching. The specific activity of glycogen determined in this fashion did not differ by more than ± 5% from values obtained by subsequent acid hydrolysis of the glycogen and chromatographic separation and analysis of the radioactive glucose.

The preparation of substrate and homogenate and the incubation procedure for the assay of monoglyceride lipase (EC 3.1.1.3) was carried out according to the method of Senior and Isselbacher (8), using monolein as the substrate. The reaction was carried out for 10 minutes (proportionality with the time was observed for 15 minutes) and was terminated by placing the reaction mixture in a boiling water bath for 3 minutes. Blanks for each assay were determined at zero time by adding boiled extract to the incubation medium. The boiled reaction mixtures were centrifuged and the clear supernatant was assayed for content of glycerol according to the method of Garland and Randle (9).

The preparation of extracts and the direct spectrophotometric assay procedure for glycerol kinase (EC 2.7.1.30) were performed according to Haessler and Isselbacher (10) using a recording spectrophotometer[5] at a temperature of 37°. Blanks contained neither ATP nor glycerol.

In those experiments in which respiratory $^{14}CO_2$ was determined following intragastric administration of the radioactive substrate, the animals were placed in radiorespirometers of the type described by Wang et al. (11). Expired CO_2 was collected in 0.25 M ethanolic hyamine hydroxide during separate successive 1-hour intervals and aliquots from each sample were added to a counting solution of toluene containing 0.4% PPO and 0.01% POPOP. The volume of 0.25 M ethanolic hyamine hydroxide required to trap all the expired $^{14}CO_2$ was determined for each age group of animals.

Protein was determined by the method of Lowry et al. (12) using rabbit serum as the standard.

Sources of chemicals are listed below.[6]

RESULTS

In preliminary experiments, feeding olive oil (and purified triolein) to animals 5 days old produced a significant rise in hepatic glycogen but no increase in glycogen in livers of postweanling rats. Oleic acid produced no increase in content of hepatic glycogen in either 5-day- or 30-

[3] Abbreviations used are PPO = 2,5-diphenyloxazole and POPOP = *p-bis*-[2-(5-phenyloxazolyl)]-benzene.

[4] Packard Model 2010, Packard Instrument Co., Inc., Downers Grove, Ill.

[5] Beckman Model DU, Beckman Instruments, Inc., Palo Alto, Calif.

[6] Glycerol-1,3-^{14}C was purchased from New England Nuclear Corp., Boston, Mass. Triolein, monolein and oleic acid were from Calbiochem, Los Angeles, Calif. Hydrocortisone sodium succinate was a product of Upjohn (Solu-Cortef), Kalamazoo, Mich. Olive oil was U.S.P. grade and glycerol reagent grade.

day-old animals. These results are similar to those reported by Hahn et al. (6). However, in contrast to the observations of Hahn et al., feeding 2.0 ml of a 2.5% solution of glycerol per 100 g body weight (50% of the glycerol equivalents in 1.1 ml olive oil per 100 g body weight) resulted in significant glycogenesis in both age groups.

The development of the glycogenic response to feeding of olive oil was studied in a controlled environment with the temperature maintained at 30 to 32°. Figure 1 shows the increase in concentration of glycogen in liver over control values as a function of body weight following oral administration of olive oil. The data are plotted as a function of body weight since in our laboratory this parameter seemed to be a better indication of maturity than the predicted age and led to more consistent results. The concentration of glycogen in the livers of animals weighing 15 to 40 g was not reduced to low constant values after a 24-hour fast. Consequently these data were variable and are not reported.

The largest increase in the content of glycogen in liver was found in 11- to 15-g animals (approximately 5 to 10 days of age); less increase in glycogen was observed in weaned 60-g animals, and very little response was obtained in postweanling rats weighing 75 to 130 g. Animals weighing approximately 5 g (0 to 2 days of age) showed a minimal response to the feeding of olive oil.

Free glycerol did not stimulate an increase in hepatic glycogen in the very young age group (fig. 2). The other results are similar to those obtained with olive oil, with the response of the young adult much less than that of animals weighing 11 to 20 g.

In an attempt to determine whether this age-dependent accumulation of hepatic glycogen following administration of glycerol was the result of a sparing action of glycerol on other 3-carbon intermediates or the direct result of glycerol incorporation into glycogen, two age groups of rats were fed ^{14}C-glycerol and the radioactivity of glycogen was determined after various time periods (table 1). Glycogen derived from glycerol is significantly greater in the 9-day-old group than in 32-day-old weaned animals, regardless of the form in which the data are expressed or the time after feeding.

Following intragastric administration of ^{14}C-glycerol to three groups of animals there were significant differences in the recovery of respiratory $^{14}CO_2$ (fig. 3). Recovery was significantly higher ($P < 0.001$) in the animals less than 48 hours of age.

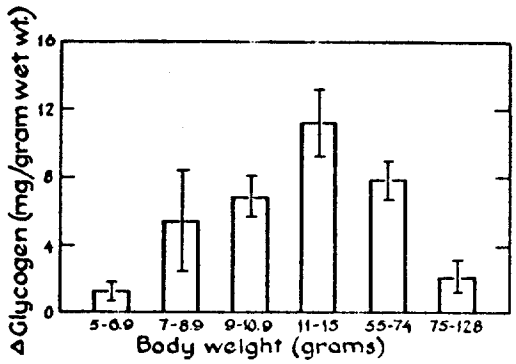

Fig. 1 Increase in hepatic glycogen in young rats following olive oil feeding. Olive oil was administered to rats intragastrically in a dose of 1.1 ml/100 g body weight following a 24-hour fast. Suckling rats were kept at 32° whereas weaned rats were studied at 22 to 24°. The concentration of hepatic glycogen was determined 4 hours after feeding and compared to control animals receiving 1.1 ml water/100 g. Results (mean ± SEM of 4 to 7 determinations) are expressed as increase in liver glycogen above control levels.

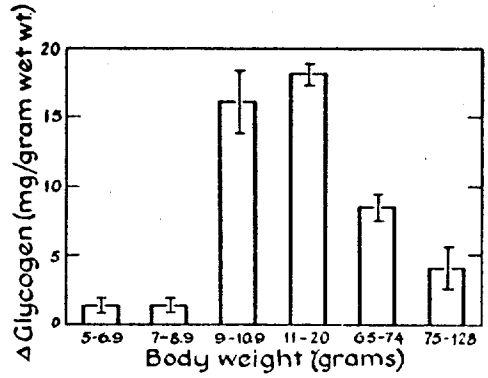

Fig. 2 Hepatic glycogen in young rats following administration of glycerol. Glycerol was administered in a dose of 2.0 ml of a 2.5% solution/100 g body weight. Temperature was controlled as in figure 1. Control animals received an equivalent volume of water. Results are expressed as increase in liver glycogen above control levels 4 hours after feeding. Each bar represents the mean ± SEM of 4 to 9 determinations.

TABLE 1

Incorporation of ^{14}C-glycerol into hepatic glycogen [1]

	Time (hr)	Age of animal: 9 days	Age of animal: 32 days
% Glycerol radioactivity incorporated into glycogen	3	12.4±1.4 (8) *[2]	8.1±1.1 (7)
	5	14.5±2.1 (6) **	5.5±0.8 (6)
% Glycogen carbon derived from ^{14}C-glycerol [3]	3	26.5±1.5 (8) **	17.1±2.4 (7)
	5	23.5±1.9 (6) *	15.2±2.3 (6)

[1] 1.0 ml of a 5% solution of glycerol-1,3-^{14}C (1.0 μCi/ml) per 100 g body weight was administered by stomach tube to 9-day-old rats fasted 24 hours (30 to 32°) and to 32-day-old animals fasted 40 hours (22 to 24°).

[2] Mean ± SEM, with number of animals in parentheses. Significance level in comparison with 32-day-old animals: * $P < 0.05$; ** $P < 0.01$.

[3] Calculated from the ratio of the specific activities of the isolated glycogen and the administered glycerol.

During the 5-hour period, animals 32 days of age converted slightly more ^{14}C-glycerol to $^{14}CO_2$ than did 9-day-old animals (not significantly different). The percentage of radioactivity expired during the first 2 hours as a fraction of the total 5-hour value was greater in the oldest group than in the younger animals, which may be a reflection of different rates of intestinal absorption of glycerol. However, the incorporation of ^{14}C-glycerol into glycogen in livers of animals of 32 days of age was no greater at 1 to 2 hours than at 3 hours (data not shown).

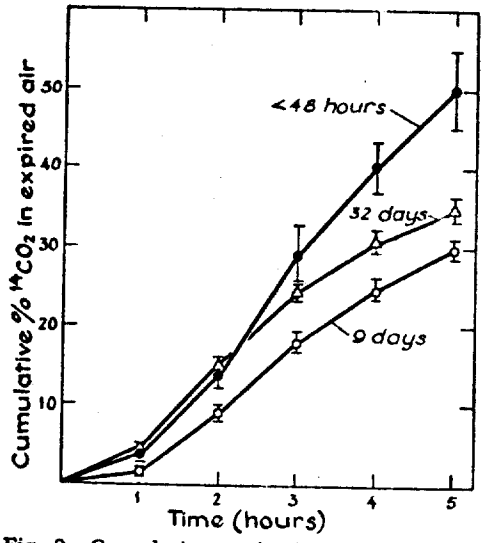

Fig. 3 Cumulative expiration of $^{14}CO_2$ following administration of glycerol-1,3-^{14}C, which was administered as in table 1, footnote 1. Animals less than 48 hours old were fasted 17 hours at 30 to 32°. Results are expressed as percentage of administered radioactivity recovered as $^{14}CO_2$ ± SEM (4 to 11 animals per age group).

Two enzymes were studied: 1) monoglyceride lipase, an intestinal enzyme involved in glycerol release from triglyceride; and 2) glycerol kinase, the enzyme responsible for the entry of glycerol into carbohydrate metabolism.

Figure 4 shows a developmental study of monoglyceride lipase in the small intestine of the rat. Newborn animals have a low activity of the enzyme which rapidly increases and attains the activity of the adult in animals weighing 25 g (15 days of age).

In addition, we studied the developmental pattern of glycerol kinase activity in both liver and intestine. In the liver very low activity was observed immediately after birth, but it rapidly attained the activity found in the adult (fig. 5). In the intestine there was no difference in the activity of glycerol kinase between infant and young adult animals.

When newborn rats 5 to 10 g in weight were injected with 0.2 mg hydrocortisone sodium succinate 4 hours prior to feeding glycerol, the glycogenic response measured 3 hours after feeding was no greater than that of control animals injected with water 4 hours prior to glycerol feeding (data not shown).

DISCUSSION

We have confirmed the finding by Hahn et al. (6) that olive oil produces a significant rise in the glycogen content of liver in fasted suckling rats but not in young adults. However, our data indicate that this glycogenic capacity is not present immediately after birth, but appears several days later and reaches a maximum at about 10 days of age. The olive oil used

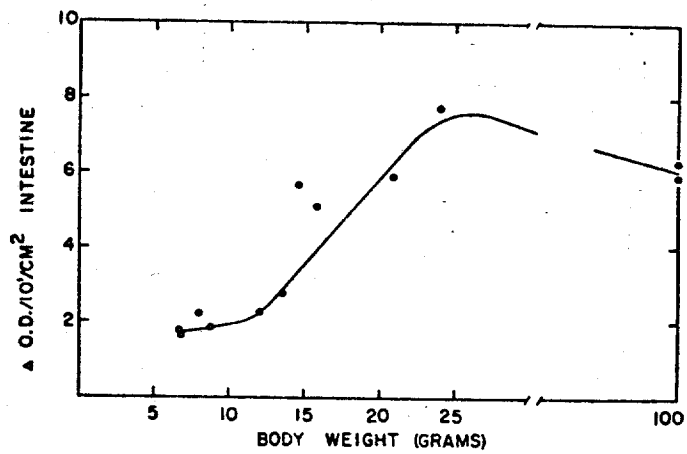

Fig. 4 Activity of monoglyceride lipase in rat intestine. Each point represents one animal. Activity is expressed as the change in optical density/10 minutes per cm² intestine. An identical curve is obtained when activity is expressed per gram body weight.

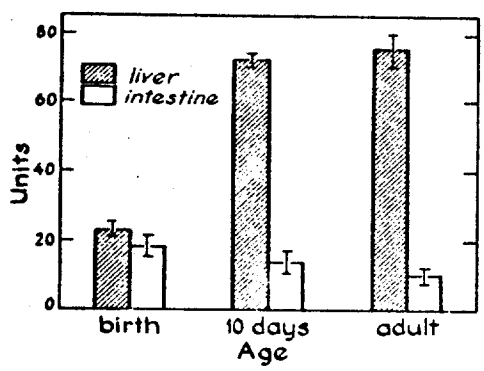

Fig. 5 Activity of glycerokinase in liver and intestine. The results are expressed in units of activity/milligram protein. A unit of activity is that amount of enzyme which causes an increase in absorbancy of 0.001 per minute. Each bar represents the mean ± SEM obtained from 4 to 6 animals.

in these experiments contained oleic acid, monolein, diolein, and predominantly triolein as shown by thin-layer chromatography. A more purified sample of triolein containing only a trace of diolein contamination produced similar results. Therefore, it would seem that this glycogenic response is truly the result of triolein administration and not due to some contaminant of olive oil.

Also, in agreement with the findings of Hahn et al., it was shown that oleic acid cannot be substituted for olive oil in augmenting the glycogen content of liver in fasted newborns. Hahn and co-workers also reported that glycerol, administered in a dose containing one-half the glycerol equivalents of their glycogenic dose of olive oil, was not glycogenic in starved infant rats. These authors speculated that possibly triglycerides are transported differently in infant rats, and are transferred across the intestinal wall intact and hydrolyzed elsewhere with the utilization of free glycerol for synthesis of liver glycogen.

We investigated the possibility that the availability of triglyceride-glycerol was greater in suckling rats than in adults. However, we found that the ability of the intestine of the suckling group to hydrolyze monoglycerides is less than that of weaned animals, so that there can be no increase in availability of triglyceride-glycerol to the suckling animals. It is still possible that complete hydrolysis at some other site, e.g., adipose tissue, liver or lung (13) results in an increased availability of triglyceride-glycerol to the infant rat. Our studies indicate that the handling of glycerol per se, regardless of any differential availability from triglycerides, is significantly different in the suckling period than after weaning.

The administration of free glycerol produces a marked increase in hepatic glycogen in animals 5 to 10 days of age, whereas the response in newborn animals and young adults is much less in magnitude. The oxidation of glycerol might spare other 3-carbon intermediates for gluconeogenesis. But when ^{14}C-glycerol was ad-

ministered to 9-day old animals a greater percentage of radioactivity was incorporated into glycogen than in 32-day-old rats. Newborn rats oxidize a high proportion of administered ^{14}C-glycerol to $^{14}CO_2$ without synthesis of glycogen.

The inability of these very young animals to form hepatic glycogen from glycerol might be a reflection of the relative acidosis in newborn mammals (14) since low pH impairs glycerol conversion to glycogen and enhances its conversion to CO_2 in slices of rat liver (15). Alternatively this inability might be caused by low activity of glycerol kinase in liver of newborn animals, with oxidation of glycerol to CO_2 by other tissues.

Our results indicate that a difference in glycerol metabolism between suckling and weaned rats accounts for the higher rate of glycogenesis in 5- to 10-day-old rats after being fed olive oil. It is not clear why Hahn et al. (6) found no increase in hepatic glycogen in 5-day-old rats following glycerol feeding whereas such increases were constant and reproducible in this study. The methodology and amount of glycerol administered were similar. They found a glycogenic response with glycerol in newborns given much greater amounts of the substrate, but this response was not comparable to that obtained in postweanling rats (5). More recently Hahn et al. (16) have reported that when ^{14}C-glyceryl-tripalmitin in olive oil is administered to newborn and adult rats, it gives rise to liver glycogen of a much higher specific activity in the younger animals. This observation supports the conclusion that the glycerol moiety of triglyceride is responsible for the glycogenic response in the infant rat.

The observed developmental differences in glycogenesis from glycerol could be explained by a relatively impaired uptake of glycerol by adult liver, manifested by low activity of glycerol kinase. Our results make this possibility unlikely. Another possibility would be relatively low reutilization of glycerol in suckling rats for synthesis of fat by the intestine. No major differences in activity of glycerol kinase in intestine were detected during development. These data are similar to those of Vernon and Walker (17) who have recently reported the activity of glycerol kinase in liver and intestine of neonatal rats.

The work of Ballard and Oliver (18–20) provides another possible mechanism for the greater synthesis of glycogen from glycerol by the liver of the 5- to 10-day-old suckling rat, as compared to the weanling animal. They have found that fructose-1,6-diphosphatase, a key enzyme in gluconeogenesis is relatively inactive in the liver of fetal rats, and the activity increases to a maximum during the first 11 days of life. The enzyme remains very active until 20 days of age and gradually decreases to the adult level (30% of peak level). Using liver slices, the incorporation of ^{14}C-pyruvate into glycogen and glucose paralleled the change in enzymatic activity. The data we have obtained with glycerol in vivo are similar to these data obtained with liver slices and indicate enhanced glyconeogenesis in 10- to 15-g rats.

The rate of formation of glucose and glycogen from glycerol increases in liver slices during the suckling period to reach a maximum at 15 to 30 days which is maintained in the adult (17). Our data obtained from intact animals indicate that the ability of the liver to convert glycerol to glycogen is greater in the suckling animals with a significant decrease in the adult. This lack of agreement between the two studies is probably a reflection of the differences between the in vitro and in vivo studies.

Since fructose-1,6-diphosphatase activity can be increased in the adult rat by administration of hydrocortisone (21–23), we measured the effect of cortisol on glycerol conversion to glycogen in fasted newborn animals, but failed to demonstrate increased glycogenesis. Yeung et al. (24) could not induce fructose-1,6-diphosphatase activity in the liver of fetal rats with triamcinolone. It is possible that either this enzyme cannot be induced by cortisol at this age or a single injection of 0.2 mg of cortisol 4 hours prior to feeding is not sufficient for induction.

Whether or not activity of fructose diphosphatase accounts for the total difference in response of infant rats and young adult animals to administration of fat cannot be stated with certainty. Although 9-

day-old rats convert more ^{14}C-glycerol into ^{14}C-glycogen than do weaned animals, this difference is not as striking as the differences observed in the concentration of hepatic glycogen after feeding glycerol or olive oil. It can be calculated that not all of the newly synthesized glycogen is derived from glycerol. It is known that free fatty acids can accelerate gluconeogenesis (25–27). The free fatty acid content of serum and liver is higher in the fed newborn rat than in postweanling rats (16); fatty acid oxidation in liver is also greater in the suckling period (4). Fatty acid oxidation inhibits mitochondrial pyruvate oxidation in liver and stimulates the pyruvate carboxylation reaction, thus enhancing gluconeogenesis (28, 29). Glycogenesis from olive oil in the suckling rat may result from a combination of increased conversion of glycerol per se into glycogen plus the synthesis of glycogen from pyruvate.

This study demonstrates marked differences in carbohydrate and fat metabolism between newborn, suckling and postweanling rat. The ability to form hepatic glycogen following the ingestion of fat is of obvious utility in an animal obtaining 60% of its calories from fat. Dietary factors are not solely responsible for this metabolic adaptation since the capacity to form glycogen is not present in the first few days after birth. Endogenous factors influencing the appearance of key gluconeogenic enzymes must also be involved in the mechanism of this response.

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THE AMERICAN
JOURNAL OF PHYSIOLOGY

VOL. 103 MARCH 1, 1933 No. 3

STUDIES ON THE PHYSIOLOGICAL ACTION OF GLYCEROL ON THE ANIMAL ORGANISM

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Received for publication November 12, 1932

As an integral part of fats, which constitute part of the normal diet of mammals, glycerol is constantly being split off in the gastro-intestinal tract and absorbed by the mucosa. It would therefore seem safe to conclude, a priori, that this substance is innocuous. Yet a number of investigations have indicated that this is not unconditionally true. Glycerol administration has been reported to lead to destruction of red blood cells and hemoglobinuria (Ustimowitsch, 1876; Plosz, 1878; Simon, 1915) even giving rise to anemia (Garibaldi, 1920); albuminuria (Ustimowitsch, 1876) and fever (Plosz, 1878, and others); and in larger doses (8 to 15 grams per kilogram body weight) to tachycardia, vomiting, muscular weakness, convulsions, and even death (Plosz, 1878, Simon, 1921). At autopsy damage to various tissues has been reported by Plosz and also by Piras (1925). It seems not to have been pointed out, however, that the route of administration may determine whether or not glycerol is injurious. Numerous examples could be cited of innocuous and even indispensable materials occurring in the gastro-intestinal tract which kill if injected subcutaneously, intraperitoneally, or intravenously. Examination of the literature with this in mind reveals the fact that it is essentially in instances of parenteral administration of glycerol that injurious consequences have been reported. This is true of the production of hemolysis by Simon (1915), anemia by Garibaldi (1920), tissue damage by Piras (1925), and death by Simon (1921). While Ustimowitsch (1876) employed both oral and parenteral administration in his extended studies, he does not clearly indicate any relation between the route of administration and the injurious effects observed. But it is significant that the protocols he publishes of instances of hemoglobinuria are of cases of intravenous injection. And furthermore in protocols of an experiment on diuresis using the oral route he says:

"der Versuch wurde unterbrochen bevor die Harnrothung in die Regel einzutreten pflegt." It seems legitimate to question whether hemoglobinuria was not the rule only when the glycerol was given otherwise than by mouth. Indeed Arnschink (1887), Leo (1903), and Nicloux (1903) were unable to obtain hemoglobinuria from feeding glycerol.

Also, it is easy to conceive of ingestion of large quantities of glycerol causing serious if not fatal changes through osmotic effects alone especially if the stomach is empty. Plosz (1878) fed dogs as much as 15 grams per kilogram body weight. Ingestion of several hundred grams of table salt or sugar would likewise produce serious changes which would in no wise place these substances in the category of toxic materials.

In the experiments of Long and Vorwald (1930) it was demonstrated that one or more daily intravenous injections of glycerol increased the susceptibility of rats to artificial infection by the tubercle bacillus. These experiments were suggested by the fact that in vitro glycerol seems to have a specific growth promoting effect on the tubercle bacillus (Nocard and Roux, quoted by Frouin and Guillaumie, 1924). Bearing in mind the fact that it has been repeatedly demonstrated that parenteral administration of glycerol may have such serious effects as hemolysis, anemia, and other tissue damage, it would seem highly probable that intravenous injection particularly might decrease the resistance of the animals to any infection, and not at all specifically to tuberculosis. Our dogs frequently displayed marked hemoglobinuria from intravenous injection of glycerol and some even died in a few minutes from a single large injection. It is significant that of the seven rats injected by Long and Vorwald one did die as a result of an injection, and two died of pneumonia. As none of the controls died of pneumonia, the results would equally well lend support to the view that glycerol (intravenously) reduces resistance to pneumonia. These authors also reported a preliminary study in which two rats fed glycerol were compared with controls in their susceptibility to artificially induced tubercle infection. Here, while the results were in the same direction, "the differences in the extent of tuberculosis . . . . were, however, not great . . . ." If these two rats, whose weights are not given, were really "fed with as much glycerol as they would tolerate," as stated, it would not be at all surprising if they were rendered less resistant to any infection.

Because of the rather extensive use of glycerol as a preservative of fruits, meat, and eggs, in pharmaceutical preparations, and even in baking, it seemed desirable to study extensively the effects of ingestion of quantities of glycerol well beyond the maximum which would be taken even if use of the material were extended. Information as to possible ill effects in human beings are most desirable, but before such studies were made extended work was done on rats and dogs.

OBSERVATIONS ON RATS. In three separate growth experiments varying quantities of starch of a standard diet were replaced by equal weights of glycerol. Diet I, the control, consisted of 61 per cent starch, 18 per cent casein, 10 per cent butter, 5 per cent yeast, 4 per cent inorganic salt mixture and 2 per cent agar. The glycerol test diets were identical, except that instead of the 61 per cent starch of diet I, diet II contained 20 per cent glycerol and 41 per cent starch, diet III had 41 per cent glycerol and 20 per cent starch, and diet IV had 61 per cent glycerol and no starch. The food was administered by the method described by Still and Koch (1928), which insures a minimum of scattering of food as well as ingestion by each group of rats of the same amount of food per gram body weight per day. Thus no variation in rate of growth can be attributed to one group eating more food than another. The cages were all equipped with wire mesh false bottoms so that all excreta dropped through. Also, the cages were cleaned and sterilized once a week. The rats themselves were dipped weekly into dilute creosote solution.

Figure 1, of the growth curves of the most prolonged experiment, run 40 weeks on 51 rats, shows that diet III (41 per cent glycerol—20 per cent starch) is at least as adequate as the control diet. It is seen that for the first 20 weeks the glycerol fed animals grew even better than the controls. That this was probably not significantly related to the diets is shown by the fact that when the diets of the two groups were switched at the twentieth week no break in either curve resulted. In two other experiments, one comparing diets I, II and III (30 rats run 25 weeks),[1] and another comparing diets I and III (10 rats run 21 weeks) the growth curves ran very much closer together. However, it is clearly evident that when all the starch is replaced by glycerol, which then constitutes 61 per cent of the total diet (diet IV), growth is greatly impaired. The rats on this high glycerol intake also appeared abnormal, for their coats lacked the smooth gloss of the controls, and they were less active.

Figure 1 also illustrates the fact that the impaired growth of these diet IV rats was not due to any toxic effect from the glycerol. For at the seventh week, when starch was added to the diet of 8 rats in the proportion of 6 grams of starch for every 10 grams of diet IV, they immediately resumed normal growth despite an unchanged daily glycerol intake. On the other hand, the nine rats not receiving this additional starch did not gain weight. At the fifteenth week a switch in the diets of these two groups confirmed the preceding findings, for again the simple addition of starch induced normal growth. At the twenty-sixth week diet IV was again resumed in all seventeen animals in this group, and the growth curves flattened out. Except, then, for a short period of growth at the

[1] This experiment was carried out by Dr. S. Szurek.

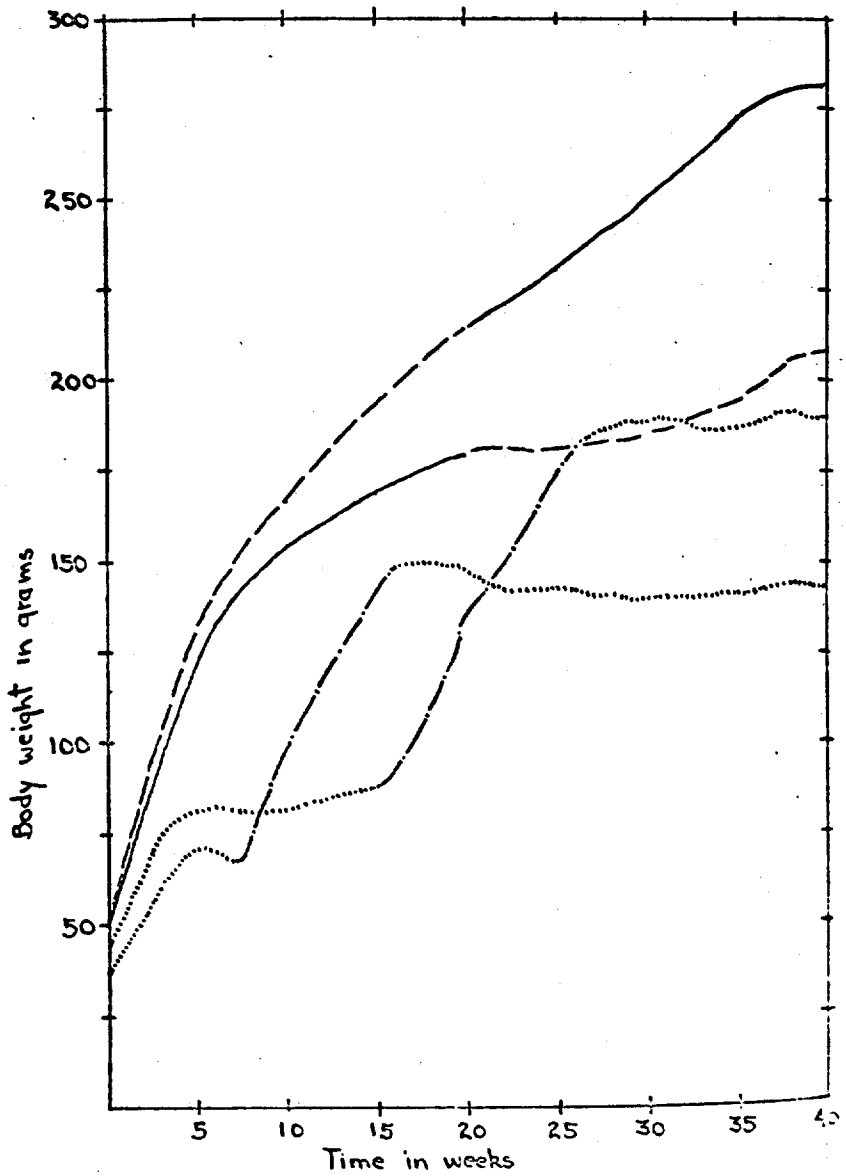

Fig. 1. Growth curves of 51 rats fed diets in which varying quantities of starch were replaced by equal weights of glycerol.

Solid line: diet I, 61 per cent starch, no glycerol.

Broken line: diet III, 20 per cent starch, 41 per cent glycerol.

Dotted line: diet IV, no starch, 61 per cent glycerol.

Dot-and-dash line: diet IV plus 6 grams of starch for each 10 grams of diet fed.

beginning of the experiment diet IV was inadequate to support growth when fed in quantities equal by weight to the growth promoting control diet. Diet IV plainly did maintain previously attained body weight, however. These findings indicate that failure to grow probably resulted not

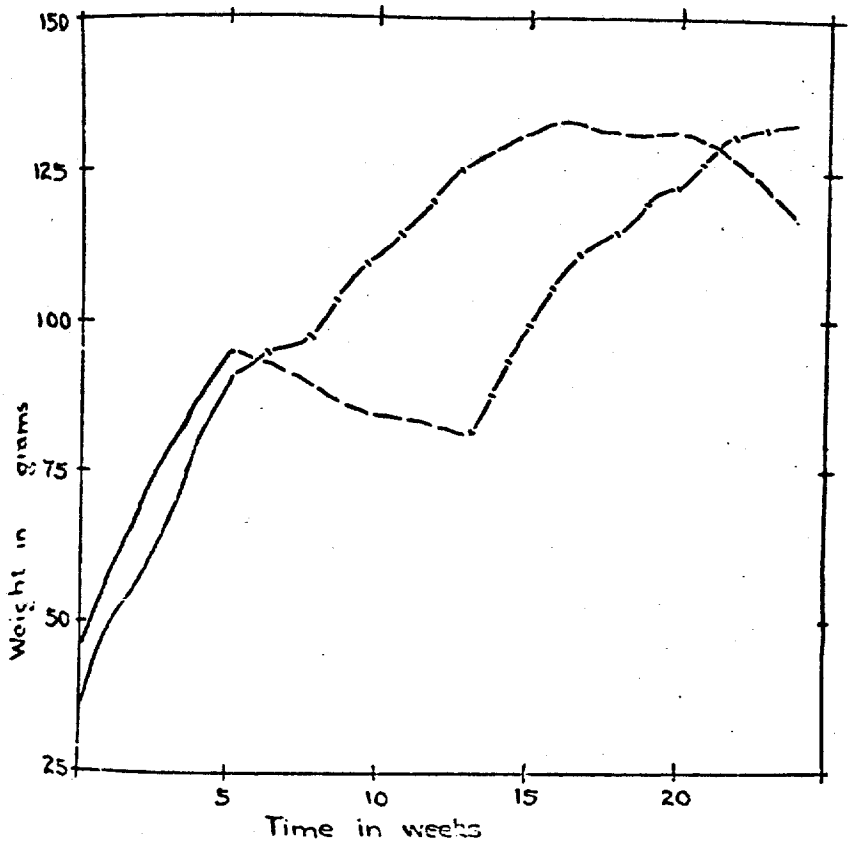

Fig. 2. Growth curves of 10 rats showing the effect of adding glycerol to a quantitatively inadequate diet.

Solid line: two groups of rats on a qualitatively and quantitatively adequate diet.

Broken line: five rats on a reduced intake of the same diet.

Dot-and-dash line: five rats on the reduced intake *plus* 12 grams of glycerol for every 10 grams of the normal diet fed.

from any toxic effect of the glycerol, but rather from inadequate quantitative intake. Confirmatory to this view is the fact that this group seemed abnormally hungry, becoming very excited when food was being administered, fighting each other for a first chance at the food and ravenously devouring it. (The reason for not feeding this group all it would eat

was that the rats were not fed ad libitum, but each group was fed proportionally to body weight as described above. Only in this way could a fair comparison of the diets be made.) Extremely rarely did this group fail to eat all it was fed. That there was no difficulty in getting the rats to eat diet IV was surprising, for the sickening sweet taste of even one meal containing 61 per cent glycerol is extremely distasteful to a human being.

To obtain data on the effects on growth from addition of glycerol to the diet, instead of replacing starch with glycerol, an experiment was performed whose results are shown in figure 2. Ten rats were fed the same control diet for five weeks, when the quantity fed to 5 was reduced just below the minimum required to maintain their weight. At the same time the remaining 5 rats received exactly the same diet qualitatively and quantitatively, plus 12 grams of glycerol for every 10 grams of the control diet. The latter group continued to grow at the normal rate. Thus not only was this large amount of ingested glycerol (over 50 per cent of the total diet) not toxic, but it actually furnished sufficient calories to make the difference between bare maintenance of weight or less, and normal growth. A reversal of the diets of the two groups at the thirteenth week fully substantiated this conclusion.

Theoretically, if combustion of the glycerol were complete the caloric yield would be slightly greater than that of an equal weight of glucose. It is probable, however, that some of the ingested glycerol is excreted in the urine either as glycerol (Nicloux, 1903) or in modified form (Plosz, 1878, and others). But the experiments described amply demonstrate that within extensive limits glycerol compares favorably with carbohydrate as a source of energy. That a large proportion of ingested glycerol is converted to sugar has been repeatedly shown in studies of liver glycogen formation (Luchsinger, 1874; Catron and Lewis, 1929, and others) and blood sugar, both on experimental animals (Voegtlin, Dunn, and Thompson, 1925a) and in humans (Ferber and Rabinowitsch, 1929). Estimates have ranged from 40 per cent conversion to glucose (Thomas, 1924) to 100 per cent (Chambers and Deuel, 1925) in diabetes. Chambers also found glycerol to be anti-ketogenic. It has further been shown that glycerol can furnish adequate protection against otherwise lethal doses of insulin in rats (Voegtlin, Dunn, and Thompson, 1925b) and also that in doses of 5 grams per kilogram, it exerts a protein sparing action in starving dogs (Starack, 1931).

Twenty-seven acute experiments were also performed in which under light ether anesthesia doses of glycerol ranging from 7 to 18 grams per kilogram were administered by stomach tube to 11 rats starved 24 hours. In no case were ill effects observed in the ensuing 24 hours.

Comparison of reproductive activity was made on the three groups receiving diet I (61 per cent starch; no glycerol), diet III (20 per cent starch;

41 per cent glycerol) and diet IV (no starch; 61 per cent glycerol), whose growth curves are given in figure 1. The number of pregnancies, young born, and young weaned were observed. Because the number of females in each group was not the same, reproductive activity is expressed not in terms of pregnancies, etc., per group, but per equal numbers of adult female rat weeks. The number of adult females in a group multiplied by the number of weeks these were housed with adult males on the same diet gives the number of adult female rat weeks. After 275 such rat weeks there were 22 pregnancies, 150 young born, and 74 young weaned in the control group, compared with 16 pregnancies, 101 born and 68 weaned in the glycerol rats. It is seen that although the number of young born to control females is considerably greater than the number born to the glycerol fed rats at the end of the experiment, the number of young weaned by the controls is only slightly greater. Furthermore, at the earlier period of 200 rat weeks all three criteria of reproduction indicate greater activity by the glycerol fed rats than by the controls. On the whole, reproduction seems not to have been hampered by substitution of starch by glycerol to a point where the latter comprises 41 per cent of the total food intake. However, no pregnancies occurred in the diet IV animals, receiving 61 per cent of their diet as glycerol. This was to be expected from the undernourished and debilitated condition in which the amounts of this diet fed kept them.

It was noticed at once that the glycerol fed rats drank more water than the controls. Measurements showed that per unit body weight diet III animals drank 30 per cent more water than the controls, and diet IV rats drank 230 per cent more than the controls. Reference to this observation will be made later, when the subject of diuresis is discussed.

At the close of the growth experiment run on 30 rats for a period of 25 weeks, the rats were killed and autopsied, and histological preparations made of the kidney, liver, and intestine. No abnormalities were found. Piras (1925) had reported congestion, small hemorrhages and cell degeneration of the liver, intestine, and kidneys as a result of small daily doses of glycerol administered over several weeks. Again, significantly enough, Piras used the subcutaneous route, which seems not to be as innocuous as the oral route.

Observations on dogs. The studies were further extended on dogs. Three pairs were used, each pair being litter mates. The control diet, fed to three dogs, consisted of 24.5 per cent protein, 52 per cent carbohydrate and 7 per cent fat, obtained from ground lungs,[2] white bread,[3] and

[2] Analysis: 17.0 per cent protein, 4.0 per cent fat, by Moulton (1922).

[3] Analysis: 7.1 per cent protein, 1.2 per cent fat, 52.3 per cent carbohydrate, by Wheeler and Bryant, from Lusk (1917).

whole milk,[4] and also 2.5 per cent yeast and 14 per cent bone meal. After this content of bone meal was arrived at the dogs never showed diarrhea, except for the occasional instances of short duration which may occur in any dog. The glycerol test diet, fed the three litter mates of the controls contained 35 per cent glycerol and 17 per cent carbohydrate as against the 52 per cent carbohydrate of the control diet. Glycerol replaced the carbohydrate of bread, and the protein of bread which was also thus withdrawn was replaced by gluten flour.[5] Control and glycerol diets contained equivalent amounts of meat (lung), milk, yeast, and bone meal. Of course the quantities fed each animal were in proportion to his body weight, so that at the end of the 50 weeks of experimentation each animal had received quantitatively and qualitatively the same food as any other animal, assuming 1 gram of glycerol equivalent to 1 gram of carbohydrate. The dogs were fed separately, but at all other times were allowed to mingle freely in a relatively large enclosed portion of the laboratory. Again it was found that the sweet taste of glycerol seemed in no wise to impair the appetite of the dogs, as had also been true of the rats.

Figure 3 shows the growth curves, commencing with the fifth week, when the puppies, previously weaned on milk and bread, were put on the experimental diets. Until the 36th week the two curves run very close together. At that time all diets were reduced in proportion to from 50 to 80 per cent of the previous intake to see whether at a caloric intake of bare maintenance level, some difference in the two diets might appear. In the ensuing 14 weeks the controls maintained their weight, whereas the glycerol dogs lost an average of 1.8 kgm., or 16 per cent of their previously attained weight. This again probably signifies that glycerol in such large quantities (35 per cent of the total food intake) is not calorically equivalent to glycose. It might be argued that with an adequate intake of other food materials the dogs were in an excellent enough physical condition to withstand a possible toxicity of glycerol, which only displayed itself in the loss in weight following reduction of the total food intake at the 36th week. In answer to this, reference may be again made to the rat experiment plotted in figure 2, in which animals receiving barely enough of a normal diet to maintain body weight were suddenly made to resume growth by the addition of sufficient quantities of the "possibly" toxic glycerol.

The dog experiments made possible a study of the previously mentioned contentions that glycerol administration produces albuminuria, red blood cell destruction, hemoglobinuria, and anemia made by Ustimowitsch (1876), Fränz (1878), Simon (1915), and Garibaldi (1920). It is recalled that

[4] Analysis: 3.2 per cent protein, 3.8 per cent fat, 5.0 per cent carbohydrate from Lusk (1917).

[5] Analysis: 81.0 per cent protein, 1.0 per cent or less fat, 4.0 per cent carbohydrate by Chicago Dietetic Supply House, manufacturers.

Simon and Garibaldi used parenteral administration, that Ustimowitsch does not clearly indicate which route was used when hemoglobinuria appeared, and that the production of hemoglobinuria upon oral administration could not be confirmed by Nicloux (1903) or Leo (1903). The erythrocyte counts for the two groups ran very nearly parallel, crossing and recrossing several times. The lowest red cell count observed in any

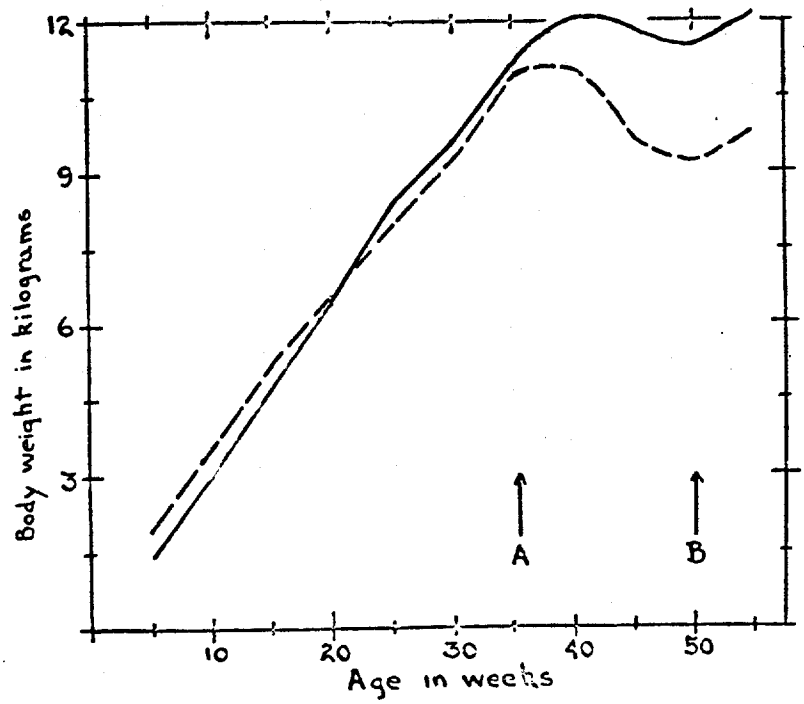

Fig. 3. Growth curves of dogs showing the effect of replacing starch by glycerol in the diet.

Solid line: weights of dogs on a diet containing 52 per cent carbohydrate, no glycerol.

Broken line: weights of dogs on a diet containing 17 per cent carbohydrate, 35 per cent glycerol.

At *A*, total daily intake of all dogs reduced.

At *B*, previous intake resumed.

glycerol dog was 5.4 millions as against a low of 5.3 millions in one of the controls.

Ustimowitsch and others have asserted that glycerol is hemolytic in vitro as well as in vivo. It may be readily demonstrated that this is a partial truth. For while an iso-osmotic solution of glycerol in distilled water will hemolyze red cells as readily as will distilled water, an iso-

hemoglobinuria occurred once. In 14 subcutaneous injections of 2.5 to 3.0 grams per kilogram in 8 dogs, albuminuria occurred twice and hemoglobinuria three times. The urine studies bring out very forcibly that whereas relatively small doses of glycerol parenterally injected may be toxic, much larger doses per os are innocuous especially if this large dose is taken with other food material. For the three dogs fed the glycerol test diet received over 9.0 grams of glycerol per kilogram per day for nearly a year, and yet suffered no demonstrable ill effects.

In the glycerol fed dogs there was observed the same increased water intake previously noticed with rats. Volume measurements of urine showed that the glycerol animals secreted approximately 5 times as much

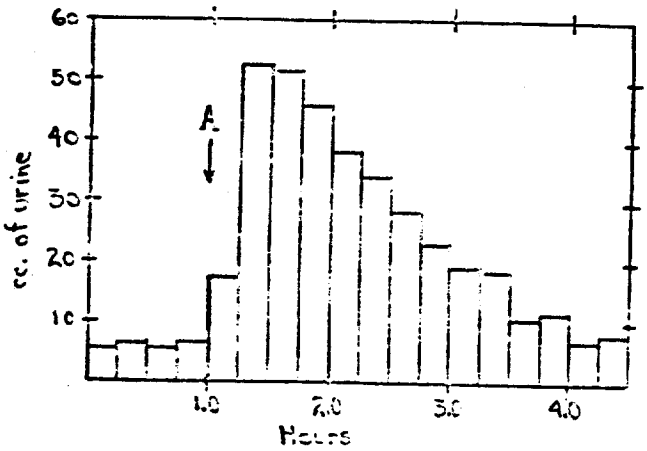

Fig. 4. Diuretic action of glycerol. Each column represents the volume of urine secreted by both kidneys in a 15 minute period. At *A* 2.5 grams of glycerol per kilogram body weight was administered by stomach tube. Figures are averages of 8 experiments on 2 dogs with bladder fistulae.

urine as dogs on the control diet, in equal periods of time. That the increased urine output was not secondary to the increased water intake was readily demonstrated by giving glycerol to dogs having no access to water. Figure 4 shows the results of administration by stomach tube of 2.5 grams of glycerol per kilogram to dogs with bladder fistulae. It is seen that in the first 15 minutes following ingestion the rate of urine flow is almost trebled, and in the second 15 minutes it is increased by nearly nine times. The diuretic effect of glycerol had already been noted by Ustimowitsch (1876) and others. Cugusi (1922) states that intravenous injection in small doses produces diuresis, but that larger doses inhibit urine secretion. This inhibition was noticed in our experiments only when the intravenous dose of glycerol produced a marked lowering of blood pressure at the same time.

Characteristically even a small dose of glycerol intravenously produces a transient fall in arterial pressure, during which period the urine flow may be diminished.

The mechanism of the diuresis is probably the same as that responsible for increased urine output in hyperglycemia and glycosuria, for despite the difficulty and uncertainty of estimating glycerol in the urine, there is an agreement by observers using different techniques, that glycerol ingestion leads to the speedy appearance of some of the material in the urine, possibly in modified form (Plosz, 1878; Nicloux, 1903; and others).

The rapidity of the onset of the diuresis suggests that glycerol may be absorbed from the stomach. Experiments on the absorption of glycerol indicated that while some of the material is absorbed from the stomach, about 50 per cent more is absorbed from the intestine in the first two to three hours after ingestion. These experiments (twelve in number) are subject to criticism, however, for the animals were anesthetized, and both the stomach and a loop of intestine were tied off. Also, the method of estimation of the unabsorbed glycerol was the crude one of measuring the volume of fluid remaining after evaporation of the water, glycerol having a boiling point considerably above that of water.

The contention of Plosz (1878) and Arnschink (1887) that glycerol ingestion leads to increase in body temperature was also tested. Numerous observations were made on the 3 chronic glycerol dogs, as well as on 13 other acute dogs fed a meal of meat-bread-milk plus 35 per cent glycerol. Readings were made at feeding, 1 hour later, and 8 hours after feeding. In no case was there an elevation of body temperature.

Throughout the period of observation the condition of the animals was good, except for an ailment early in the experiment which affected controls and glycerol fed animals alike. There was no consistent difference in the behavior of the two groups. Careful autopsy revealed no gross differences in the two groups. Microscopic studies on the stomach, intestine, lung, spleen, kidney and liver revealed normal organs. Formalin fixation and fat stains of the liver revealed no degeneration. Thus there is again a failure to confirm the findings of cell degeneration reported by Plosz (1878) and Piras (1925), the latter worker at least, having employed the subcutaneous route of glycerol administration.

Observations on man. The work so far carried out seemed to warrant continuation of the experiment on the human species. Fourteen graduate students in physiology (10 men, 4 women) served as subjects. For a period of 50 days each subject drank 30 cc. of 95 per cent glycerol with orange juice after each of the three daily meals.[5] Preceding and following

[5] After some experimentation in disguising the sweet taste of glycerol by taking it with coffee, milk, or lemon juice the following method of ingestion was adopted. A layer of orange juice was floated on about 35 cc. of glycerol. Immediately following

this period were ten day control periods. No rigid control of the diet was attempted, each subject eating his customary diet throughout, with these exceptions: each day, in the control as well as glycerol ingestion periods the juice from three oranges was drunk: also any tendency to reduce the intake of normal foods during the glycerol ingestion period was to be avoided, the aim being to test the effects of addition of glycerol to a normal diet. The experimental period fell entirely within a school quarter, so that all subjects were at work normally, and no vacation periods interrupted.

The daily ingestion of 90 cc. or about 110 grams of glycerol amounted to from 1.3 grams to 2.2 grams per kilogram body weight, depending upon the weight of the subject. If all the glycerol ingested were utilized it would yield theoretically about 440 calories per day, which would be at least one tenth of the normal caloric intake of a student.

There was a slight tendency toward an increase in body weight during glycerol ingestion. At the end of the glycerol period five people had gained from 1.5 to 4 pounds and 4 had lost from 1 to 4 pounds, four remaining practically unchanged.

Both red and white blood cell counts and hemoglobin estimations (Sahli) were made twice during each control period and 6 times during the glycerol period, on each subject. The results are summarized in table 1. It is seen that there are no significant changes in the blood picture, which extends and completely substantiates the previous findings in dogs.

Analyses of urine samples were made on each subject twice during each control period and from 5 to 7 times during the glycerol period. Reducing material was found in 12 per cent of 80 analyses of urine from the glycerol period as contrasted with 4 per cent of 47 samples taken in control periods. This may be another manifestation of the conversion of glycerol to sugar, evidence for which has been briefly reviewed above.

Hemoglobin was never found in the urine of these subjects. A trace of albumin was detected once in the glycerol period (subject J. N., female) and twice in the second control period (D. C., female, and R. S., male). These transitory instances of albuminuria are again probably cases of benign albuminuria. The numerous causes for the appearance of this phenomenon are reviewed by Fishberg (1931).

Glycerol ingestion has been stated to give rise to increased uric acid excretion. Rosenfeld (1924) found that glycerol per os in man led to an increase in uric acid excretion from 580 to 695 mgm. per day. Lewis and

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the rapid drinking of this, leaving about 5 cc. of the viscous material adhering to the sides of the tumbler, a swallow or two of orange juice from another glass was taken. Thus orange juice immediately preceded and followed the glycerol, and there was little to be tasted except the orange juice.

Corley (1923) followed the hourly uric acid excretion after ingestion of 50 grams of glycerol in subjects on a purine-free diet. Calculating from the figures of their experiment in which the increase in uric acid excretion was greatest, one obtains the result that ingestion of 50 grams of glycerol gives rise to an added uric acid excretion of 92 mgm. This would increase the 24 hour excretion calculated from their figures, from 513 to 606 mgm. In two of our subjects duplicate determinations of the uric acid content of 24 hour urine samples were made twice in each control period and 5 times during glycerol ingestion. The results, expressed as averages, show that the excretion was 523 mgm. per day before, 512 mgm. during, and 506

TABLE 1

Table showing the effect on the blood picture of ingestion of 110 grams of glycerol daily in man

	NUMBER OF SUBJECTS F: FEMALE M: MALE	CONTROL 10 DAYS AVERAGE OF 2 DETERMINATIONS	INGESTION 50 DAYS AVERAGE OF 6 DETERMINATIONS	CONTROL 10 DAYS AVERAGE OF DETERMINATIONS
Red blood cell counts in millions per cu. mm.	4 f.	5.0	4.9	5.1
	10 m.	5.7	5.8	5.6
	All 14	5.5	5.6	5.5
	M.L.* m.	6.3	5.7	6.1
	K.R.† m.	5.1	5.6	4.9
Grams of Hb. per 100 cc. blood (Sahli)	4 f.	15.5	16.4	14.7
	10 m.	17.0	17.6	17.8
	All 14	16.6	17.2	16.9
	R.S.* m.	17.4	16.9	18.2
	F.G.† f.	16.2	17.6	15.1
White cell count in thousands	All 14	7.3	7.1	7.0
	F.G.* f.	8.1	5.3	7.1
	J.H.† m.	5.4	6.9	4.3

*Shows greatest decrease, † shows greatest increase during glycerol ingestion.

mgm. after the glycerol ingestion period. It must be borne in mind that there was no rigid control of food intake in these experiments.

Until more is known of the metabolic processes involved in the production of the uric acid of the urine, the implications and significance of increases of the order of magnitude obtained by Rosenfeld and by Lewis and Corley cannot be stated. However, the interest of one group of workers in this phenomenon centers about the possibility of formation of uric acid urinary calculi. Rosenfeld (1924) reports that glycerol feeding leads to the appearance in the urine of uric acid crystals, concretions, and sediment, resulting from the alleged capacity of glycerol to precipitate the uric acid of the urine as crystals of sediment. On the other hand Hansen and Nemm

(1926) assert that uric acid and sodium urate are soluble equally well in water, or glycerol, or mixtures. They even believe that glycerol is useful as a therapeutic agent in ridding the bladder of uric acid stone. It does this by virtue of the fact that calcium oxalate, which they say constitutes the "framework" of even uric acid stones, is more soluble in glycerol than in water. Thus the appearance in the urine of uric acid sediment would be due to the disintegration of preëxisting stones rather than to the formation of new stones. These workers add that the sedimentation time of ground-up uric acid crystals is much prolonged in urine or serum to which glycerol has been added. But without drawing final conclusions as to any such possible beneficial action of glycerol, it does seem that there is little or no justification for the assertion that glycerol ingestion favors the formation of urinary calculi.

Urine output comparisons were made on two subjects to determine whether the diuresis observed in the rats and dogs occurs in man. The average 24 hour secretion was 1450 cc. before, 1380 cc. during, and 1300 cc. after glycerol ingestion. This absence of diuresis is probably due to the smaller quantities of glycerol ingested by man. Lewis and Corley (1923) obtained similar results. Of their five subjects receiving 50 grams of glycerol three showed no diuresis. Correlated with this lack of diuresis it was found in our observations that the specific gravity of the urine remained practically constant.

On six subjects curves were made of the variations in body temperature throughout the day. Examination of curves in which average figures are charted reveals the fact that not only is there no consistent rise in body temperature, but also that the curves remain unaltered in shape. The results from each individual are similar to this, each of the three curves taken before, during, and after glycerol ingestion, crossing and recrossing one another repeatedly.

Basal metabolic rate determinations yielded average figures of 66.1 calories per hour before, 61.9 during, and 64.5 after glycerol ingestion in 3 subjects whose average "normal" rate was 65.1 calories per hour. It is seen that the average rate during glycerol ingestion is just under (by 5.2 per cent) the control rate. This decrease was mostly due to the fall in rate of subject D. C., who normally shows a definite plus basal rate. The tendency in her case for the rate to approach the normal rate (for her) helped bring the averages down to a point below the normal rate for the group. Subject F. G. had a sub-normal rate throughout. Rate variations of the order of magnitude found are probably insignificant. These results are in conformity with those of McCann and Hannon (1923) who showed that in the two hours following ingestion of 40 to 100 grams of glycerol three subjects (one diabetic, two normal) showed a slight increase in oxygen consumption, and two (one diabetic, one normal) showed a slight

decrease. In other words, no consistent change resulted from glycerol ingestion.

No dietary experiment on human beings would be complete without a study of the effect of the diet on the activity of the large intestine. Neither rats nor dogs had shown any changes in bowel activity, but the most dependable information was obtained from the human studies. A record of the number of bowel movements per day was kept by each subject. Average figures of the results showed that no significant change occurred in any subject, and the group as a whole remained fairly constant, there being an average of 1.6 bowel movements per day before, 1.5 during, and 1.4 after the glycerol ingestion period. In three subjects the irregularity in number of movements from day to day was slightly greater during the glycerol period than during the control periods.

Even more important than the number of daily bowel movements is their consistency. An analysis of the distribution of the stools into the 5 categories of loose, soft, normal, firm, and hard showed that there was a slight tendency toward softer stools and a very slight decrease in firmer stools during glycerol ingestion. While this displays itself in the records, the changes were not marked enough for any of the subjects to list a tendency to looser stools amongst the effects they themselves had observed, except for one individual who thought that glycerol ingestion relieved a slight tendency toward constipation.

The subjective effects noticed included: increased thirst occasionally (2 subjects), an occasional sensation of warmth in the stomach after ingestion, especially in those exceptional instances when the glycerol was taken not with a meal, but on an empty stomach (4 subjects), and even "a feeling of particular well being and exceptional freedom from colds" (1 subject). There were no reports of the sleeplessness, talkativeness and excitement resembling alcohol intoxication described by Lewin (quoted by Schmey, 1909) from ingestion of 40 to 60 grams of glycerol daily. Schmey also quotes from Battenstein, describing a case of glycerol addiction, in which the victim took from 100 to 1000 grams of glycerol daily, the latter amount for a month. Thus far no signs of addiction have displayed themselves in our subjects.

SUMMARY

1. In studies of replacement of carbohydrate by glycerol in the diet of rats (40 weeks) to a point where glycerol constituted 41 per cent by weight of the total food intake, and in the diet of dogs (50 weeks) until it amounted to 35 per cent of the intake, normal growth occurred. Reproduction in rats was unaffected.

2. Addition of glycerol to a rat diet qualitatively adequate but quantitatively just sufficient for maintenance of body weight caused a remarkably-

osmotic solution of glycerol in 0.9 per cent NaCl does not do so. It is common knowledge that an identical experiment can be performed using urea instead of glycerol, and both experiments are to be explained by the fact that the red cell "membrane" is freely permeable to both urea and glycerol. Consequently these materials fail to exert any osmotic effects on the red cells and exposure of the cells to iso-osmotic solutions of glycerol or urea in distilled water is practically equivalent to exposing them to distilled water. Simon (1915) recognized that a 1.0 per cent solution of NaCl protects erythrocytes from hemolysis by glycerol.

Examination of the urine of each dog for hemoglobin and albumin was done repeatedly from the 13th to the 45th week of the experiment and in no instance was hemoglobin or albumin detected even in traces. The analyses were made on urine samples collected from 1 to 24 hours after feeding. In addition to this, 71 acute experiments on 22 other dogs were done, in which the glycerol was given per os either with a lung-bread-milk meal, or separately by stomach tube in doses which ranged from 2 to 4 grams per kilogram. Eight of these experiments were on 6 dogs which had been starved 18 to 24 hours. However, the emetic action of glycerol when administered to dogs on an empty stomach, described by Plosz (1878), often rendered it impossible to give the material by stomach tube without first depressing the animal somewhat with morphine and atropine subcutaneously. It is noteworthy that when an emetic dose of glycerol was given by mouth along with lung, bread and milk, no emesis occurred. In none of the 71 tests were albumin or hemoglobin detected in the urine.

There are two reasons for having repeated so frequently these urinalyses on many dogs. In the first place, early in the work one dog not included in the above group did show an albuminuria after a dose of 2 grams of glycerol per kilogram body weight in 50 per cent solution given per os on an empty stomach. Repeated attempts on the same animal failed to elicit a similar response. In the light of the failure to find albumin in the many other acute experiments performed, as well as in the repeated examinations of the urine of the chronic dogs, it would seem justifiable to conclude that in the single exceptional instance of albuminuria (impossible to confirm later) some other significant factor besides the glycerol ingestion was involved. That benign albuminuria may occur from a variety of causes has been repeatedly demonstrated. Perhaps the most extensive study, made by Maclean (1919), showed that 5 per cent of 50,000 normal subjects showed albuminuria in the morning and that this percentage increased with exercise.

In the second place, it was found possible to obtain not only albuminuria but also hemoglobinuria by parenteral glycerol administration. In two intra-peritoneal injections of 3.0 grams of glycerol per kilogram in one dog

tion of normal growth. Addition of 110 grams of glycerol per day (50 days) to an adequate diet in man produced a slight tendency toward increased body weight.

3. Ingestion by growing dogs of 9 grams of glycerol per kilogram per day for nearly a year did not change the red blood cell count. Ingestion by man of 110 grams of glycerol per day for 50 days did not affect the red or white cell counts or the hemoglobin content of the blood.

4. In neither man nor dogs was albuminuria or hemoglobinuria produced from glycerol taken orally, although both these phenomena were produced in dogs upon parenteral administration.

5. Glycerol per os in relatively large quantities exerts a diuretic action in dogs and rats, but in the quantities fed to man it lacked this effect.

6. The body temperature of man and dogs was unaffected by glycerol ingestion.

7. No adverse subjective effects in man and no changes in the general appearance and behavior of rats and dogs throughout the observation periods, resulted from glycerol ingestion in the quantities used.

8. In man the basal metabolism and uric acid excretion were not significantly affected.

9. Activity of the large intestine of man as regards daily number and consistency of stools, was unaffected by the quantities of glycerol fed.

10. No gross or microscopic pathology was detected in rats and dogs reared (for nearly a year) on diets of high glycerol content.

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