

Monograph on Succinic Acid

8/10/73

SUCCINIC ACID #87

I28

I28

MONOGRAPH  
ON  
SUCCINIC ACID

TR-72-1552-33

Submitted Under:  
Contract No. FDA 72-104

August 10, 1973

INFORMATICS INC.  
6000 Executive Boulevard  
Rockville, Maryland 20852

## SUCCINIC ACID

### Table of Contents

Summary	1
Chemical Information	3
Biological Data	
Acute Toxicity	6
Short Term Studies	6
Long Term Studies	6
Special Studies	6
Biochemical Aspects	8
Bibliography	12
Documents	46

## SUCCINIC ACID

### Summary

The human body is regularly exposed to succinic acid as a constituent of fresh meat and certain vegetables and contains succinic acid, in the mitochondria, functioning as an intermediary metabolite in the citric acid cycle. Although its role in the citric acid cycle has been studied extensively, little information on other aspects of its biochemistry or toxicology are available.

Several authors have reported that succinic acid is utilized by the intact organism (435, 360, 224, 225, 19), however, the pathway of utilization has not been elucidated. Although succinic acid has long been established as an intermediary metabolite in the citric acid cycle, no studies have been reported which indicate that it is incorporated into the cycle as such.

The injection of succinate has been reported by Brahmachari and Sarma (47) to produce a hypoglycemic response in rabbits and by Gessa et al. (134) to result in a depressant effect on the central nervous system and a significant increase in brain dopamine in male rats.

The absorption by man of iron from orally administered ferrous sulphate was found by Brise and Hallberg (51) to be increased by the addition of succinic acid to the iron dose, while Boddy and Will (40) reported that the effect of oral or intravenous succinic acid on the absorption of iron, from ferrous succinate, by non-anemic, symptomless, adult subjects was not sufficiently large compared with individual biological variation to be statistically significant. Brahmachari and Sarma (47) reported that in vitro mixing of sodium succinate and insulin prior to injection into rabbits decreases the effect of the insulin, although simultaneous but separate injection of the two compounds has little or no effect on the insulin activity. Chick embryo tests by Landauer and Sopher (207) indicate that succinate decreases the teratogenicity of 3-acetylpyridine, 6-aminonicotinamide and sulfanilamide and potentiates the teratogenicity of insulin.

The available information on the acute toxicity of succinic acid is very limited in amount and is of questionable value. The remaining toxicological information consists of one article by Dye et al. (100) which includes reports of three studies. In a short-term study 15 rats were injected s.c. at 7 days of age with a dosage of 0.5 mg of succinic acid (in sesame oil) and daily thereafter with increasing doses until they were receiving 2.0 mg/day at four weeks of age (100). This level of dosage was continued until the rats reached 60 days of age (100). As controls, 90 rats received similar doses of sesame oil only (100). No significant differences between the treated and control rats in growth, time of hair appearance, tooth eruption, eye

opening or vaginal opening were observed, except possibly in the opening of the vagina (57.2 days vs. 49.4 days for the controls) (100). In another study, daily vaginal smears were made on 15 two-month-old female rats for a period of 2 weeks, after which 5 of the rats were ovariectomized (100). After a post-operative period of 7 days the 5 spayed rats were injected s.c. with 5.0 mg succinic acid (in sesame oil) daily for 3 weeks (100). Daily vaginal smears and microscopic examination of sections of uterine horn, cervix and vagina from animals sacrificed at the end of the injection period revealed no significant changes (100). In a chick embryo test, Dye et al. (100) injected 0.05 ml of a solution made by dissolving 1.0 mg of succinic acid in 1.0 ml of phosphate buffer solution (pH 7.4) into the air sac of 40 fertile eggs on the tenth day of incubation. Although the treated chicks exhibited slightly lower body weight than the controls, no abnormalities of development were noted at hatching and of those chicks which were saved, all developed to maturity without incident (100).

# SUCCINIC ACID

## Chemical Information

### I. Nomenclature

#### A. Common Names

1. Succinic Acid
2. Amber Acid
3. Ethylenesuccinic Acid

#### B. Chemical Names

1. 1,4-Butanedioic Acid

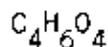
#### C. Trade Names

None

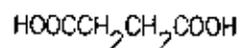
#### D. Chemical Abstracts Registry Number

000110-15-6

### II. Empirical Formula



### III. Structural Formula



### IV. Molecular Weight

118.09

### V. Specifications

#### Food Chemical Codex

Assay	Not less than 99.0% of $C_4H_6O_4$
Melting range	Between 185 degrees and 190 degrees
Limits of impurities	
Arsenic (as As)	Not more than 3 ppm
Heavy metals (as Pb)	Not more than 10 ppm
Residue on ignition	Not more than 0.025%

## VI. Description

### A. General Characteristics

Succinic acid exists in the form of colorless or white, odorless, minute monoclinic prisms having a very acid taste.

### B. Physical Properties

The following values have been established for the physical properties of succinic acid:

Melting Point	185-187 degrees
Boiling Point	235 degrees with partial conversion into the anhydride
pH of 0.1 M aq soln <sup>15</sup>	2.7
Specific gravity, $d_4^{15}$	1.564
Bulk Density	55 165/cu. ft.
Ionization Constants	
$K_1$	$6.5 \times 10^{-5}$
$K_2$	$2.3 \times 10^{-6}$
Heat of Combustion, $\Delta E$	-357.1 kcal/mole
Std. free energy of anion formation ( $\Delta F^\circ$ degrees f), 25 degrees C	-164.97 kcal for aq soln
Buffering Index	2.90
Solubility	One gram dissolves in 13 ml cold water, 1 ml boiling water, 18.5 ml alcohol, 6.3 ml methanol, 36 ml acetone, 20 ml glycerol, 113 ml ether; practically insol in benzene, carbon disulfide, carbon tetrachloride, and petroleum ether.

### C. Stability

Store in well-closed containers.

## VII. Analytical Methods

The official method for the determination of succinic acid in eggs and egg products involves extraction with ether, separation by column chromatography and, finally, titration with  $Ba(OH)_2$  (see AOAC 16.037) (14).

## VIII. Occurrence

### A. Plants

Succinic acid is found in broccoli, rhubarb, beets, sauerkraut, fungi and lichens.

## B. Animals

Fresh meat extracts contain succinic acid.

## C. Synthetics

Succinic acid is manufactured by the catalytic hydrogenation of maleic or fumaric acid. It has also been produced commercially by aqueous alkali or acid hydrolysis of succinonitrile. Succinonitrile is derived from ethylene bromide and potassium cyanide.

## D. Natural Inorganic Sources

Succinic acid occurs in fossils.

## SUCCINIC ACID

### Biological Data

#### I. Acute Toxicity

Substance	Animal	Sex & No.	Route	Dosage mg/kg Body Weight	Measurement	Ref.
Succinic Acid	Frog	-	s.c.	2000	MLD	246

Herman et al. reported that rabbits can tolerate 0.54 g of succinic acid when injected i.v. as an 0.5 N solution or 1.63 g of an 0.25 N solution (160).

On the basis of tests in which rabbits were given up to 4 g of neutralized succinic acid subcutaneously, Rose concluded that succinic acid is non-nephropathic to rabbits. It was suggested that the failure of succinic acid to produce nephropathic effects results from the rapid oxidation of succinic acid within the organism (309).

#### II. Short-Term Studies

##### Rats

In a study by Dye et al., 15 rats were injected s.c. at 7 days of age with a dosage of 0.5 mg of succinic acid (in sesame oil) and daily thereafter with increasing doses until they were receiving 2.0 mg per day at four weeks of age. This level of dosage was continued until the rats reached 60 days of age. As controls, 90 rats received similar doses of sesame oil only. The rats were weighed and measured weekly and the time of hair appearance, tooth eruption, eye opening, and vaginal opening were recorded for each rat. No significant differences between the treated and the control rats were observed, except possibly in the opening of the vagina. The average age of opening in the test rats was 57.2 days as compared to 49.4 for the controls (100).

#### III. Long-Term Studies

None

#### IV. Special Studies

##### Reproduction

Using rats, Dye et al. conducted an experiment to test for possible estrogenic properties of succinic acid. Daily vaginal smears were made on 15 two-month-old female rats for a period of 2

weeks, after which 5 of the rats were ovariectomized. After a post-operative period of 7 days the 5 spayed rats were injected s.c. with 5.0 mg succinic acid (in sesame oil) daily for 3 weeks. Daily vaginal smears were made and at the end of the injection period all of the animals were sacrificed and microscopic sections were made of the uterine horn, cervix and vagina. The daily vaginal smears showed no changes in the diestrus smear of the ovariectomized rats as compared to the controls and microscopic examination of the tissue sections revealed no significant changes (100).

#### Chick embryo

To test the effect of succinic acid on chick embryos, Dye et al. injected 0.05 ml of a solution made by dissolving 1.0 mg of succinic acid in 1.0 ml of phosphate buffer solution (pH 7.4) into the air sac of 40 fertile eggs on the tenth day of incubation. Two groups of 120 eggs were maintained as controls; one group received phosphate buffer alone and the other was left intact. At hatching, the average weight of the chicks treated with succinic acid was 39.5 g as compared with 42.3 g for the intact controls and 44.3 g for the controls injected with phosphate buffer solution alone. No abnormalities of development were noted at hatching and of those chicks which were saved (one-fourth), all developed to maturity without incident (100).

## SUCCINIC ACID

### Biochemical Aspects

#### I. Breakdown

No Information Available

#### II. Absorption - Distribution

No Information Available

#### III. Metabolism and Excretion

In tests using weanling male rats, Yoshida et al. determined the available energy value of succinic acid to be 3.4 kcal/g when fed as 5% of the diet. The gross energy of succinic acid was reported as 2.99 kcal/g (435).

According to Stoehr, feeding of 0.15 g succinic acid neutralized with  $\text{Na}_2\text{CO}_3$  to fasted young male rats resulted in 4 hours in a marked increase of liver glycogen (360).

The results of tests by Mackay and Barnes indicate that small doses of succinic acid (42.4 mM) are entirely converted to dextrose in the phloridzinized dog. When larger doses (424 mM) are given a smaller percentage is excreted in the urine as sugar, but its anti-ketogenic and nitrogen sparing activity indicate its conversion to dextrose before being burned (224).

Mackay et al. reported that when fed in quantities of 10-50 g per day succinic acid has no antiketogenic activity in the human diabetic when measured by the ketonuria, while in the normal fasting person it is as antiketogenic as an equivalent amount of glucose. The authors suggest that the antiketogenic action of succinic acid in the intact organism indicates its conversion to glucose. They also found that the ketosis of fasting rats which previously had been receiving a fatty liver producing diet is reduced in the same degree by glucose as by an equivalent amount of succinic acid and that these compounds are almost equally good glycogen formers (225).

After consuming 5 g of succinic acid, Balassa collected his urine for 24 hours and tested it for succinic, malic and fumaric acid. Analysis revealed no succinic or malic acid and only small quantities of fumaric acid, amounting to less than 1% of the administered dose. The urine, which is normally acid or neutral, reacted alkaline to litmus, indicating that the succinic acid was burned (19).

#### IV. Effects on Enzymes and Other Biochemical Parameters

Brahmachari and Sarma reported that the injection of 100 mg of sodium succinate into rabbits (average weight 2 kg) produces a hypoglycemic response amounting to a 22.5% reduction in blood sugar (47).

Gessa et al. reported that intraperitoneal injection of succinic acid (4 g/kg) resulted in a depressant effect on the central nervous system and a significant increase in brain dopamine in male Long-Evans rats weighing 180-220 g. The mechanism by which these effects were produced was not determined (134).

#### V. Drug Interaction

The absorption by man of Iron from orally administered ferrous sulphate was found by Brise and Hallberg to be increased by the addition of succinic acid to the Iron dose, the degree of increase being directly related to the quantity of succinate added. On the basis of their tests, the authors concluded that the promoting effect on Iron absorption was due to a direct action on the transfer of Iron across the mucosal cells. It was suggested that succinic acid exerts its action by increasing the intracellular mucosal metabolism (51).

Boddy and Will reported that the effect of oral or intravenous succinic acid on the absorption of iron, from ferrous succinate, by non-anemic, symptomless, adult subjects was not sufficiently large compared with individual biological variation to be statistically significant (40).

Brahmachari and Sara reported that in vitro mixing of sodium succinate (100 mg) and insulin (2 units) prior to injection into rabbits decreases the effect of the insulin. Simultaneous but separate injection of the two compounds, however, produced little or no change in the insulin activity (47).

In tests in which succinate was injected into the yolk sac of chick embryos simultaneously with certain teratogens, Landauer and Sopher determined that succinate decreased the teratogenicity of 3-acetylpyridine, 6-aminonicotinamide, and sulfanilamide and potentiated the teratogenicity of insulin. The authors suggested that succinate inhibits the teratogens, which exert their effects by interference with mitochondrial energy production in the tissues for which they have specific affinity, by being fed into the respiratory chain of the mitochondria and thereby providing the affected tissues with an extra burst of cellular energy, compensating for damage done by the teratogens (207).

## VI. Consumer Exposure

Succinic acid is added to food products as a buffer or neutralizing agent.

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 succinic acid in foods and table 11 reports the annual poundage data. Table 13 reports the possible daily intake per food category and total dietary based on food consumption by total sample.

TABLE 2 -- USAGE LEVELS REPORTED FOR NAS APPENDIX A SUBSTANCES (GROUP 1) USED IN REGULAR FOODS(1)

SUBSTANCE NAME (IDENTIFY NO.)	FOOD CATEGORY NO. NAME	# FIRMS REPORTING	*** USUAL USE *** MTD. REPR. %	*** MANIP. USE *** MTD. REPR. %
SUCCINIC ACID NAS 0214 FEMA 3569	15 MEAT PRODS(1) 15 CONDM. RELSHEN	* *	00010 08400	00010 *****

TABLE 11, PART A -- ANNUAL POUNDAGE DATA FOR NAS APPENDIX A SUBSTANCES (GROUPS I & II)

SUBSTANCE NAME (IDENTIFY NO.)	# REPORTS TO NAS 1960/1970	POUNDRAGE REPORTED TO NAS (MATCHING REPORTS FOR BOTH YEARS) 1960 1970	TOTAL 1970 POUNDRAGE REPORTED TO NAS	# REPORTS TO FEMA	POUNDRAGE REPORTED TO FEMA-- 1970 ONLY	TOTAL 1970 POUNDRAGE NAS + FEMA
SUCCINIC ACID NAS 0214 FEMA 3569	* / *	0 150	150	*	233	383

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 (IDENTIFY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS (ACT)	***** POSSIBLE DAILY INTAKE, MG. AVERAGE HIGH	***** POSSIBLE DAILY INTAKE, MG. HIGH
SUCCINIC ACID NAS 0214	15 MEAT PRODS(1)	*	0-5 MO. 1067100	176900
			6-11 MO. 1,362700	3,403000
			12-23 MO. 1,842200	3,165900
			2-65+ YR. 4,702400	7,534100
SUCCINIC ACID NAS 0214	15 CONDM. RELSHEN	*	0-5 MO. *****	100000
			6-11 MO. 1,579000	1,940000
			12-23 MO. 2,802000	6,354000
			2-65+ YR. 7,352000	17,600000
SUCCINIC ACID NAS 0214	ALL CATEGORIES	*	0-5 MO. 1067100	280000
			6-11 MO. 1,934700	5,281100
			12-23 MO. 4,102000	6,546900
			2-65+ YR. 12,174400	25,744100

## SUCCINIC ACID

### Bibliography

1. Aarna, A., and J. Kann. 1966. Fractional crystallization of binary mixtures of dibasic aliphatic carboxylic acids with urea from aqueous solutions. Tr. Tallin. Politekh. Inst. Ser. A No. 238:117-27.
2. Ababei, L., G. Chera, E. Raileanu, L. Raileanu, G. Leporda, D. Lacatis, M. Trandafirescu, M. Filip, and C. Zerner. 1969. Variations of glycolytic activity and tissue respiration in the liver of biotin-deficient rats. Fiziol. Norm. Patol. 15(1):69-75.
3. Abdurazakova, S. Kh., and G. V. Esebua. 1970. Chemical composition of pumpkins. Izvestiya Vysshikh Uchebnykh Zavedenii Pishchevaya Tekhnologiya (6):16-18.
4. Abe, T., and H. Tsuyuki. 1969. Studies on organic acids in "shottsuru" (fish sauce). J. of Food Science & Technology (Nihon Shokuhin Kogyo Gakkai-shi) 16(12):560-63.
5. Adler, A. 1927. The effect of acids on the water miscibility of serum. Kolloid-Ztschr. 43:313-28.
6. Aizenberg, V. Ya., I. O. Davtyan, and A. K. Gulamiryan. 1966. Chemical composition of some grape breeds. Izv. Sel'skokhoz. Nauk. Min. Sel. Khoz. Arm. SSR (6):51-7.
7. Albonico, F., F. Addeo, M. Ameno, and D. Tosco. 1969. Contribution to the knowledge of the composition of Solopaca wines. Industrie Agrarie 7(11/12):411-23.
8. Amerlung, D., H. J. Huebener, and L. Roka. 1953. Enzymic transformations of steroids. III. The introduction of the 11-hydroxy group and the 11-keto group by the liver. Hoppe-Seyler's Z. Physiol. Chem. 294:36-48.
9. Anagnostopoulos, C. 1953. Phosphatase of the prostate gland. I. Mechanism of the influence of citric acid. Bull. Soc. Chim. Biol. 35:575-93.
10. Andrew, R. L., and L. G. Neubauer. 1940. Succinic acid in beer. Analyst 65:455.
11. Anet, E. F. L. J., and T. M. Reynolds. 1953. Isolation of l-quinic acid from the peach fruit. Nature 172:1188-9.
12. Annau, E. 1936. Fumaric acid catalase and the behavior of pyroracemic acid in the liver. Hoppe-Seyler's Z. Physiol. Chem. 244:145-49.

13. Asao, Y., and T. Yokotsuka. 1961. Flavor substances in soy sauce. XVIII. Isolation of C<sub>7</sub>H<sub>12</sub>O<sub>5</sub> compound in soy sauce, and its structure. *Nippon Nogei Kagaku Kaishi* 35:831-7.
- \* 14. Association of Official Agricultural Chemists. 1965. *Methods of analysis*. A.O.A.C. Washington, D.C.,
15. Austen, K. F., and W. E. Brocklehurst. 1961. Anaphylaxis in chopped guinea pig lung. I. Effect of peptidase substrates and inhibitors. *J. Exptl. Med.* 113:521-39.
16. Babin, R. 1953. Identification and colorimetric determination of succinic acid. *Bull. Soc. Pharm. Bordeaux* 92:173-4.
17. Bach, D., and J. Lambert. 1937. The action of an antiseptic on the dehydrogenase of *Staphylococcus aureus*. *C. R. Seances Soc. Biol. Filiales Associees* 126:300-302.
18. Bakowski, J., S. H. Schanderl, and P. Markakis. 1964. Nonvolatile acids of green beans, *Phaseolus vulgaris*, cv. Green Crop x Romano. *Mich. State Univ., Agr. Expt. Sta., Quart. Bull.* 47(2):149-52.
- \* 19. Balassa, G. 1937. Succinic: fate in human organism. *Ztschr. F. Physiol. Chem.* 249:217-219.
20. Barnabas, T., and M. G. Badve. 1954. Kawath fruit, *Feronia elephantum*. II. Nutritive value of the protein of Kawath fruit. *J. Univ. Poona Sci. and Technol.* 6:93-7.
21. Barr, C. G. 1948. Investigations on the fluorometric determination of malic and succinic acids in apple tissue. *Plant Physiol.* 23(4):443-454.
22. Barreda, P. 1935. Glycogen formation in the liver. *Arch. Exp. Path. u. Pharmacol.* 178(3):333-341.
23. Bartonicek, B., and S. Lukac. 1970. Gas-chromatographic estimation of products of the radiolysis of succinic acid. II. Use of polymer porous beads (Synachrom) for the analysis of free fatty and dibasic acids. *Chromatographia* (3):108-12.
24. Bayfield, E. G., and G. L. Lannuler. 1962. Flour brew studies. II. Effects of acids, pH, and oxidants upon brew fermentation and the resulting bread. *Baker's Dig.* 36(1):34-8, 77; cf. *ibid.* 35:34-42.
25. Beatty, C. H., and E. S. West. 1955. Effect of succinic and malic acids and fructose on ketosis in alloxan-diabetic rats. *J. Biol. Chem.* 215(2):661-668.
26. Beatty, C. H., E. S. West, and R. M. Bocek. 1958. Effect of succinate, fumarate, and oxalacetate on ketone-body production by liver slices from nondiabetic and diabetic rats. *J. Biol. Chem.* 230:725-33.

27. Becker, E. 1954. Paper chromatographic detection of water-soluble organic acids in foods. *Z. Lebensm.-Untersuch. u. -Forsch.* 98:249-57.
28. Bekirski, D. M. 1969. Amino acids and organic acids in Armenian peaches. *Biologicheskii Zhurnal Armenii* 22(10):116.
29. Beilucci, G., and B. Grigatti. 1970. Separation and determination of some organic acids formed during the spontaneous aerobic fermentation of tomatoes. *Industria Conserve* 45(1):11-14.
30. Berg, H. W., F. Filipello, E. Hinreiner, and A. D. Webb. 1955. Evaluation of thresholds and minimum difference concentrations for various constituents of wines. I. Water solutions of pure substances. *Food Technol.* 9(1):23-26.
31. Bergstermann, H. 1948. Increasing susceptibility of thiol-containing enzymes to poisons during substrate and vitamin deficiencies. *Klin. Wochschr.* 26:435-6.
32. Bernhard, K., and M. Andrae. 1937. Metabolic tests with dicarbonic acids. *Hoppe-Seyler's Z. Physiol. Chem.* 245:103-06.
33. Bethea, S. 1970. Note on determination of free succinic acid in eggs by gas-liquid chromatography. *J. of the Assoc. of Official Analytical Chemists* 53(3):468-70.
34. Beznak, M., I. Hajdu, and Z. Korenyi. 1942. The role of the increase in the concentration of certain blood constituents in the production of suprarenal hypertrophy caused by muscular exercise. *Arch. Intern. Pharmacodynamie* 67:242-56.
35. Bilionozhko, G. E. 1963. Separation of organic acids in winter crops by paper chromatography. *Fiziol.-Biokhim. Osnovi Pldvlshchennya Produktivnosti Roslin* Sb. 418-20.
36. Black, M. M., and I. S. Kleiner. 1951. Succinate stimulation of normal and tumor tissue slice metabolism measured by reduction of tetrazolium chloride. *Proc. Soc. Exper. Biol. & Med.* 76:437-441.
37. Bleiweis, A. S., H. C. Reeves, and S. J. Ajl. 1967. Rapid separation of some common intermediates of microbial metabolism by thin-layer chromatography. *Anal. Biochem.* 20(2):335-8.
38. Blixenkron-Moller, N. 1938. Carbohydrate and ketone body formation from fatty acid in the artificially perfused cat liver. *Hoppe-Seyler's Z. Physiol. Chem.* 252:137-50.
39. Blundstone, H. A. W., and D. Dickinson. 1964. Chemistry of edible rhubarb. *J. Sci. Food Agr.* 15(2):94-101.

- \* 40. Boddy, K. 1967. Succinic acid and iron absorption. *Scot. Med. J.* 12(5):183-185.
41. Boehmer, T. 1968. Formation of propionylcarnitine in isolated rat liver mitochondria. *Biochim. Biophys. Acta* 164(3):487-97.
42. Bohnsack, H. 1967. Essential oils, scented and flavored substances. XX. Experiments on natural raspberry fruit oils. 3. Analysis of raspberry residue oil extracts. *Riechst., Aromen, Koerperpflagem.* 17(12):514-16.
43. Borntraeger, A. 1925. The organic acids of tomatoes, especially the citric acid and its compounds. *Ztschr. F. Unters. Nahrge. U. Genussmittel* 50:273-300.
44. Borntraeger, A. 1928. The organic acids of tomatoes. II. *Ztschr. Unters. Lebensmittel* 55:112-43.
45. Bose, S., and A. S. Datta. 1962. Nonnitrogenous organic acids of sugar cane. I. A chromatographic analysis of nonnitrogenous organic acids present in raw juice, clarified juice, and molasses. *Shankara* 5:46-52.
46. Bourzeix, M., J. Guitraud, and F. Champagnol. 1970. Identification of organic acids and determination of their individual amounts in grapejuices and wines by chromatography and photodensitometry. *J. of Chromatography* 50(1):83-91.
- \* 47. Brahmachari, H. D., and G. Raghupathy Sarma. 1963. Effect of some intermediary metabolites on the exogenous insulin response of normal rabbits. *Proc. Rajasthan-Acad. Sci.* 10(1):36-9.
48. Braunstein, A. E., and M. G. Kritzman. 1937. Formation and decomposition of amino acids by intermolecular exchange of amino groups. *Nature (London)* 140:503-04.
49. Breusch, F. L. 1938. Hydrogen transport in carcinoma tissue. *Biochem. Z.* 295:125-31.
50. Brill, C. 1954. Enzymic micro-determination of succinate and fumarate in tissue homogenates. *Biochim. et Biophys. Acta* 15(2):258-262.
- \* 51. Brise, H., and L. Hallberg. 1962. Effect of succinic acid on iron absorption. In: *Iron absorption studies II.* *Acta Med. Scand.* 171(Suppl. 376):59-73.
52. Broman, T. 1930. The use of succinodehydrogenase in muscle extract for detection of succinic acid. *Skand. Arch. Physiol.* 59(1/2):25-32.

53. Brozovic, M., et al. 1967. The excretion of methylmalonic acid and succinic acid in vitamin B 12 and folate deficiency. *Brit. J. Haemat.* 13:1021-32.
54. Bruemmer, J. M. 1967. Qualitative and quantitative analysis of grain, flour, and bread ingredients. *Brot Gebaeck* 21(6):112-14.
55. Brugsch, T., and H. Horstern. 1925. Studies on intermediate carbohydrate conversion. XIII. *Biochem. Ztschr.* 164:247-56.
56. Brun, S., and C. Grau. 1968. Determination of organic acids in wines by thin-layer chromatography on flexible sheets. *Kodak chromatogram. Qual. Plant. Mater. Veg.* 16(1-4):197-9.
57. Buch, M. L., E. C. Dryden, and C. H. Hills. 1955. Chromatographic comparison of nonvolatile acids of fresh and stored apple-juice concentrate. *J. Agr. Food Chem.* 3:960-4.
58. Buchloh, G., and M. Hane. 1958. Determination of fumaric acid and some other organic acids in apple fruits. *Gartenbauwissenschaft* 23:507-11.
59. Busch, H., R. B. Hurlbert, and V. R. Potter. 1952. Anion exchange chromatography of acids of the citric acid cycle. *J. Biol. Chem.* 196(2):717-727.
60. Califano, L. 1937. Fatty acid oxidation by the liver. *Biochem. Z.* 289:354-64.
61. Calvet, F. 1965. Regulation of hepatic xanthine dehydrogenase. *Arq. Port. Bioquim.* 8:183-206.
62. Canic, V. D., and N. Perisic-Janjic. 1970. Separation of organic acids by thin-layer chromatography on starch. *Tehnika (Belgrade)* 25(2):330-2.
63. Caprano, V. 1942. Influence of peroral administration of succinic acid on the elimination of ketone bodies during muscular activity. *Boll. Soc. Ital. Biol. Sperim.* 17:286-287.
64. Carangal, A. R., Jr., S. R. Arnaldo, and E. A. Tucay. 1956. Chemical studies on coffee grown in the Philippines. I. The nonnitrogenous organic acids of the leaves, shells, and beans. *Philippine Agriculturist* 40:99-103.
65. Carles, J., and A. Alquier-Bouffard. 1962. The organic acids of grape vines and their gradients. *Compt. Rend.* 254:925-7.
66. Carles, J., and M. Lamazou-Betbeder. 1958. Principal differences between the organic and free amino acids in grape and in wine. *Compt. Rend.* 247:2181-4.
67. Castex, M. R., L. E. Camponovo, F. E. Labourt, and J. Firmat. 1951. Effect on alcoholic intoxication. *Prensa Med. Argent.* 38:55-61.

68. Castino, M. 1969. Succinic acid in wine. I. Spectrophotometric determination. *Vini d'Italia* 11(63):509, 511, 513, 515, 517, 519 & 521.
69. Castino, M. 1970. Succinic acid in wines. II. Factors affecting its formation. *Vini d'Italia* 12(67):289-97.
70. Chattopadhyay, H., and S. Freeman. 1965. Carbon-14-labeled glucose metabolism by bone from normal and parathyroid-treated rats. *Am. J. Physiol.* 208(5):1036-41.
71. Chetverikova, E. P. 1959. Effect of succinic acid on medication sleep and certain oxidation processes in tissues. *Vop. Med. Khim.* 5:429-34.
72. Claborn, H. V., and W. I. Patterson. 1948. The determination and identification of lactic and succinic acid in foods. *J. Assoc. Offic. Agric. Chem.* 31(1):134-139.
73. Clark, B., et al. 1964. Determination of succinic acid by an enzymic method. *Biochem. J.* 93:210-220.
74. Clarke, B. J., F. V. Harold, R. P. Hildebrand, and A. S. Morieson. 1962. Trace volatile constituents of beer. IV. Volatile acids. *J. Inst. Brewing* 68:179-87.
75. Clutterbuck, P. W. 1927. Experiments on formation of succinic acid in body; determination of succinic acid and its formation in muscle and liver pulp. *Biochem. J.* 21:512-521.
76. Clutterbuck, P. W. 1928. The determination of succinic acid in blood. *Biochem. J.* 22(3):745-748.
77. Colpa-Boonstra, J. P., and K. Minnaert. 1959. Myoglobin in heart-muscle preparations and the effect of nitrite treatment. *Biochim. et Biophys. Acta* 33:527-34.
78. Copenhaver, J. H., Jr., and R. P. Forster. 1958. Displacement characteristics of intracellularly accumulated p-aminohippurate in a mammalian renal transport system in vitro. *Am. J. Physiol.* 195(2):327-330.
79. Cordonnier, R., and C. Bizeau. 1960. Ion-exchange chromatographic separation and determination of lactic, succinic and malic acids in wine. *Ann. Inst. Natl. Rech. Agron., Ser. E Ann. Tech. Agric.* 9(4):349-362.
80. Corley, R. C., and W. C. Rose. 1926. The kidney damaging effect of dicarbonic acids and their derivatives. *J. Pharm. and Exp. Therapeutics* 27:165-80.
81. Corrao, A., A. M. Gattuso, and V. Cilluffo. 1969. Balance of secondary products of alcoholic fermentation in Sicilian wines. *Industrie Agrarie* 7(9/10):365-76.

82. Cortes, I. M., and C. Diez de Bethencourt. 1962. Coloring materials and acids in wines. *Chim. Anal. (Paris)* 44:527-32.
83. Crompton, D. W. T., and P. F. V. Ward. 1967. Lactic and succinic acids as excretory products of *Polymorphus minutus* (Acanthocephala) in vitro. *J. Exp. Biol.* 46:423-430.
84. Cruz-Coke, E., H. Niemeyer, and M. Maric. 1947. Action of thiourea on the oxygen consumption of tissues. *Bol. Soc. Biol. Santiago Chile* 4:14-19.
85. Czerwinski, W. 1967. Separation of homologs of oxalic acid on a strongly acidic cation exchange resin. *Chem. Anal. (Warsaw)* 12(3):597-606.
86. Das, N. B. 1945. Growth of *Bact. coli anaerogenes*. *Sci. and Culture* 11(6):321-322.
87. Das, S. K., P. Markakis, and C. L. Bedford. 1965. Nonvolatile acids of red tart cherries. *Mich. State Univ., Agr. Exp. Sta., Quart. Bull.* 48(1):81-88.
88. Daukas, K., and M. Finkelsteinaitė. 1959. Conductometric determination of organic acids. *Vilniaus Univ. Mokslo Darbai. Chem.* 28(1):23-28.
89. Davis, D. R., J. F. Gallander, J. Hacskaylo, W. A. Gould. 1963. Chemical composition of maple sugar sand. *J. Food Sci.* 28(2):182-90.
90. de Bobadilla, G. F., and E. Navarro. 1950. Acids of Jerez wines from maturity of the grape to the aging of the wine. *Bol. Inst. Nacl. Invest. Agron. (Madrid)* 9:473-519.
91. Delaney, P. F., and P. F. Fenton. 1967. Role of growth hormone and dietary adaptation on incorporation of leucine into a mitochondrial protein. *Proc. Soc. Exp. Biol. Med.* 125(3):832-4.
92. De Lindemann, L. 1970. Separation of mono- and dibasic fatty acids by gas chromatography. *J. Chromatogr.* 51(2):297-300.
93. Delmas, J., N. Poitou, and B. Levadou. 1963. Chromatographic separation of the principal organic acids in grape leaves. *Chim. Anal. (Paris)* 45:63-5.
94. Deuel, H. J., S. Murray, and L. Hallman. 1937. Comparison of ketolyte activity of succinic acid and glucose. *Proc. Soc. Exp. Biol. Med.* 37:413-414.
95. Dienst, S. G., and N. Greer. 1967. Plasma tricarboxylic acids in salicylate poisoning. *J. Maine Med. Ass.* 58(1):11-12, 14.

96. Dietrich, P., E. Lederer, M. Winter, and M. Stoll. 1964. Flavors. XI. Cocoa flavor. *Helv. Chim. Acta* 47(6):1581-90.
97. Dittman, J. 1968. Thin-layer chromatography of carboxylic acids on cellulose. *J. Chromatogr.* 34(3):407-10.
98. Drews, E. 1953. Detection of organic acids and preservatives by means of paper chromatography. *Getreide u. Mehl* 3:85-8, 90-1.
99. Dryer, R. L., J. R. Paulsrud, D. J. Brown, K. Mavis. 1970. Comparative oxidation of palmitic, oleic, and succinic acids by rat and bat brown adipose tissue homogenates as a function of temperature. *Lipids* 5(1):15-22.
- \* 100. Dye, W. S., Jr., M. D. Overholster, and C. G. Vinson. 1944. Injections of certain plant-growth substances in rats and chick embryos. *Growth* 8:1-11.
101. Edson, N. L. 1936. Ketogenesis - antiketogenesis. III. Metabolism of aldehydes and dicarbonic acids. *Biochemical J.* 30:1855-61.
102. Ege, R. 1924. Studies on the permeability of anions in contact with the membrane of red corpuscles. *C. R. Soc. de Biologie* 91:779-781.
103. Eichholtz, V. F., and E. Brandes. 1937. Iron catalysis. *Arch. Exptl. Path. Pharmacol.* 187:215-20.
104. Elliott, K. A. C., and E. F. Schroeder. 1934. Metabolism of lactic acid and succinic acid in normal and tumor tissue. I. *Biochemical J.* 28:1920-39.
105. Emmrich, R., P. Neumann, and I. Emmrich-Glaser. 1941. Behavior of certain alkylized malonic, succinic and glutaric acids in animal body. *Ztschr. F. Physiol. Chem.* 228-241.
106. Endo, K. 1969. Effects of succinate on antigen-antibody reactions in guinea pig. *Aerugi* 18(9):758-71.
107. Eshchenko, N. D. 1967. Level and intensity of succinic acid metabolism in the brain during the interruption of oxidative phosphorylation. *Nerv. Sist., Leningrad. Gos. Univ., Fiziol. Inst.* 8:64-7.
108. Fare, G., D. C. H. Sammons, F. A. Seabourne, and D. L. Woodhouse. 1967. Lethal action of sugars on ascites tumor cells in vitro. *Nature* 213(5073):308-97.
109. Fauconnier, B. 1955. Development of influenza virus and pH of the allantoic fluid of embryonated eggs. IV. Inhibiting action of acid substances. *Ann. Inst. Pasteur* 89:101-10.

110. Fenech, G. 1955. Chromatography of the acids in wine. *Il Farmaco (Pavia) Ed. Sci.* 10:438-43.
111. Feng, C., and W. H. Adolph. 1949. Organic acids in Chinese leaf vegetables. *J. Chinese Chem. Soc.* 16:100-4.
112. Fernandez-Fernandez, M. J. E., C. Liaguno-Marchena, and B. Inigoleal. 1966. Yeasts metabolism. II. Assimilation of malic, citric and succinic acids. *Rev. Cienc. Apl.* 20(41):315-336.
113. Ferraz, F. G. P., and M. E. A. Relvas. 1961. Organic acids in biological fluids. I. Differential colorations as a function of their concentration. *J. Chromatog.* 6:505-13.
114. Flanzy, C., P. Andre, M. Flanzy, and Y. Chambroy. 1967. Quantitative variation in nonketonic nonvolatile stable organic acids in grape berries under carbonic anaerobiosis. I. The effect of temperature. *Ann. Technol. Agr.* 16(1):27-34.
115. Flaschentraeger, B., and G. Loehr. 1928. Does succinic acid occur in cattle blood? Contribution to the knowledge of fat metabolism. *Ztschr. Physiol. Chem.* 174:302-05.
116. Flesch, P., and D. Jerchel. 1955. Paper chromatography and ion exchange for determination of organic acids in wine. *Wein-Wiss., Beihefte Fachzeit. Deut. Weinbau* 9(2):5-23.
117. Forssman, S., and T. Lindsten. 1946. A contribution to the pharmacodynamics of malonic-, succinic-, citric-, aconitic-, alpha-ketoglutaric and glutamic acid. *Acta Pharmacol. et Toxicol.* 2(4):329-342.
118. Foster, J. M., M. L. Terry, and H. Gunther. 1967. The energy metabolism of human leukocytes. I. Oxidative phosphorylation by human leukocyte mitochondria. *Blood* 30(2):168-75.
119. Franzen, H., and F. Helwert. 1923. The chemical constituents of green plants. 25. Apple acids. (*Pirus malus*). *Ztschr. F. Physiol. Ch.* 127:14-38.
120. Frohman, C. E., J. M. Orten, and A. H. Smith. 1951. Chromatographic determination of the acids of the citric acid cycle in tissues. *J. Biol. Chem.* 193:277-83.
121. Fujie, S. 1961. Nutritional studies on fluorine. IV. The determination of the acids of the citric acid cycle by silica gel column chromatography. *Nippon Nogeï Kagaku Kaishi* 35:892-5.
122. Furchgott, R. F., and E. Shorr. 1946. Sources of energy for intestinal smooth muscle contraction. *Proc. Soc. Exptl. Biol. Med.* 61:280-6.

123. Furchgott, R. F., and E. Shorr. 1948. The effect of succinate on respiration and certain metabolic processes of mammalian tissues at low oxygen tensions in vitro. *J. Biol. Chem.* 175:201-15.
124. Furukawa, S. 1963. Nonvolatile organic acids in vinegar. I. Contents of nonvolatile organic acids in commercial vinegars. *Hakko Kogaku Zasshi* 41:14-19.
125. Furukawa, S., T. Takeuchi, and R. Ueda. 1967. Nonvolatile organic acids in vinegar. II. Alcohol fermentation in cider vinegar production. *Hakko Kogaku Zasshi* 45(3):204-10.
126. Gadzhiev, D. M. 1941. A new method for determining tartaric, lactic, malic and succinic acids in wine. *Vinodelie i Vinogradstvo* 9-10:21-2; *Khim. Referat. Zhur.* 4(7-8):75.
127. Gaeumann, E., S. Naef-Roth, and H. Kobel. 1952. Synergism in toxins from *Fusarium lycopersici*. *Compt. Rend.* 234:276-8.
128. Gautheron, D., C. Godinot, and N. Pialoux. 1967. Inhibition of oxidative phosphorylations in porcine cardiac mitochondria by coenzyme A. *Bull. Soc. Chim. Biol.* 49(5):551-67.
129. Gavrilova, L. V. 1965. Biogenic stimulators and the productivity of sugar beets. *Byul. Gl. Botan. Sada* No. 57:98-100.
130. Genevois, L., and J. Baraud. 1959. By-products from the alcoholic fermentation--fusel oil composition. *Inds. Aliment. et Agr. (Paris)* 76:837-44.
131. Genevois, L., E. Peynard, and J. Ribereau-Gayon. 1948. Balance sheet of secondary products from alcoholic fermentation in Bordeaux red wines. *Compt. Rend.* 226: 439-440.
132. Gepshtain, A., and A. Lifshitz. 1970. Organic acids of orange juice. *Lebensmittel-Wissenschaft und Technologie* 3(6):115-17.
133. Gerundo, M. 1950. Probable role of succinic acid in bacteriostasis by succinylsulfathiazole (sulfonamide). *M. Rec.* 163:23-34.
- \* 134. Gessa, G. L., P. F. Spano, L. Vargiu, F. Crabal, A. Tagliamonte, and L. Mameli. 1968. Effect of 1,4-butanediol and other butyric acid congeners on brain catechol amines. *Life Sci. (Oxford)* 7(5), (Pt. 1):289-98.
135. Gevorkyan, D. M. 1966. Effect of gamma-aminobutyric acid on citric acid level in the presence of malonate and hydroxylamine. *Vop. Biokhim. Mozga, Akad. Nauk Arm. SSR, Inst. Biokhim.* 2:74-7.

136. Geyer, R. P., E. J. Bowle, M. S. Gongaware, and M. A. Ryan. 1955. Studies on the metabolic interplay of fatty acids, succinate, and electrolytes. *Arch. Biochem. and Biophys.* 55(1):104-113.
137. Gordon, E. E. 1958. Effect of polycarboxylic acids on blood clotting. *Proc. Soc. Exptl. Biol. and Med.* 99(1):192-194.
138. Gozsy, B., and A. Szent-Gyorgyi. 1934. The mechanism of primary respiration in pigeon breast muscle. *Hoppe-Seyler's Zeitschr. Physiol. Chem.* 224(1/2):1-10.
139. Graves, C. N., G. W. Salisbury, and J. R. Lodge. 1967. Mammalian spermatozoan metabolism in the presence of thalidomide. *J. Anim. Sci.* 26(5):1082-6.
140. Graig, M. E., M. P. Munro, and K. A. C. Elliott. 1939. Lactic and succinic acid metabolism in normal and tumor tissue. VI. Beef retina and chick embryo. *Biochemical J.* 33:443-53.
141. Grimlund, K. 1936. The effect of lactic, pyruvic, succinic, fumaric, and glycerophosphoric acids on the work ability of the mono-bromacetic acid poisoned muscles and heart of frogs. *Skand. Arch. Physiol.* 73(1/3):109-122.
142. Gross, D. 1956. High-voltage paper electrophoresis of nonvolatile organic acids and their mixtures with amine acids. *Nature* 178:29-31.
143. Gross, D. 1959. High-voltage paper electrophoresis of organic acids and determination of migration rates. *Chem. & Ind. (London)* 1219-20.
144. Gross, F., and H. Kaufmann. 1954. Inhibition of the analgetic action of morphine and clidion by different sugars and decomposition products of sugars. *Helvetica Physiol. et Pharmacol. Acta* 12(4):284-292.
145. Guillemet, R. 1937. Lactic acid, succinic acid and bread preparation. *C. R. Seances Soc. Biol. Filiales Associees* 125:906-08.
146. Haarhoff, K. N. 1969. Use of multivariate nonlinear regression analysis in fitting enzyme kinetic models. Empirical study of the inhibition of aspartate aminotransferase by dicarboxylic acid substrate analogs. *J. Theor. Biol.* 22(1):117-50.
147. Hahn, A., and W. Haarmann. 1928. Dehydrogenation of succinic acid. *Zeitschr. Biol.* 87(2):107-114.
148. Hahn, A., and W. Haarmann. 1929. Dehydrogenation of succinic acid. II. *Zeitschr. Biol.* 89(2):159-166.

149. Hallman, N., and P. E. Simola. 1938. Studies on the formation of citric acid in various organs of animals. Suomen Kemistilehti 11:8:21-22.
150. Hanajima, Y. 1964. Food seasonings. Japan. Pat. 70,14,871.
151. Hara, T. 1967. Studies on the isomer of succinic acid: II. Anticancer activity of the isomer of succinic acid (rat, mouse). Jap. J. Vet. Sci. 29(3):117-131.
152. Harris, P. N., M. E. Krahl, and G. H. A. Clowes. 1947. P-Dimethylaminoazobenzene carcinogenesis with purified diets varying in content of cysteine, cystine, liver extract, protein riboflavin, and other factors. Cancer Research 7:162-75.
153. Harrow, B., A. Mazur, E. Borek, and C. P. Sherwin. 1937. Acetylation. II. Effect of different substances on the formation of p-aminobenzoic acid in rabbits. Biochem. Z. 293:302-4.
154. Hashizume, T., Y. Horie, and T. Mitsui. 1952. Chemical constituents of beer. I. Identification of amino acids of beer by paper partition chromatography. Mem. Research Inst. Food Sci., Kyoto Univ. No. 4:12-18.
155. Hautala, E., and M. L. Weaver. 1969. Separation and quantitative determination of lactic, pyruvic, fumaric, succinic, malic, and citric acids by gas chromatography. Anal. Biochem. 30(1):32-39.
156. Hayashi, T., H. Hoshino, and T. Ootsuka. 1965. Chemoreceptors in brain to gamma-hydroxybutyrate through cerebrospinal fluid of dogs. Olfaction Taste II, Proc. Inst. Symp., 2nd, Tokyo 599-608.
157. Hayashida, M., R. Ueda, and S. Teramoto. 1967. Organic acids in sake-brewing. V. Comparison of the organic acid content of four types of seed mash. Hakko Kogaku Zasshi 45(2):103-9.
158. Hayashida, M., R. Ueda, and S. Teramoto. 1968. Organic acids in sake brewing. VII. Diversity of organic acid contents in sake. Hakko Kogaku Zasshi 46(2):85-91.
159. Henle, W., and G. Szpíngler. 1936. Metabolism of isolated fat tissue. 3. The real respiration quotient and the influence on it of nutrients in vitro and vivo. Arch. Exp. Path. u. Pharmakol. 180(5/6):672-689.
- \* 160. Hermann, S., R. Neiger, and M. Zentner. 1938. Effect and fate of acids in the organism. I. Determination of pH in circulating rabbit blood after intravenous injection of free acids. Arch. Exptl. Path. Pharmakol. 188:526-32.

161. Hillig, F. 1950. Report on decomposition in eggs. *J. Assoc. Offic. Agr. Chemists* 33:722-7.
162. Hinsberg, K. 1943. Is the butyric acid of normal serum changed or not? *Z. Krebsforsch.* 54:270-8; *Chem. Zentr.* 1944. 11:328-9.
163. Hoberman, H. D., and L. Prosky. 1967. Pathways of hydrogen from succinate and malate in the energy-linked and non-energy-linked reduction of acetoacetate. *J. Biol. Chem.* 242(17):3944-50.
164. Hoelscher, H. A. 1950. Demonstration of dehydrogenases in tumor cells with tetrazolium salts. *Z. Krebsforsch.* 56:587-95.
165. Hollaender, A., W. K. Baker, and E. H. Anderson. 1951. Effect of oxygen tension and certain chemicals on the x-ray sensitivity of mutation production and survival. *Cold Spring Harbor Symposia Quant. Biol.* 16:315-26.
166. Hood, J. E., and P. R. Saunders. 1957. Oxidation of carbon-14-labeled glucose, pyruvate, and succinate by isolated contracting myocardium. *Am. J. Physiol.* 190:425-8.
167. Horiguchi, Y. 1960. Biochemical studies on *Pinctada martensii* and *Hyprlopsis schlegelii*. XI. The organic acids in tissues of the shellfish. *Nippon Suisangaku Kaishi* 26:695-700.
168. Houston, D. F., B. E. Hill, V. H. Garrett, and E. B. Kester. 1963. Organic acids of rice and some other cereal seeds. *J. Agr. Food Chem.* 11(6):512-17.
169. Hulpiou, H. R., R. B. Forney, and H. P. Onyett. 1954. The failure of succinate to alter metabolism of ethyl alcohol in dogs and rabbits. *Quart. J. Alc.* 15:9-15.
170. Imai, S., and K. Suzuki. 1969. Components of shoyu mash brewed at different temperatures in the early stage. *Seasoning Science* 16(5):1-5.
171. Impo, A., et al. 1966. On the effects of succinic acid on the isolated heart. *Atti. Accad. Med. Lombard* 21:596-7.
172. Ina, K., T. Ikeno, and T. Nakabayashi. 1965. Flavor compounds of oyster sauce. *Nippon Shokuhin Kogyo Gakkaishi* 12(7):288-91.
173. Inoue, H., M. Uchida, and T. Miyabe. 1966. Lactic acid bacteria isolated from mirin liquor. II. The composition of organic acids of ordinary mirin liquor and acid-putrefied mirin liquor. *Hakko Kogaku Zasshi* 44(3):71-6.
174. Isonaga, K. 1953. The microdetermination of succinic acid in urine. *Hukuoka Acta Med.* 44:679-93.

175. Ivy, A. C., and G. B. McIlvain. 1923. The stimulation of stomach secretion by application of substances on the duodenal and jejunal mucosa. *Am. J. Physiol.* 67:124-40.
176. Iwata, T., K. Nakagawa, and K. Ogata. 1969. Physiological studies of chilling injury in Citrus natsudaidai fruit. II. *Engel Gakkaï Zasshi* 38(1):93-100.
177. James, H. P., H. W. Elliott, and E. W. Page. 1948. Oxygen uptake of human placental tissue as affected by selected substrates and drugs. *Proc. Soc. Exptl. Biol. Med.* 67:130-2.
178. Jarczyk, A. 1968. Isolation and determination of nonvolatile acids in apple products. *Zesz. Nauk. Szk. Gł. Gospod. Wlejsk. Warszawe, Technol. Rolno-Spozyw* 5:21-38.
179. Johnston, F. B., and M. M. Hamill. 1968. The nonvolatile organic acids of some fresh fruits and vegetables. *Can. Inst. Food Technol. J.* 1(1):3-5.
180. Jolivet, E., and M. Zdenka. 1962a. Organic acids of dahlia tubers. Differences from potato and Jerusalem artichoke tubers. *Compt. Rend.* 254:2056-8.
181. Jolivet, E., and M. Zdenka. 1962b. Evolution of organic acids in the tuber of Jerusalem artichoke in the course of an annual cycle--comparison with the potato tuber. *Compt. Rend.* 254:721-3.
182. Juries, E. W. 1968a. Determination of succinic acid in fruits. *Elelmiszervizsgalati Közlem.* 13(3):112-20.
183. Juries, E. W. 1968b. Determination of succinic acid in fruit. *Z. Lebensm.-Unters. Forsch.* 137(6):373-9.
184. Kamysheva, A. S. 1969. Influence of adenosine diphosphate on the respiratory intensity of rabbit brain mitochondria in postnatal ontogenesis. *Byull. Eksp. Biol. Med.* 67(4):43-6.
185. Kano, F., and S. Saito. 1965. Biochemical studies on the storage of vegetables. I. Effects of blanching. *Kaseigaku Zasshi* 16(2):61-5.
186. Kazanskaya, L. N., I. M. Loginova, O. V. Afanas'eva, A. I. Vasil'eva, and L. K. Levando. 1967. Intensification of fermentation and improvement in the quality of bread. *Khlebopek. Konditerskaya Prom.* 11(11):4-6.
187. Kazinik, E. M., N. V. Novoruskaya, L. M. L'vovich, and G. A. Gudkova. 1969. Gas-liquid chromatography of high-boiling aliphatic dicarboxylic acids. *Zh. Anal. Khim.* 24(10):1592-4.

188. Kellogg, H. M., E. Brochmann-Hanssen, and A. B. Svendsen. 1964. Gas chromatography of esters of plant acids and their identification in plant materials. *J. Pharm. Sci.* 53(4):420-3.
189. Kim, C. J. 1963. Quantitative changes of organic acid and sugars during the fermentation of takju. *Daehan Hwahak HwoeJee* 4:33-42.
190. Kirk, J. E., T. J. S. Laursen, and R. Schaus. 1955. Studies on the succinic dehydrogenase of human aortic tissue. *J. Gerontol.* 10(2):178-181.
191. Kiyokawa, K. 1951. Effects of several fatty acids and salts upon renal gaseous metabolism, blood flow, and urinary output. *Japon.* 47:32-49.
192. Klauer, H., and W. Specht. 1937. Succinic acid in toxicological analysis. *Deut. Z. Ges. Gerichtl. Med.* 28:265-9.
193. Klein, W. 1925. Gasometric and spectroscopic observations of hemoglobin formations. *Biochem. Ztschr.* 156:323-33.
194. Knyaginichev, M. M., and G. L. Derkovskaya-Zelentsova. 1953. Rapid and simplified method for quantitative determination of lactic, the sum of succinic and malic, and citric and tartaric acids in leaven, dough, and bread. *Trudy Vsesoyuz. Nauch.-Issledovatel. Inst. Khlebopekarnoi Prom..* 5:209-13; Referat Zhur., Khim. 1954. No. 43511.
195. Kollas, D. A. 1964. The influence of controlled storage on the organic acids of apples. *Nature* 204(4960):758-9.
196. Kondrashova, M. N., et al. 1971. Succinic acid in skeletal muscles during intensive activity and during rest. *Dokl. Akad. Nauk. SSSR* 198:243-6.
197. Konosu, S., M. Shiboota, and Y. Hashimoto. 1967. Concentrations of organic acids in shellfish, with particular reference to succinic acid. *Elyo To Shokuryo* 20(3):186-9.
198. Konosu, S., K. Fujimoto, Y. Takashima, T. Matsushita, and Y. Hashimoto. 1965. Constituents of the extracts and the amino acid composition of the protein of short-necked clam. *Nippon Suisan Gakkaishi* 31(9):680-6.
199. Kozukue, N. 1970. Quality changes and the mechanism of chilling injury in peppers stored at low temperature. *Food Industry (Shokuhin Kogyo)* 13(16):90-93.
200. Krebs, H. A., and W. A. Johnson. 1937. The roll of citric acid as intermediary in metabolism in animal tissues. *Enzymologia* 4:148-56.

201. Krueger, R. C. 1955. Effect of beta-keto acids on the action of tyrosinase. *Arch. Biochem. and Biophys.* 56(2):394-404.
202. Krylov, A. V., and G. A. Tarakanova. 1962. Transformations of methyl and ethyl alcohols in fruits. *Fiziol. Rast.* 9:480-2.
203. Kuvaev, B. E., and N. S. Imyanilov. 1967. Determination of monobasic and dibasic acids by paper chromatography. *Zavod. Lab.* 33(11):1382-3.
204. Kuwabara, K. 1957. Studies on the uptake of p-aminohippurate (PAH) by slices of the renal cortex of a rabbit. *Folia Pharmacol. Japonica* 53(6):1036-1048.
205. Lafon, M. 1955. Formation of secondary products of alcoholic fermentation. *Ann. Inst. Natl. Recherche Agron., Ser. E., Ann. Technol. Agr.* 4:169-221, 241-92.
206. Lambion, R., and R. Meskhi. 1957. Bacteria of malolactic fermentation. *Rev. Fermentations et Inds. Aliment.* 12:131-44.
- \* 207. Landauer, W., and D. Sopher. 1970. Succinate, glycerophosphate, and ascorbate as sources of cellular energy and as antiteratogens. *J. Embryol. Exp. Morphol.* 24 (Pt. 1):187-202.
208. Landes, D. R., and L. E. Dawson. 1971. Organic acid patterns in fresh and incubated whole egg products. *Poultry Science* 50(2):641-642.
209. Larson, P. S., J. K. Finnegan, and H. B. Haag. 1956. Effect of chemical configuration on the edema-producing potency of acids, aldehydes, ketones, and alcohols. *J. Pharmacol. Exptl. Therap.* 116:119-22.
210. Laufberger, V. 1925. The effect of intermediate products on the gaseous metabolism of rabbits. *Biochem. Ztschr.* 158:259-77.
211. Leferenz, H. 1948. Influence of succinic acid on the blood lactic acid level after work. *Biochem. Z.* 319:184-95.
212. Le Heux, J. W. 1921. Choline as hormone for intestinal motility. III. Participation of choline in the action of various organic acids in the intestines. *Pfluegers Arch. d. Physiol.* 190:280-300.
213. Leloir, L. F., and J. M. Munoz. 1938. Ethyl alcohol metabolism in animal tissues. *Biochem. J.* 32:299-307.

214. LeRoux, P. 1961. Some quantitative data on the organic acids of the field mushroom (*Agaricus campestris*). *Compt. Rend.* 252:205-7.
215. Lesaint, C., C. Tendille, and J. L. Papin. 1962. Malonic acid, the predominant acid in dahlia petals. *Compt. Rend.* 255:1002-3.
216. Lewis, M., G. R. Lee, G. E. Cartwright, and M. M. Wintrobe. 1967. Glycine decarboxylation in the porcine erythrocyte; its relation to aminolevulinic acid synthesis. *Biochim. Biophys. Acta* 141(2):296-309.
217. Lin, Y. D., F. M. Clydesdale, and F. J. Francis. 1970. Organic acid profiles of thermally processed spinach puree. *J. of Food Science* 35(5):641-44.
218. Lin, Y. D., F. M. Clydesdale, and F. J. Francis. 1971. Organic acid profiles of thermally processed, stored spinach puree. *J. of Food Science* 36(2):240-42.
219. Lincke, H. 1945. The metabolic behavior of alkylated succinic acids in the dog; the biological decomposition of the fatty acid chain. Zurich. 91pp.
220. Lo, T. -Y., and C. Wu. 1944. The peptization of rice proteins by organic acids and organic salts. *Chinese J. Sci. Agr.* 1:173-8.
221. Luciani, S., A. R. Contessa, and C. Bortignon. 1967. Effect of papaverine on succinate oxidation. *Boll. Soc. Ital. Biol. Sper.* 43(8):409-10.
222. Lynen, F. 1940. The respiration of animal tissue after freezing in liquid air. *Hoppe-Seyler's Zeitschr. Physiol. Chem.* 264(3/4):146-152.
223. Macharashvili, G. I. 1970. The organic acid composition of apple juice and wines. *Vinodelie i Vinogradarstvo SSR* 30(5):24-26.
- \* 224. MacKay, E. M., and R. H. Barnes. 1938. Conversion of succinic acid to glucose in the phloridzinated dog. *Proc. Soc. Exp. Biol. and Med.* 38(3):417-419.
- \* 225. MacKay, E. M., J. W. Sherrill, and R. H. Barnes. 1939. The antiketogenic activity of succinic acid. *J. Clin. Invest.* 18(3):301-305.
226. MacKenzie, K. G., and M. C. Kenny. 1965. Nonvolatile organic acid and pH changes during the fermentation of distiller's work. *J. Inst. Brewing* 71(2):160-5.

227. Maeda, S., A. Mukai, N. Kosugi, and Y. Okada. 1962. Flavor components of honey. *Nippon Shokukhim Kogyo Gakkaishi* 9(7):270-4.
228. Maeda, Y. 1964. Organic acids in rhubarb. *Nippon Shokuhin Kogyo Gakkaishi* 11(10):444-6.
229. Maiafaya-Baptista, A., J. A. Gulmarais, J. Garrett, and W. Osswald. 1957. Physiopharmacological aspects of the motility of the stomach. *Arch. Internat. Pharmacodyn.* 111(2):191-214.
230. Maltabar, V. M., Zh. N. Frolova, and Z. A. Mamakova. 1971. Effect of the titratable acidity of processed wine on the content of ester in cognacs. *Sadovodstvo, Vinogradarstvo i Vinodelie Moldavii* 26(3):28-30.
231. Manabe, T. 1969. Studies on the constituents of and processing methods for Japanese chestnut. V. Organic acids in the edible portion of Japanese chestnut. *J. of Food Science & Technology (Nihon Shokuhin Kogyo Gakkaishi)* 16(2):81-86.
232. Marinelli, L., M. F. Feil, and A. Schait. 1968. The isolation and identification of organic acids in beer. *Proceedings. Amer. Soc. of Brewing Chemists* 113-19.
233. Markakis, P., A. Jarczyk, and S. P. Krishna. 1963. Nonvolatile acids of blueberries. *J. Agr. Food Chem.* 11:8-11.
234. Markova, A. V., and V. A. Smirnov. 1969. Separation of chloroacetic, acetic, and succinic acids by a partition chromatographic method. *Zh. Anal. Khim.* 24(8):1271-2.
235. Markova, J. 1970. Qualitative and quantitative analysis of organic acids separated from rye flour, pre-ferment, dough and mix bread. *Sbornik Vysoke Skoly Chemicko-Technologicke v Praze, E-Potraviny* 29:123-30.
236. Marquardt, P. 1938. Inactivation of adrenaline by succinic acid. *Klin. Wochschr.* 17:1445-6.
237. Marquardt, P. 1939. The succinic acid destruction of adrenaline and its biological relations. *Klin. Wochschr.* 18:252-3.
238. Martin, G. E., J. G. Sullo, and R. L. Schoeneman. 1971. Determination of fixed acids in commercial wines by gas-liquid chromatography. *J. of Agricultural and Food Chemistry* 19(5):995-998.
239. Martin, G. J., and W. Stenzel. 1944. The influence of various chemicals and vitamin deficiencies on the excretion of glucuronic acid in the rat. *Arch. Biochem.* 3:325-31.

240. Matsubara, I., K. Morita, and S. Kinoshita. 1960. Quantitative analysis of blackstrap molasses. V. The content of amino acids. *Hakko Kyokaishi* 18:73-77.
241. Matsui, H., and S. Sato. 1963. Organic acids in poor sake. *Nippon Jozo Kyokai Zasshi* 58(7):635-8.
242. Mayer, K., I. Busch, and G. Pause. 1964. Succinic acid formation during wine fermentation. *Z. Lebensm.-Untersuch.-Forsch.* 125(5):375-81.
243. Mazza, F. P. 1936. The biological oxidation of 2-basic aliphatic acids and the oxidation of monobasic fatty acids. *Arch. Scienze Biol.* 22:307-26.
244. McFeeters, R., S. H. Schanderl, and P. Markakis. 1966. Nonvolatile acids of green peas. *Mich. State Univ., Agr. Expt. Sta., Quart. Bull.* 48(3):417-20.
245. McGavran, J., and M. Rheinberger. 1933. The question of specificity of intracellular dehydrogenase. IV. The valuation of hydrogen ion concentration. *J. Biol. Chemistry* 100:267-69.
- \* 246. Merck and Co., Inc. 1968. *The Merck Index. An encyclopedia of chemicals and drugs.* Merck and Co., Inc. Rahway, N.J.
247. Miller, A., and J. K. Rocks. 1966. High bloom strength algin gel. U.S. Pat. 3,266,906.
248. Miyaji, K. 1929. Tyramine as a constituent of Japanese vinegar. *Ztschr. Physiol. Chem.* 184:157-162.
249. Miyaji, K., and Y. Saeki. 1956. Action of sodium succinate on the heart. *Kyushu J. Med. Sci.* 7:92-100.
250. Miyazaki, S., Y. Suhara, and T. Kobayashi. 1969. Separation of aliphatic dibasic acids by thin-layer chromatography. *J. Chromatogr.* 39(1):88-90.
251. Miyoshi, S. 1959. Changes produced in the steady potential from the cortical surface of the brain after administration of metabolism-promoting or -inhibiting agent. *Fukuoka Igaku Zasshi* 50:969-84.
252. Morard, P., and E. Bourrier. 1971. Quantitative determination of several organic acids in plant tissues by gas chromatography. *Chimie Analytique* 53(5):315-322.
253. Morgan, J. F., S. Tolnal, and G. F. Townsend. 1960. In vitro antitumor activity of fatty acids. II. Saturated dicarboxylic acids. *Can. J. Biochem. Physiol.* 38:597-603.

254. Mori, S., and T. Takeuchi. 1970. Thin-layer chromatography of diamines, dicarboxylic acids, and omega-amino acids. Application to the analysis of copolyamides. *J. Chromatogr.* 47(2):224-31.
255. Moriguchi, S., H. Ishikawa, R. Ueda, and M. Hayashida. 1961. Organic acids in soysauce. III. Composition of organic acids in wheat and wheat bran. *Hakko Kogaku Zasshi* 39:293-7.
256. Moriguchi, S., and H. Ishikawa. 1964. Organic acids in soy sauce. VI. Changes in amounts of organic acids during the koji-making process. *Hakko Kogaku Zasshi* 42(1):35-40.
257. Mosca, A. 1954. Preserving chocolate and its substitutes. *Ital. Pat.* 494,153.
258. Mountney, G. J., and J. O'Malley. 1965. Acids as poultry meat preservatives. *Poultry Sci.* 44(2):582-6.
259. Moussatache, H., and A. Prouvost-Danon. 1957. The effect of succinate and malonate on the chemical release of histamine from guinea-pig-lung slices. *Naturwissenschaften* 44:637-8.
260. Mushahwar, I. K., A. Alter, and A. R. Schulz. 1967. Some metabolic studies of beta-aminoisobutyric acid. *Life Sci. (Oxford)* 6(12):1317-30.
261. Nagano, K. 1961. Glucose metabolism of rat brain. I. The effect of succinate. *Gunma J. Med. Sci.* 10:135-43.
262. Needham, D. M. 1927. Quantitative study of succinic acid in muscle; metabolic relationships of succinic, malic and fumaric acids. *Biochem. J.* 21:739-750.
263. Needham, D. M. 1930. Quantitative studies of succinic acid in muscle. III. Glutamic and aspartic acids as sources of succinic acid. *Biochemical J.* 24:208-27.
264. Nelson, E. K., and C. A. Greenleaf. 1929. A study of sugar cane sirup distillation discharge. *Ind. Engin. Chem.* 21:857-59.
265. Nelson, E. K., and H. H. Mottern. 1931a. The organic acids of spinach, broccoli, and lettuce. *J. Am. Chem. Soc.* 53:1909-12.
266. Nelson, E. K., and H. H. Mottern. 1931b. Some organic acids in honey. *Industr. and Engineer. Chem.* 23(3):335-336.
267. Nesty, G. A. 1970. Food acidulant. *U.S. Pat.* 3,523,024.

268. Nielsen, N., and J. Dagys. 1940. Growth substance effect of amino acids. VI. Studies on the effect of citric acid and further studies on the action of beta-alanine. *Compt. Rend. Trav. Lab. Carlsberg (Copenhagen) Ser. Physiol.* 22:447-480.
269. Nordmann, R., O. Gauchery, J. P. du Ruisseau, Y. Thomas, and J. Nordmann. 1954. Identification of the organic acids of urine by paper chromatography. *Compt. Rend.* 238:2459-61.
270. Nossal, P. M. 1948. The metabolism of erythrocytes. I. Respiration in the absence and presence of methylene blue. *Australian J. Exptl. Biol. Med. Sci.* 26:123-38.
271. Nuccorini, R., and A. Zaccagnini. 1930. Chemical studies of fruits. The determination of tartaric, citric, malic and succinic acids in fruit. *Ann. Sperimentazione Agrar.* 4:301-06.
272. NV Lijm- en Gelatinefabriek 'Delft'. 1970. Method for preparing a dry gelatin product and method for using this for a gelatin pudding powder. Netherlands Pat. 6,809,670.
273. Nygaard, P. 1967. Two-way separation of carboxylic acid by thin-layer electrophoresis and chromatography. *J. Chromatogr.* 30(1):240-3.
274. Nyhan, W. L., and H. Busch. 1958. Metabolic patterns for succinate-2-C<sup>14</sup> in tissues of tumor-bearing rats. *Cancer Res.* 18(10):1203-1208.
275. Ohara, A., K. Endo, and M. Yoshioka. 1964. Inhibitors of thiol enzymes. V. Influence of dibasic acids on the activity of papain. *Kyoto Yakka Daigaku Gakuho No.* 12:32-5.
276. Orten, J. M., W. Gamble, C. B. Vaughn, and K. C. Shrivastava. 1968. A micromethod for silica-gel column chromatography of urinary organic acids. *Microchem. J.* 13(2):183-92.
277. Palladina, L. I., and A. M. Gudina. 1956. The effect of substances of the tricarboxylic acid cycle on the oxidative processes of regenerating tissues. *Ukrain. Blokhim. Zhur.* 28:329-36.
278. Panzani, R., G. Fargepallet, A. Goupil, and Mme. Fargepallet. 1954. Comparative study of variations of different expiratory constants before and after acetylcholine and succinic acid. *Marseille-Med.* 91:175-177.
279. Paoletti, C. 1969. Determination of succinic acid in wine and in fermentation products of yeasts, moulds and bacteria: modification of the method of Marignan and Peynaud. *Industria Agraria* 7(2):48-50.

280. Pausescu, E., A. Dinca, and R. Chirvasie. 1967. Leukocyte metabolism in transplantation immunity. II. Effect of immunosuppressive and cytostatic substances on the oxidative metabolism of leukocytes during kidney homotransplant rejection. *Rev. Roum. Physiol.* 4(4):283-92.
281. Pinnow, J. 1929. On currant wine. II. *Ztschr. Unters. Lebensmittel* 58:331-42.
282. Pires, R., and K. Moehler. 1970. Enzymic determination of succinic acid in wine. *Z. Lebensm.-Unters.-Forsch.* 143(2):96-9.
283. Porter, W. L., M. L. Buch, and C. O. Willets. 1951. Maple sirup. III. Preliminary study of the nonvolatile acid fraction. *Food Research* 16:338-41.
284. Pray, L. W., and J. J. Powers. 1966. Acidification of canned tomatoes. *Food Technol.* 20(1):87-91.
285. Preobrazhenskii, A. A., and L. M. Bobkova. 1970. Contents of organic acids during fermentation of grape must. *Vinodelie i Vinogradarstvo SSSR* 30(1):15-17.
286. Prey, V., and H. Polleres. 1969. Thin-layer chromatographic determination of lactic acid in raw juices obtained in sugar manufacture. *Z. Zuckerind.* 19(9):510-11.
287. Primo, E., A. Casas, J. Alberola, M. Martinez, and M. P. Cornejo. 1969. Detection of adulteration in citrus juices. XV. Identification of carboxylic acids present in orange juice, commercial sucrose and citric acid by gas-liquid (GLC) and thin layer chromatography (TLC). *Revista de Agroquimica y Tecnologia de Alimentos* 9(3):415-22.
288. Primo Yufera, E., J. Sanchez, and J. Alberola. 1963. Detection of adulteration in citrus juices. I. Methods for the identification of acids in orange juice by thin-layer chromatography and gas-liquid chromatography. *Rev. Agroquim. Tecnol. Alimentos* 3:346-49.
289. Primo Yufera, E., J. S. Parared, and J. Alberola. 1963. Detection of citrus juice adulteration. I. Processes for determination of acids in orange juice through thin-layer chromatography. *Rev. Agroquim. Tecnol. Alimentos* 3:349-56.
290. Primo Yufera, E., J. Sanchez, and J. Alberola. 1965. Detection of adulteration in citrus juices. III. Identifications of nonvolatile acids in orange juices from the United States. *Rev. Agroquim. Tecnol. Alimentos* 5(1):121-4.
291. Procopio, M., and M. Antona. 1961. Determination of the most important organic acids of wine by use of ion-exchange resins. Determination of succinic acid. *Vini Ital.* 3:217-19.

292. Pucher, G. W., and H. B. Vickery. 1941. Determination of succinic acid in plant tissue. *Indust. and Engineer. Chem. Analyt.* Ed. 13(6):412-415.
293. Quagliariello, E., and F. Palmieri. 1968. Control of succinate oxidation by succinate uptake by rat liver mitochondria. *Eur. J. Biochem.* 4(1):20-7.
294. Quagliariello, E., F. Palmieri, and M. Cistemino. 1967. Intramitochondrial concentrations of respiratory substrates in various functional states. *Boll. Soc. Ital. Biol. Sper.* 43(6):297-300.
295. Raveux, R., and J. Bove. 1957. Separation and determination of carboxylic acids from C1 to C6 by partition chromatography on a silica column. I. General study. *Bull. Soc. Chim. France* 369-76.
296. Reagan, J. G., L. R. York, and L. E. Dawson. 1971. Improved methods for determination of certain organic acids in pasteurized and unpasteurized liquid and frozen whole egg. *J. of Food Science* 36(2):351-54.
297. Rees, K. R. 1954. Aerobic metabolism of the muscle of *Locusta migratoria*. *Biochem. J.* 58(2):196-201.
298. Rennhard, H. H. 1968. Polysaccharides for foods. *Fr. Pat.* 1,538,007.
299. Rentschler, H., and H. Tanner. 1954. Composition of fruit acids of Swiss fruit juices. I. Fruit acids of Swiss cider pears. *Mitt. Lebensm. Hyg.* 45:142-58.
300. Reznikov, V. M., and I. I. Savina. 1968. Chromatographic analysis of organic acids in wood hydrolyzates. *Sb. Tr. Vses. Nauch.-Issled. Inst. Gidroliza Rast. Mater.* 17:53-60.
301. Ribereau-Gayon, P. 1953. A simple and positive method to distinguish the malic acid-lactic acid fermentation of wines. *Compt. Rend. Acad. Agr. France* 39:807-9.
302. Richardson, L. R., and J. V. Hallick. 1957. Feed spoilage. Heat-inhibiting activity of various compounds and commercial products. *Texas Agr. Expt. Sta., Bull. No. 879*, 6 pp.
303. Roberts, E. J., and L. F. Martin. 1954. Identification and determination of nonnitrogenous organic acids of sugar cane by partition chromatography. *Anal. Chem.* 26:815-18.
304. Rodgers, K. 1961. Estimation of succinic acid in biological materials. *Biochem. J.* 80(2):240-244.

305. Rodopulo, A. K. 1953. Partition and determination of organic acids in grape wine and grape must by chromatographic methods. *Biokhimiya* 18:544-7.
306. Rodopulo, A. K. 1954. Determination of di- and tricarboxylic acids in grapes, ciders, and wines. *Vinodelie i Vinogradarstvo SSSR* 14(3):5-9.
307. Roper, R., and T. S. Ma. 1968. Microchemical investigation of medicinal plants. II. The antitubercular activity of some plant acids and their hydrazides. *Mikrochim. Acta* (1):212-18.
308. Rosculet, G. 1969. Metabolic products of yeast fermentation: organic acids, isolation, separation, and characterization. *Am. Soc. Brew. Chem., Proc.* 127-38.
- \* 309. Rose, W. C. 1924. The toxic effect on the kidney of dicarboxylic acids and their derivatives. *J. Pharm. and Exp. Therapeutics* 24:123-46; 147-58.
310. Rosenthal, O. 1937. The intensity of succinic acid oxidation in surviving liver tissue. *Biochemical J.* 31:1710-18.
311. Roujansky, O. C. A. 1971. Digestion-aiding acidified foods. French Pat. 1,603,568.
312. Rous, S., L. Aubry, and F. Bonini. 1970. Microsomal succinic dehydrogenase activity and mode of succinate-2,3-14C incorporation into rat liver microsomal fatty acids in vitro. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 7(1):32-4.
313. Rubinstein, R., A. V. Howard, and O. M. Wrong. 1969. In vivo dialysis of feces as a method of stool analysis. IV. Organic anion component. *Clin. Sci.* 37(2):549-64.
314. Sakaguchi, K., and S. Tada. 1940. On the formation of succinic acid by *Bacterium succinicum* nov. sp. *Zentralbl. Bakt.* II. Abt. 101(18/20):341-354.
315. Sakakibara, S., J. Shimakawa, H. Imaseki, and I. Uritani. 1965. Production of organic acids in vegetables during frozen storage. *Nippon Shokuhin Kogyo Gakkaishi* 12(2):39-42.
316. Salwin, H., and J. F. Bond. 1969. Quantitative determination of lactic acid and succinic acid in foods by gas chromatography. *J. of the Assoc. of Official Analytical Chemists* 52(1):41-47.
317. Samisch, Z., and A. Cohen. 1949. Composition of oranges in Israel. Jewish Agency Palestine, Agr. Research Sta., Rehovot, Bull. 51.
318. Sanders, A. P., W. D. Currie, and B. Woodhall. 1969. Protection of brain metabolism with glutathione, glutamate, gamma-aminobutyrate, and succinate. *Proc. Soc. Exp. Biol. Med.* 130(3):1021-7.

319. Sanderson, G. W., and R. R. Selvendran. 1965. Organic acids in tea plants. Nonvolatile organic acids separated on silica gel. *J. Sci. Food Agr.* 16(5):251-8.
320. Sato, K., T. Suzuki, and Y. Sahash. 1966. Biogenesis of vitamin B in *Candida albicans* 4888. IV. Incorporation of radioactive carbon of succinic acid into vitamin B. *Vitamins (Kyoto)* 33(4):357-360.
321. Scheffer, F., R. Kickuth, and H. Lorenz. 1965. The separation and the determination of organic acids in plants. *Qualitas Plant. Mater. Vegetabiles* 12(4):342-8.
322. Schenck, G., and C. H. Brieskom. 1944. The components of balm mint. *Arch. Pharm. u. Ber. Deutsch. Pharm. Ges.* 282(1/2):1-9.
323. Schmitz, H. 1950. Demonstration of dehydrogenases in mice ascites tumor with Thunberg's method. *Z. Krebsforsch.* 56:596-600.
324. Schopeer, J. F. 1971. Correction of the acidity of musts and wines. *Bulletin de l'Office International du Vin* 44(479):55-57.
325. Schormueller, J., and H. Langner. 1960. Quantitative determination of organic acids in foods. *Z. Lebensm.-Untersuch. u. -Forsch.* 113:104-12.
326. Schormueller, J., W. Brandenburg, and H. Langer. 1961. Organic acids in coffee substituents, dried powder extracts of coffee substitutes, and coffee. *Z. Lebensm.-Untersuch. u.-Forsch.* 115:226-35.
327. Schormueller, J., K. Lehmann, and H. Langner. 1960. The composition of the "tear fluid" of Emmentaler cheese. *Z. Lebensm.-Untersuch. u. -Forsch.* 112:364-75.
328. Schramm, R. 1961. Paper chromatography of organic acids in storage roots of some Umbelliferae. *Acta Soc. Botan. Polon.* 30:285-92.
329. Schramm, R., and M. Piatkowska. 1961. Paper chromatography of organic acids in broad bean. *Acta Soc. Botan. Polon.* 30:381-9.
330. Schulze, K., and A. Melle. 1938. Drug investigation. *Deut. Apoth.-Ztg.* 53:1536-8.
331. Seidel, C. F., H. Schinz, and M. Stoll. 1958. Compounds with aroma. II. Strawberry aroma. *Helv. Chim. Acta* 41:372-7.
332. Semlchon, L., and M. Flanzy. 1932. Use of chromic acid oxidation on a dibasic acid. *Compt. Rend. Acad. Sciences* 194:2063-65.

333. Sendarovich, B. P., and R. M. Klyachina. 1962. Organic acids in onion bulbs. *Tr. Vost.-Sibirsk. Filiala, Akad. Nauk SSSR* 35:30-2.
334. Sequelra, R. M. 1970. A method for the determination of organic acids in sugar beets and factory juices by gas liquid chromatography. *J. of the Am. Soc. of Sugar Beet Technologists* 16(2):136-141.
335. Seto, K., T. Tsuda, and M. Umezu. 1955. Metabolism of lower fatty acids in the rumen mucosa. 1. Influence of tricarboxylic acid cycle-related compounds on the formation of ketone bodies from lower fatty acids in the rumen mucosa of oxen. *Seikagaku* 27:213-17.
336. Shapiro, I. A., and A. N. Panteleev. 1965. Organic acids of the pumpkin and the vegetable marrow. *Vestn. Leningr. Univ.* 20(15):, Ser. Biol. No. 3:92-6.
337. Shibaoka, H., and I. Furusawa. 1964. Effect of manganese ions on the IAA-induced elongation of *Avena* coleoptile sections inhibited by some organic acids. *Plant Cell Physiol. (Tokyo)* 5(3):273-80.
338. Shibazaki, K., and K. Aso. 1951. Utilization of apples. VI. Recovery of volative apple flavor. *J. Fermentation Technol. (Japan)* 29:352-7.
339. Shimizu, T. 1921. The chemical composition of the brain. *Biochem. Ztschr.* 117:252-62.
340. Shiring, E. R., and A. Ya. Zagorul'ko. 1969. Products of the decomposition of sucrose during vaporization, and associated substances in ammonia water. *Sakh. Prom.* 43(4):16-20.
341. Silverberg, H. D. 1957. Succinic acid as an index of decomposition in spinach. *J. Assoc. Offic. Agr. Chemists* 40:414-17.
342. Simola, P. E., and T. Kosunen. 1938. The excretion of citric acid by rats after administration of various organic acids. *Suomen Kemistilehti* 11B:22-3.
343. Simola, P. E. and F. E. Krusius. 1938. The formation of alpha-ketoglutaric acid in animal metabolism. *Suomen Kemistilehti* 11B:9.
344. Simola, P. E., F. E. Krusius, and H. Alapeuso. 1938. Citric acid formation in rats. *Suomen Kemistilehti* 11B:18.
345. Simonart, P., and J. Mayaudon. 1956. Chromatographic study of cheese. II. Aliphatic acids. *Neth. Milk Dairy J.* 10:156-61.
346. Simonyan, A. A., and R. A. Stepanyan. 1966. Polarographic study of oxidation in liver mitochondria of chick embryos during embryogenesis. *Biol. Zh. Armenii* 19(1):58-63.

347. Singh, V. K., and T. Kristoffersen. 1969. Accelerated ripening of Swiss cheese curd. *J. of Dairy Science* 52(6):899-900.
348. Smith, R. M., and G. R. Russell. 1967. Metabolism of propionate by sheep-liver mitochondria. Evidence for rate control by a specific succinate oxidase. *Biochem. J.* 104:460-72.
349. Souty, M., P. Andre, and L. Breuils. 1965. Nonvolatile organic acid content of sweet peppers. *Ann. Technol. Agr.* 14(4):357-64.
350. Spanyol, P., and I. Szeredy. 1962. Smoking of foods. IV. Available acids in smoke and smoked products. *Z. Lebensm. Untersuch. Forsch.* 118:293-9.
351. Spettoli, P., and V. Bolcato. 1971. Action of *Acetobacter aceti* on some constituents of wines. *Industrie Agrarie* 9(7/8):261-266.
352. Stadie, W. C. 1945. The intermediary metabolism of fatty acids. *Physiol. Rev.* 25(3):395-441.
353. Stanimirovic, S. G., D. L. Stanimirovic, and D. R. Nastic. 1963. Identification of organic acids and saccharides in fruits and leaves of medlar (*Mespilus germanica*). *Glasnik Hem. Društva, Beograd.* 28(5-6):327-35.
354. Stare, F. J., E. S. Gordon, and M. J. Musser. 1938. Effect of succinic acid on the respiratory of normal human muscle and various myopathies. *Nature* 141:831.
355. Staruszkiewicz, W. F., Jr. 1969. Collaborative study on the quantitative gas chromatographic determination of lactic and succinic acids in eggs. *J. Ass. Offic. Anal. Chem.* 52(3):471-476.
356. Staruszkiewicz, W. F., Jr. 1970. Collaborative study on the GLC and AOAC methods for the quantitative determination of lactic and succinic acids in egg. *J. of the Assoc. of Official Analytical Chemists* 53(1):28-35.
357. Staruszkiewicz, W. F., Jr., J. F. Bond, and H. Salwin. 1970. Quantitative gas chromatographic determination of beta-hydroxybutyric acid with application to eggs. *J. of Chromatography* 51(3):423-32.
358. Steinhauer, J. E., and L. E. Dawson. 1969. Quantitative determination of lactic and succinic acids in frozen whole eggs by gas-liquid chromatography. *J. Food Sci.* 34(1):37-42.
359. Stinson, E. E., M. H. Subers, J. Petty, and J. W. White, Jr. 1960. The composition of honey. V. Separation and identification of the organic acids. *Arch. Biochem. Biophys.* 89:6-12.

- \* 360. Stoehr, R. 1933. Note on glycogen formation and succinic acid. Hoppe-Seyler's Zeitschr. Physiol. Chem. 217(3/4):153-155.
361. Stoll, U. 1969. Examination of nonfatty and fatty acids extractable from muscle tissue and meat products by thin-layer and gas chromatography. J. Chromatogr. 44(3-4):537-46.
362. Stoll, U. 1970a. Combination of thin layer and gas chromatography for the identification of aliphatic hydroxy and dicarboxyl acids. J. Chromatography 52(1):145-51.
363. Stoll, U. 1970b. Changes of acid composition in meat and meat products during processing and storage. Z. Lebensm.-Unters.-Forsch. 143(1):1-5.
364. Stoppani, A. O. M. 1945. The effect of citric and dicarboxylic acids on the metabolism of phosphorus compounds. Anales Asoc. Quim. Argentina 33:188-99.
365. Suchy, V., and K. Rada. 1967. Centrifugal thin-layer chromatography. Sugars and organic acids. Ceskoslov. Farm. 16(7):340-2.
366. Sukeho, T., R. Suzuki, and S. Tsuiki. 1967. Oxidative decarboxylation pathway of succinate oxidation in Ehrlich ascites tumor cells. Sci. Rep. Res. Inst. Tohoku Univ., Ser. C 14(3-4):158-63.
367. Sulli, E. 1969. Respiratory behavior of coliform bacteria grown aerobically and anaerobically on various substrates. Riv. Med. Aeronaut. Spaz. 32(1):19-41.
368. Supran, M. K., J. J. Powers, P. V. Rao, T. P. Dornseifer, and P. H. King. 1966. Comparison of different organic acids for the acidification of canned pimientos. Food Technol. 20(2):117-22.
369. Suzuki, S. 1934. The influence of some amino acids on the production of oxalic acid in the animal organism. Japanese J. Med. Sci. 11. Biochem. 2(3):413-425.
370. Svendsen, A. B. 1950. Paper chromatography as a qualitative procedure for the phytochemical analysis of organic acids. Pharm. Acta Helv. 25:230-6.
371. Svendsen, A. B. 1953. The phytochemistry of radix angelicae. Blyttia 11:96-104; Biol. Abstr. 28:2172, 1954.
372. Sweeney, J. P., V. J. Chapman, and P. A. Hepron. 1970. Sugar, acid and flavour in fresh fruits. J. of the Am. Dietetic Assoc. 57(5):432-35.

373. Szekeres, L., G. Lenard, and J. Soti. 1958. The action of various metabolic intermediates on oxygen consumption of normal and hypoxic heart muscle slices. *Arch. Internat. Pharmacodyn.* 115(1/2):141-149.
374. Szent-Gyorgyi, A. 1935. Mechanism of respiration. *Nature*, London 135:305.
375. Take, T., and H. Itsuka. 1966. Flavor compounds in various foods. VI. Soybean sprout. *Kaseigaku Zasshi* 17(4):213-17.
376. Takizima, Y. 1949. The chemical components of Japanese apricot, *Prunus mume*. *J. Agr. Chem. Soc. Japan* 23:8-10; *Kagaku no Ryoiki (J. Japan. Chem.)* 2:81-2, 1948.
377. Tamura, T., E. Miyazawa, and S. Miyasawa. 1968. Toxicity of drugs. V. Subchronic toxic tests on mice with the use of amber acid (succinic acid) and magnesium phosphate drugs. *J. Nippon Univ. Sch. Dent.* 10(3):83-90.
378. Tang, F., and I. A. Sytlnskil. 1964. Quantitative determination of organic acids in sweet potato tubers. *Fiziol. Rast.* 11(5):930-1.
379. Tavant, H., and M. Mange. 1965. Determination of free organic acids in leaves of *Begonia semperflorens* and *Cicer arietinum* by silica-gel chromatography. *Ann. Sci. Univ. Besancon Botan.* (3):2:24-8.
380. Tavernier, J., and P. Jacquin. 1947. Organic acids of apple must. *Compt. Rend.* 225:1373-4.
381. Taylor, N. W. 1928. The penetration of acid in living tissue. *J. Gen. Physiol.* 11:207-19.
382. Temperli, A. 1958. Application of Warburg's manometric method to cheese-production bacteriology. *Schweiz. Milchztg.* 84:445-8.
383. Teramoto, S., H. Taguchi, and R. Ueda. 1962. Organic acid constituents of Japanese fermented food products. *Proc. Intern. Congr. Food Sci. Technol., 1st, London* 2:549-55.
384. Terrell, A. W. 1938. Succinic acid and glucose in pituitary ketonuria. *Proc. Soc. Exp. Biol. and Med.* 39(2):300-301.
385. Thomson, J. E., G. J. Banwart, D. H. Sanders, and A. J. Mercuri. 1967. Effect of chlorine, antibiotics, beta-propiolactone, acids, and washing on *Salmonella typhimurium* on eviscerated fryer chickens. *Poultry Sci.* 46(1):146-51.
386. Thorn, W., G. Gercken, and P. Huerter. 1965. Function, substrate supply and metabolite content of rabbit heart perfused in situ. *Am. J. Physiol.* 214(1):139-45.

387. Thoukis, G., M. Ueda, and D. Wright. 1965. The formation of succinic acid during alcoholic fermentation. *Am. J. Enol. Viticult.* 16(1):1-8.
388. Ting, S. V., and H. M. Vines. 1966. Organic acids in the juice vesicles of Florida Hamlin orange and Marsh Seedless grapefruit. *Proc. Am. Soc. Hort. Sci.* 88:291-7.
389. Toenniessen, E. 1930. The formation of succinic acid from pyroracemic acid by in vivo perfusion of mammal muscles. *Klin. Wchschr.* 9:211-13.
390. Toenniessen, E., and E. Brinkmann. 1930. The oxidative decomposition of carbohydrates in mammal muscle, especially the formation of succinic acid from pyroracemic acid. *Z. Physiol. Chem.* 187:137-159.
391. Torley, D. Y. 1942. The amount of organic acids in Hungarian wines. *Mezogazdasagi Kutatasok* 15:310-20.
392. Tripod, J., A. Moncada, R. Jaques, and E. Wirz. 1955. Intermediary metabolites and the isolated rabbit heart. *Arch. Intern. Pharmacodynamie* 104:121-36.
393. Tsukamoto, K. 1959. Influence of organic acids of tricarboxylic acid cycle on the liver functions. *Fukuoka Igaku Zasshi* 50:136-51.
394. Tudoranu, G., I. Foni, P. Toporas, G. Creteanu, and C. Pavelescu. 1950. Chemical anticoagulating agents. *Le Sang* 21:511-14.
395. Turowska, M. 1960. Biacridine derivatives as indicators in titration of weak acids. *Chem. Anal. (Warsaw)* 5:815-21.
396. Udo, S., and T. Sato. 1962. Composition and flavor of clams and cuttlefish. *Nippon Nogei Kagaku Kaishi* 36(10):838-41.
397. Ueda, R., M. Hayashida, and S. Teramoto. 1963. Organic acids in beer. I. Composition of organic acids in commercial beer. *Hakko Kagaku Zasshi* 41:10-14.
398. Ueda, R., M. Hayashida, and S. Teramoto. 1964a. Organic acids in beer. II. Composition of organic acids in barley, malt, and wort. *Hakko Kagaku Zasshi* 42(1):22-7.
399. Ueda, R., M. Hayashida, and S. Teramoto. 1964b. Application of organic acid analysis to the control of production in fermentation industry. *Kogyo Kagaku Zasshi* 67(5):753-6.
400. Ueda, R., S. Nagai, and S. Moriguchi. 1959. Organic acids in soysauce. I. Determination of the organic acids in soysauce. *Hakko Kagaku Zasshi* 37:94-9.

401. Ueno, T., and T. Kuramochi. 1961. Semi-chemical soy sauce. VII. Volatile components produced by HCl hydrolysis of defatted soybean. *Nippon Nogei Kagaku Kaishi* 35:454-8.
402. Ulrich, R., and O. Thaler. 1957. Qualitative and quantitative variations of sugars, organic acids, and amino acids content during the development of the Williams pear. *J. Agric. Trop. et Bot. Appliquee* 4(1/2):12-30.
403. Unilever NV. 1968. Method for preparing artificial flavourings for giving foodstuffs a meaty flavour. Netherlands Pat. 6,707,232.
404. Unilever Ltd. 1970. Artificial flavourings. British Pat. 1,205,882.
405. Unilever Ltd. 1971. Meat-like flavours. British Pat. 1,221,482.
406. Uruguay, Instituto Uruguayo de Normas Tecnicas. 1970. Wines: method for determination of succinic acid content. Uruguayan Standard UNIT 248-70 5 pp.
407. Van Dame, H. C. 1954. Decomposition in tomato products. Determination of acetic, formic, succinic, and lactic acids. *J. Assoc. Offic. Agr. Chemists* 37:572-4.
408. Vartiainen, E. 1966. The combination of ferrous succinate and succinic acid in the treatment of anemia during pregnancy. *Duodecim*. 82(17):842-9.
409. Vavruch, I. 1952. Chromatographic study of bee honey. *Chem. Listy* 46:116-17.
410. Veeger, C., and W. P. Zeylemaker. 1969. Determination of succinate with succinate dehydrogenase. *Methods Enzymol.* 13:524-5.
411. Vesce, C. A. 1939. Succinic acid and work in diabetic persons. *Arch. Studio Fisiopatoi. Clin. Ricambio* 7:331-50.
412. Vishwakarma, P., and W. D. Lotspeich. 1959. The excretion of l-malic acid in relation to the tricarboxylic acid cycle in the kidney. *J. Clin. Invest.* 38(2):414-423.
413. Vogt, H. 1951. The role of succinic acid in biological cycles. *Pharm. Ztg.-Nachr.* 87:402-5.
414. Von Fellenberg, T. 1922. A procedure for the determination of single acids of wine. *Mitt. Lebensmittelunters. u. Hyg.* 13:1-45.

415. Wagner, H. G., and F. A. Isherwood. 1961. Silica gel chromatography of organic acids from plant tissue. *Analyst* 86:260-6.
416. Walker, C. H. 1967. Isolation and recovery of dicarboxylic acids. *Separ. Sci.* 2(3):399-400.
417. Wall, J. S., L. C. Swange, D. Tessari, and R. J. Dimler. 1961. Organic acids of barley grain. *Cereal Chem.* 38:407-22.
418. Webb, J. L. 1950. The actions of metabolic substrates and inhibitors on the rabbit auricle. *Brit. J. Pharmacol.* 5:87-117.
419. Weil-Mahlherbe, H. 1937a. Studies on cerebral metabolism. II. Formation of succinic acid. *Biochemical J.* 31:299-312.
420. Weil-Mahlherbe, H. 1937b. Studies on cerebral metabolism. III. Dismutation of alpha-keto acids. *Biochemical J.* 31:2202-16.
421. Westcott, D. E., G. E. Livingston, W. B. Esselen, and C. R. Fellers. 1955. Non-enzymatic discoloration of green bean puree. *Food Res.* 20(2):149-159.
422. Whereat, A. F., F. E. Hull, M. W. Orishimo, and J. L. Rabinowitz. 1967. The role of succinate in the regulation of fatty acid synthesis by heart mitochondria. *J. Biol. Chem.* 242(18):4007-12.
423. Wieland, H., O. B. Claren, and B. N. Pramanik. 1933. The mechanism of the oxidation process. XXXVI. The enzymatic dehydration of lactic acid, pyroracemic acid and methyl glyoxal by yeast. *Liebigs Ann. Chem.* 507:203-12.
424. Williams, M. W., and M. E. Patterson. 1964. Nonvolatile organic acids and core breakdown of Bartlett pears. *J. Agr. Food Chem.* 12(1):80-3.
425. Williamson, J. R., and B. E. Corkey. 1969. Assays of intermediates of the citric acid cycle and related compounds of fluorometric enzymic methods. *Methods Enzymol.* 13:434-513.
426. Wohnlich, J. J. 1967. Direct paper chromatography of some alpha-keto and some di- and tricarboxylic acids. *Bull. Soc. Chim. Biol.* 49(7):900-4.
427. Woodman, J. S., A. Giddey, and R. H. Egli. 1968. Carboxylic acids of brewed coffee. *Int. Colloq. Chem. Coffee* 3:137-43.

428. Woods, M. W. 1956. The influence of succinate and 2,4-dinitrophenol on the respiratory activity of mouse cancers and normal tissues. *J. Natl. Cancer Inst.* 17:615-29.
429. Worbe, J. F., P. Mottaz, and A. Sadeghi. 1969. Effect of succinic and alpha-ketoglutaric acids on the metabolic activity of rat small intestine. *C. R. Soc. Biol.* 163(2):431-6.
430. Yamasaki, H., S. Muraoka, and K. Endo. 1960. Energy metabolic aspect of histamine release in anaphylaxis. *Japan. J. Pharmacol.* 10:21-9.
431. Yatazawa, M., and K. Ogawa. 1955. Organic acid metabolism in plants. I. Organic acid in leaf blade of paddy rice. *J. Sci. Soil Manure Japan* 25:203-6.
432. Yee, C., and H. P. Cohen. 1968. Modification of the kinetic procedure for the microassay of succinate. *Anal. Biochem.* 22:530-3.
433. Yokotsuka, T. 1954. Flavoring substances in soy sauce. XIII. The fungistatic substances in brewed soy sauce and the increase of the fungistatic nature by heating the soy sauce. *J. Agr. Chem. Soc. Japan* 28:114-18.
434. York, L. R. 1971. Microbial counts and organic acid quantitation as quality indices of egg products. *Dissertation Abstracts International, Section B, The Sciences & Engineering* 32(6)3427: Order No. 71-31338.
- \* 435. Yoshida, M., H. Ikuno, and O. Suzuki. 1971. Evaluation of available energy of aliphatic chemicals by rats: An application of bioassay of energy to mono-gastric animal. *Agr. Biol. Chem.* 35(8):1208-1215.
436. Zagrodzki, S., and A. Kurkowska. 1967. Application of anion exchange paper for separation and determination of certain acids. *Chem. Anal. (Warsaw)* 12(1):159-63.
437. Zagrodzki, S., and K. Sz wajcowska. 1966. Determination of organic acids present in beet sugar factory juices. *Zeszytyy Probl. Postepow Nauk Rolniczych* No. 62b:175-7.
438. Zaura, D. S., and J. Metcalf. 1969. Quantitation of seven tricarboxylic acid cycle and related acids in human urine by gas-liquid chromatography. *Anal. Chem.* 41(13):1761-8.
439. Zblnovsky, V., and R. H. Burris. 1954. New techniques for adding organic acids to silicic acid columns. *Anal. Chem.* 26:208-10.

440. Anon. 1970. Acidulants - the search for sours. Canadian Food Industries 41(11):42-43.
441. Anon. 1970. Methods of analysis and constituents of wines. Report of the 12th assembly of the subcommittee on the unification of methods of analysis and appreciation of wines, Paris 20-21, April 1970. Bulletin de l'Office International du Vin 43(473/74):767-98.

## On the fate of succinic acid in the human body\*.

by G. Balassa

According to the views of older authors (1), succinic acid is burned not only by normal, but also by diabetic organisms. More recently Flaschentraeger and Bernhard (2) studied the excretion of dicarbonic acid by the dog and by man. From their investigations it seems that up to C<sub>5</sub> the dicarbonic acids are not excreted, but are probably degraded. Flaschentraeger and Bernd did not find the administered succinic acid or its esters in the urine. Since succinic acid is quickly oxidized to fumaric acid in the tissues and this is converted through fumarase to an equilibrium mixture of fumaric and malic acids, there exists the possibility that the incorporated succinic acid is excreted as fumaric or malic acid. For these reasons, I investigated in self-tests the fate of succinic acid and determined in addition fumaric and malic acid in the urine. Twice I took 5 g of succinic acid as the sodium salt and tests the urine excreted in 24 hours. No succinic or malic acid could be determined in the urine. Small quantities of fumaric acid could be found in both tests and indeed in the first test 36 mg and in the second 26 mg were found, representing 0.72% and 0.52%, respectively, of the consumed quantity. The normally acid or neutral reacting urine was alkaline to litmus after sodium succinate intake, indicating that the succinic acid was burned.

## Tests

In order to be able to carry out the determination, there must be a) a method for the extraction of the substance concerned, b) an appropriate micro-method for the determination of fumaric acid.

a) The extraction of the organic acid was carried out by the Widmarkschen (3) principle - namely extraction of the acid solution with ether and removal of the organic acids in alkaline water. Fifteen ml of urine which was evaporated to 1/5 of its original volume was strongly acidized with H<sub>2</sub>SO<sub>4</sub> and put into a glass cylinder 16 cm high and 12 cm wide with a countersunk stopper (a glass for anatomical preparations). I put the lye (50% NaOH) in a 4 cm wide, 1 cm high Petri dish, which stood on a 0.5 cm high glass stand. Higher stands are unsuitable. Then I overflowed it with excess ether, such that the Petri dish was evenly covered. Possible emulsification could be prevented through the addition of several ml of alcohol. The entire apparatus was shaken on an oscillating machine (10-15° in both directions, approximately 40 periods per minute). After termination of the tests, I neutralized the lye with concentrated HNO<sub>3</sub> and carried out the fumaric acid determination. Several examples of such tests:

(1) Eingewogenes Fumarat in mg	Wiedergewonnenes Fumarat in mg	Bestimmung dauer in 24 St.	(2) Anmerkungen
15,00	14,50	6	(3) Am. d. l. W. ...
10,00	3,86	4	(4) Am. auf 1/5 eing. ... von H <sub>2</sub> O
	2,14	6	
	0,77	8	
	0,71	10	
	1,27	13	
	1,25	16	
	0,20	19	
	0,70	19	

1. Weighed fumaric acid in mg
2. Determined fumaric acid in mg
3. Extraction time in hours
4. Remarks
5. From distilled water
6. From urine evaporated to 1/5 volume

b) For the micro-determination of fumaric acid the method of von Szegedy (4) must be adapted for small quantities. The procedure was as follows: To yield a solution to be determined (neutral; volume approximately 5 ml) containing less than 5 mg fumaric acid, 2.5 ml, previously 5 ml, of 10% mercurio-nitrate solution (in 5% HNO<sub>3</sub>) was added to a centrifuge tube. This solution was mixed with 10% HNO<sub>3</sub>, the volume of which was equal to the volume of the solution to be analyzed. This solution was heated on a water bath, then the heated fumaric acid-containing solution added, and the mixture again heated on the water bath; if the separation of the precipitate took place slowly several ml of alcohol were added. The precipitate was centrifuged and stored in a refrigerator 10-12 hours. The supernatant was drained from the precipitate through a filter. The precipitate was washed twice with 3 ml of 5% HNO<sub>3</sub> and twice with an equal amount of distilled water, the liquid drained through the filter. Then 5 ml of hot n-NaCl solution (containing 10 ml of n-NaCl in a liter) was added, the shredded filter paper of the precipitate added and again heated. After cooling it was filtered into a 100 ml mounted Erlenmeyer flask with a countersunk stopper and washed 3 times with 3 ml of hot distilled water. After addition of 2 ml 0.2m KH<sub>2</sub>PO<sub>4</sub> and 0.5 ml of 0.2m Na<sub>2</sub>PO<sub>4</sub>, 1 ml of bromine solution (0.1n in n-KBr) was added from a microburette. With opening of the stopcock a stop watch was started. After 60 seconds the flask was closed. The solution was stored for exactly 2 hours in the dark, then 0.1 g KJ in 5 ml of ice-cold n-HCl was added and titrated with 0.1n or 0.05n Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>. The titer of bromine solution was determined in a blank test. Each ml of bound bromine solution corresponded to 0.0058 g fumaric acid. For example:

① Weighed Fumaric acid in mg	② Determined Fumaric acid in mg	③ Weighed malic acid in mg	④ Determined malic acid in mg	⑤ Calculated Fumaric acid in mg
4.00	4.05	5.00	10.00	4.95
4.00	4.00	5.00	10.00	5.00
4.00	4.01	1.00	2.00	1.01
4.00	4.24	1.00	2.00	0.55
4.00	4.14			
4.00	4.02			

1. Weighed fumaric acid in mg
2. Determined fumaric acid in mg
3. Weighed malic acid in mg

The essential difference from the procedure of von Szegedy lies in that I carried out the titration in buffer solution, since the pH must be accurately restrained for a micro-determination. This is supported through the following example: 10 mg of fumaric acid were titrated at various pH values. Findings were:

pH	Conc. Fumaric acid	pH	Conc. Succinic acid	pH	Conc. Fumaric acid	pH	Conc. Succinic acid
6.23	9.76	7.17	11.50	8.29	16.15	8.97	21.31
6.24	10.15	7.39	12.50	8.09	22.92	11.08	20.70
6.29	10.55	in 0.2 M phosphate buffer		in 0.2 M Borate buffer			

1. mg of fumaric acid
2. In 0.2m phosphate buffer
3. In 0.2 borate buffer

I titrated these results at pH 6.24, because here small pH changes influence the test results only a little.

The malic acid determinations were carried out according to Straub (5), the succinic acid determinations according to Moyle (6).

\* This work was supported by the Josiah Macy, Jr. Foundation.

#### Literature

1. For example, Neuberg, The Urine, 1, 276(1910).
2. This journal, 159, 297(1929); 245, 103(1937); 216, 133(1937).
3. Skand. Arch. Physiol. 48, 61.(1926); Biochem.Z. 215, 434(1929).
4. Z. anal. Chem. 108(1937).
5. This journal, 244, 117(1936).
6. Biochemic. J. 18, 351(1924).

## SUCCINIC ACID AND IRON ABSORPTION

K. Boddy and G. Will

Glasgow Royal Infirmary and The Scottish Universities' Research Reactor, 117 K. 1967

**I**n a study of oral iron uptake using solutions of 14 iron preparations Brise and Hallberg (1962a) demonstrated that there were great differences in the absorbability of different iron compounds. More iron was absorbed from a solution of ferrous succinate than from any other compound, including ferrous sulphate. The absorption of iron from tablets containing ferrous succinate was not greater than from ferrous sulphate tablets, however.

Because of these findings it was suggested that succinic ions *per se* influenced the absorption of iron and further experiments were carried out to test this hypothesis (Brise & Hallberg, 1962b). In these studies the addition of succinic acid to solutions of ferrous succinate was shown to increase iron absorption, the increase being proportional to the amount of succinic acid added. Intravenously administered succinic acid was also shown to increase the absorption of iron from ferrous sulphate solutions given orally.

In an attempt to utilise these observations in practical therapy a commercial preparation ('Ferromyn-S'—Calmic) containing ferrous succinate and succinic acid in tablet form has recently become available.

This paper describes an investigation of the effect of succinic acid on the oral uptake of iron, using  $Fe^{59}$  labelled ferrous succinate and a whole body monitor to measure iron absorption.

## MATERIALS AND METHODS

Liquid preparations of ferrous succinate were used to avoid the possible effect on iron absorption of variable integration of a pill coating. The ferrous succinate was labelled with  $Fe^{59}$  so that each dose contained 100  $\mu c$ . radioactivity.

Absorption was studied at two levels of iron dosage, 20 mg. elemental iron (20 mg. ferrous succinate) and 150 mg. elemental iron (150 mg. ferrous succinate). To one half of the doses of ferrous succinate, free succinic acid was added in amounts based on the optimum amounts (Brise & Hallberg, 1962b) so that there were in all 4 separate preparations containing: (1) 20 mg. ferrous succinate; (2) 20 mg. ferrous succinate with 15 mg. succinic acid; (3) 150 mg. ferrous succinate; and (4) 150 mg. ferrous succinate with 110 mg.

succinic acid. A small quantity of ascorbic acid was added to each dose to prevent oxidation of the ferrous ion.

Non-anaemic, asymptomatic, adult, 8 subjects were selected for the investigation.

The doses of iron were given in the early morning after an overnight fast. The iron solution was taken directly from a small beaker which was then rinsed with tap water and the rinse water also taken. The rinsing procedure was repeated so that the total volume ingested was 150 ml. No food or drink was taken for 2 hours afterwards. The percentage absorption of iron was calculated from the 4 hour and 20 day whole body  $Fe^{59}$  radioactivity counts as previously described (Will & Boddy, 1967).

Each subject was first given a solution containing ferrous succinate only and the iron uptake measured. After an interval of about 2 months the uptake study was repeated, the subject being given on the second occasion a solution containing the same quantity of ferrous succinate as before but with the appropriate amount of free succinic acid added. The two iron uptake results were then compared.

To study the effect of intravenous succinic acid on oral iron absorption a sterile solution of pH 7 containing in each millilitre 30 mg. of succinic acid and 5 mg. of sodium chloride was used. The subject was first given an oral dose of 5 mg. ferric iron labelled with 10  $\mu c$ . of  $Fe^{59}$  and the percentage iron absorption measured by whole body counting. Two months later a similar oral dose of  $Fe^{59}$  labelled iron was given but on this occasion it was preceded by the slow intravenous injection of 5 ml. of the succinic acid solution, the injection starting 5 minutes before the administration of the oral iron dose.

## RESULTS

The absorption figures of the 5 subjects who were given the 20 mg. doses of ferrous succinate are shown in Table I. In 4 subjects iron absorption was increased by the addition of succinic acid. The mean absorption from the

Table I. The effect of 15 mg. succinic acid on the absorption of 20 mg. ferrous succinate.

Case	Iron absorption (%)	
	20 mg. ferrous succinate	20 mg. ferrous succinate + 15 mg. succinic acid
1	1.0	1.2
2	0.1	8
3	0.1	3.0
4	1.1	0
5	0.1	0

doses containing ferrous succinate only was 8.5 per cent (range 3.0 to 13.1%) and the mean absorption from the doses containing 15 mg. of added succinic acid was 13.0 per cent (range 5.9 to 17.2%). Statistical analysis of the 2 sets of results using the Student *t* test gave a value for *t* of 1.82, which indicates that the differences are not statistically significant.

In 6 subjects iron absorption from solutions containing 150 mg. ferrous succinate was studied. Only 2 showed increased absorption from the doses containing the added succinic acid. In the remaining 4 the percentage iron absorption fell. The mean iron absorption from the solutions containing 150 mg. ferrous succinate only was 7.2 per cent (range 0.2 to 15.0%) and the mean absorption from the doses containing 150 mg. ferrous succinate plus 110 mg. succinic acid was 6.0 per cent (range 2.0 to 8.2%). The results are shown in Table II. The Student *t* test gave a value for *t* of -0.46 showing that no statistically significant difference was observed.

Table II. The effect of 110 mg. succinic acid on the absorption of 150 mg. ferrous succinate.

Case	Iron absorption (%)	
	150 mg. ferrous succinate	150 mg. ferrous succinate plus 110 mg. succinic acid
1	5.7	2.0
2	2.5	7.3
3	0.2	8.2
4	10.7	8.0
5	9.8	3.9
6	15.0	6.7

Table III. The effect of intravenous succinic acid on oral iron absorption.

Case	Iron absorption (%)	
	5 mg. ferric iron	150 mg. succinic acid I.V. followed orally by 5 mg. ferric iron
1	9.0	13.2
2	11.5	13.4
3	9.1	14.1
4	12.5	2.4
5	12.3	7.6
6	12.1	7.0

The results obtained from the 6-subject who took part in the intravenous succinic acid absorption test are shown in Table III. The mean absorption from the 5 mg. doses of ferric iron was 12.8 per cent (range 9.1 to 19.3%). When the oral dose of iron was preceded by the intravenous injection of 150 mg. succinic acid the subjects showing a decrease in iron absorption. The Student *t* test gave a value for *t* of -1.12, indicating that any effect was statistically insignificant.

Two patients with rheumatoid arthritis were given oral doses of 5 mg. labelled ferric iron both with and without intravenous succinic acid. In one the iron absorption remained virtually unchanged, 1.7 per cent without succinic acid and 1.9 per cent following intravenous succinic acid. The second patient had 10.7 per cent iron absorption from the 5 mg. ferric iron alone falling to 0.6 per cent when the oral dose was preceded by intravenous succinic acid.

## DISCUSSION

The investigation was designed to assess the influence of free succinic acid on the oral absorption of iron at both physiological and pharmacological dosage levels.

Five mg. was chosen as the physiological dose because this is roughly the iron content of a normal full meal (Pirzio-Biroli *et al.*, 1958), while 37 mg. was regarded as a satisfactory therapeutic dose of iron (Kerr & Davidson, 1958).

All doses were of uniform volume and were given to the fasting subject to eliminate as far as possible the influence of local gastrointestinal factors on iron absorption.

Earlier comparative studies of iron absorption have usually been based on the determination of haemoglobin regeneration in iron deficient patients during iron therapy (Fullerton, 1934; Haler, 1952; Will & Vilter, 1954). The haemoglobin regeneration rate is, however, influenced by many factors, including the severity of anaemia, state of the iron stores, continued bleeding, the presence of infection, which make interpretation of the results difficult. By using a whole body counter to obtain a direct estimate of iron absorption in non-anaemic subjects we have avoided these main sources of error.

While our results would appear to contradict those of Brise and Hallberg, attention should be drawn to the difference in the methods used to measure iron 'absorption'. The measurement of iron uptake with the whole-body monitor represents total body intake. As Brise and Hallberg emphasise, their method does not estimate total absorption but measures only the fraction of absorbed iron utilised in haemoglobin formation. In this difference may lie the apparent conflict of the findings. Brise and Hallberg's observed increase in iron utilisation could be explained by succinic acid increasing either the availability of iron to the marrow or the rate of utilisation of iron by the marrow. Their findings that intravenous succinic acid increases the utilisation of orally administered iron also suggests that the effect of succinic acid is not a local one on the gastro-intestinal tract.

The administration of a single tracer dose as in the present studies may require comparison with the 10 doses each given on successive days as reported by Brise and Hallberg. The possibility that successive doses of succinic acid may have an increasing effect cannot be excluded. Further, these authors showed that individual variations in utilisation may be about  $\pm 16$  per cent with successive doses and they estimated that with single doses the variations may be about  $\pm 35$  per cent. If similar conclusions apply to absorption, the effects of succinic acid may not be sufficiently large compared with the biological variation to be readily detected. To test these hypotheses, a new series of investigations is in progress which will involve the administration

of successive daily doses of iron and the simultaneous measurement of utilisation and absorption.

**ACKNOWLEDGEMENTS.** The excellent technical assistance of Miss P. King and Mr D. Dunn is gratefully acknowledged. Messrs. Calnic, E.L.C. Co., Ltd., generously supplied the lithium ferrous succinate and Technical Measurements Corporation, Franklin, kindly provided a 400-channel pulse height analyser. Thanks are due to Dr A. Brown, Royal Infirmary, Glasgow, and Professor H. W. Wilson, Director of the Reactor Centre, for their interest and encouragement.

#### REFERENCES

- Brise, H., Hallberg, R. (1967a). Absorbability of different iron compounds. *Acta med. scand. Suppl.*, **376**, 23.
- Brise, H., Hallberg, R. (1967b). Effect of succinic acid on iron absorption. *Acta med. scand. Suppl.*, **376**, 59.
- Fullerton, H. W. (1934). The treatment of hypochromic anaemia with soluble ferrous salts. *Edinb. med. J.*, **41**, 90.
- Haler, D. (1952). The therapeutic response of secondary anaemias to organic and inorganic iron salts. *Brit. med. J.*, **2**, 1241.
- Kerr, D. N., Davidson, L. S. P. (1958). Gastrointestinal intolerance to oral iron preparations. *Lancet*, **2**, 489.
- Pirzio-Biroli, G., Bottoelli, T. H., Flach, C.A. (1958). Iron absorption. II. The absorption of radio iron administered with a standard meal in man. *J. Lab. clin. Med.*, **51**, 37.
- Will, G., Boddy, K. (1967). Iron turnover estimated by a whole body monitor. *Scot. med. J.*, **12**, 157.
- Will, J. J., Vilter, R. W. (1954). A study of the absorption and utilisation of an iron chelate in iron deficient patients. *J. Lab. clin. Med.*, **44**, 499.

# Effects of some intermediary metabolites on the 'exogenous insulin response' of normal rabbits

H. D. BRAHMACHARI & G. RAGHUPATHY SARMA  
Biochemistry Section, Birla College, Pilani

## INTRODUCTION

Continued daily injections of sodium  $\beta$ -hydroxybutyrate in gradually increasing doses has been found to initially increase the insulin content of the pancreases of guinea-pigs and then bring about a gradual decrease of active insulin/gm. of pancreas tissue<sup>1</sup>. Similar experiments by Schwartz *et al.*<sup>2</sup> have recently confirmed these findings in both guinea-pigs and albino rats.

These findings have lent fresh support to the hypothesis put forward by Nath and Brahmachari<sup>3,4</sup> that the pathological accumulation of some intermediary metabolites, especially keto-acids like acetoacetic acid,  $\beta$ -hydroxybutyric acid, etc., may produce a continual stimulating effect on the  $\beta$ -cells of the islets of Langerhans, thus producing an initial hypertrophy of the insulin-secreting cells and increased production of insulin. This is finally followed by an eventual overwork atrophy and degeneration of the  $\beta$ -cells, accompanied by a decreased insulin secretion and consequent clinical symptoms of diabetes mellitus.

Detailed studies have, therefore, been undertaken in this laboratory to investigate the effects of various intermediary metabolites on the mechanism of insulin action. The preliminary results of the early investigations on this line have been published elsewhere<sup>5</sup>. The present communication gives the detailed results of (a) the immediate effects of injections of the metabolites alone and combined with exogenous insulin *in vivo* and *in vitro*; and (b) the effects of prolonged daily injections of some metabolites on the exogenous insulin response of normal rabbits.

## MATERIALS AND METHODS

(i) *Effects of injections of the metabolites alone and in combination with exogenous insulin 'in vivo' and 'in vitro'*—Pure sodium salts of the metabolites concerned and crystalline insulin solution (Lily) were used in the experiments on healthy normal rabbits. Five rabbits (average weight 2 kg.) were used in each case and each experiment was repeated twice as in cross-over tests in insulin assay<sup>6</sup>.

In one set of experiments sodium salts of metabolites concerned (dissolved in 5 c.c. of normal saline) were injected in fasting (18 hr) normal rabbits and the blood sugar level studied at hourly intervals for a period of 3 hr.

In the second set of experiments, the metabolites concerned were injected simultaneously but separately with two units of insulin (in normal saline) in the two legs of the rabbits to study the *in vivo* effect on the insulin response.

In a third set of experiments, the metabolites concerned were mixed *in vitro* with two units of insulin (diluted to 5 c.c. with normal saline), the mixture incubated at 37°C. for 30 min. and the clear solution injected in one leg of the rabbits. The blood sugar level was then examined as in the previous cases.

Control experiments were carried out simultaneously with each set of the above experiments to study the blood sugar response on injection of two units of insulin in 5 c.c. of normal saline alone.

The micro method of Folin and Mahros<sup>9</sup> was used in estimating blood sugar in samples of blood drawn from the marginal ear veins of the experimental rabbits. The results of these experiments are summarized in Table I.

(b) *Effects of prolonged daily injections of some metabolites on the exogenous insulin response of normal rabbits*—Four rabbits (average weight, 1.5 kg.) for each set of experiments and one set of controls (injected with 5 c.c. of normal saline only) were used. The substances studied were the sodium salts of DL- $\beta$ -hydroxybutyric acid and pyruvic acid.

Initially 1.5 units of crystalline insulin (Lily) were injected into normal healthy young rabbits and their normal insulin response calculated by the standard method of Marks (*loc. cit.*). Groups of such animals were then given daily injections of the substances concerned dissolved in 5 c.c. of normal saline in gradually increasing doses starting from 150 mg./animal/day and the insulin response to 1.5 units of insulin was studied at regular weekly intervals for five weeks to determine the effect of these injections on the response of such animals to exogenous insulin. The results are summarized in Table II.

## RESULTS

TABLE I — EFFECTS OF INJECTIONS OF METABOLITES ON THE INSULIN RESPONSE OF NORMAL RABBITS

Sl. No.	Substance injected	Average percentage reductions in blood sugar			
		2 units of insulin only	100 mg. of substance	100 mg. and 2 units of insulin <i>in vivo</i>	100 mg. and 2 units of insulin <i>in vitro</i>
1.	Insulin (control)	56.9 ± 3.4	—	—	—
2.	Sod. $\alpha$ -ketoglutarate	—	8.67 ± 1.4	62.50 ± 3.6	48.22 ± 3.1
3.	Sod. pyruvate	—	8.98 ± 1.1	66.78 ± 3.2	50.80 ± 3.4
4.	Sod. fumarate	—	9.83 ± 2.3	60.94 ± 2.8	55.21 ± 3.6
5.	Sod. $\beta$ -hydroxybutyrate	—	12.84 ± 1.5	66.75 ± 3.5	53.43 ± 2.1
6.	Sod. iso-citrate	—	17.12 ± 2.0	57.51 ± 3.3	49.00 ± 2.8
7.	Sod. succinate	—	22.54 ± 1.8	56.14 ± 3.2	46.00 ± 3.0
8.	Sod. maleate	—	27.92 ± 4.0	64.49 ± 4.8	51.41 ± 3.6
9.	Sod. citrate	—	30.34 ± 3.0	51.98 ± 3.1	40.83 ± 2.8

TABLE II — EFFECTS OF PROLONGED INJECTIONS OF  $\beta$ -HYDROXYBUTYRATE AND PYRUVATE (SODIUM SALTS) ON THE RESPONSE TO EXOGENOUS INSULIN INJECTIONS IN RABBITS

Sl. No.	Substance injected	Average response to 1.5 units of injected insulin in terms of percentage reduction of blood sugar					
		0 days	7 days	14 days	21 days	28 days	35 days
1	Control	47.22 $\pm$ 1.9	49.10 $\pm$ 1.7	46.38 $\pm$ 2.1	48.00 $\pm$ 1.7	48.42 $\pm$ 1.5	45.92 $\pm$ 2.0
2	Sod. $\beta$ -hydroxybutyrate	45.59 $\pm$ 2.1	46.94 $\pm$ 1.8	56.55 $\pm$ 1.6	49.23 $\pm$ 2.2	56.55 $\pm$ 1.4	31.12 $\pm$ 1.7
3	Sod. pyruvate	48.72 $\pm$ 2.0	52.90 $\pm$ 2.2	55.16 $\pm$ 1.5	57.44 $\pm$ 1.7	57.45 $\pm$ 1.8	58.74 $\pm$ 2.1

### DISCUSSIONS

From Table I, it can be surmised that all the metabolites when injected alone produce a hypoglycaemic response which is greatest in the case of citrate and progressively lesser in the following order:

Citrate > maleate > succinate > iso-citrate >  
 $\beta$ -hydroxybutyrate > fumarate > pyruvate >  $\alpha$ -ketoglutarate.

This effect may be due to a direct or indirect stimulation of the insulin activity produced by these substances. The results given in Table II also support this stimulation hypothesis for sodium  $\beta$ -hydroxybutyrate and sodium pyruvate. While  $\beta$ -hydroxybutyrate seems to produce an initial stimulation followed by a decrease in insulin activity within 35 days, the stimulating effect of pyruvate seems to be more prolonged since it has continued till the end of the period of observation, i.e. 35 days. These observations have corroborated the previous findings on the effects of  $\beta$ -hydroxybutyrate and pyruvate on the production of initial hypertrophy followed by an overwork atrophy of the  $\beta$ -cells of the islets of Langerhans (Nath & Brahmachari, loc. cit.).

The results obtained in the *in vivo* and *in vitro* experiments are difficult to correlate with the generalized observations given above. *In vitro* mixing of  $\alpha$ -ketoglutarate, pyruvate, iso-citrate, succinate and citrate appears to decrease the potency of exogenous insulin, while that of fumarate,  $\beta$ -hydroxybutyrate and maleate appears to have no effect on such activity.

On the other hand, the *in vivo* effect of  $\alpha$ -ketoglutarate, pyruvate, fumarate,  $\beta$ -hydroxybutyrate and maleate seems to enhance the potency of exogenous insulin, while that of iso-citrate, succinate and citrate seems to produce almost no effect.

It may be interesting to observe here that some of these metabolites, particularly sodium succinate and sodium citrate, have been reported to be successfully used in the treatment of diabetes mellitus in human beings<sup>10</sup>. This is supported by the present observations of the effects of injections of succinate and citrate alone on the reduction of the blood sugar level of normal fasting rabbits.

## SUMMARY

1. The effects of injections of some intermediary metabolites alone and in combination with insulin *in vivo* and *in vitro* have been studied in normal fasting rabbits.
2. The effects of prolonged daily injections of sodium  $\beta$ -hydroxybutyrate and sodium pyruvate on the 'exogenous insulin response' of normal fasting rabbits has also been studied.

## ACKNOWLEDGEMENT

The authors wish to thank Dr. S. M. Mitra, Principal, Birla College, Pilani, for his interest in the work and enthusiastic support.

The award of the senior Government of India scholarship to one of us (G. R. S.) is gratefully acknowledged.

## REFERENCES

1. BRAHMACHARI, H. D. & MAHENDRA KUMAR (1959). *Nature*, **183**: 51.
2. SCHWARTZ, A., *et al.* (1960). *Nature*, **185**: 771.
3. NATH, M. C. & BRAHMACHARI, H. D. (1944). *Nature*, **154**: 487.
4. *Idem* (1946). *Nature*, **159**: 336.
5. *Idem* (1948). *Nature*, **161**: 18.
6. *Idem* (1950). *Science (U.S.A.)*, **112**: 92.
7. BRAHMACHARI, H. D. & RAGHUPATHY SARMA, G. (1961). *Nature*, **191**: 491.
8. MARKS (1928). League of Nations Report on Insulin Standardization, C.H. 396.
9. FOLIN & MALMROS (1929). *J. Bio-Chem.*, **83**: 115.
10. YAVERBAUM, P. M. (1958). *Patoziologov, chita, Sbornik*, 158-161.

FROM THE DEPARTMENT OF MEDICINE II (HEAD: PROFESSOR ERIK WASSÉN), UNIVERSITY OF GÖTEBORG,  
SÄLLGRENSKA KÖRKHUSK, GÖTEBORG, SWEDEN.

## EFFECT OF SUCCINIC ACID ON IRON ABSORPTION

By

HANS BRISE AND LEIF HALLBERG

### INTRODUCTION

In a previous paper it was reported that there were great differences in absorbability of different iron compounds<sup>1</sup>. It was hypothesized that, with different iron compounds, different iron ion concentrations were obtained in the gastrointestinal tract. This hypothesis could explain the lower absorption of iron from ferric compounds and from such ferrous compounds in which a considerable part of the iron was expected to be present as complex ions in the gastrointestinal tract. However, no explanation could be given for the

observation that more iron was absorbed from a solution of ferrous succinate than from solutions of quite dissociated iron compounds as e.g. ferrous sulphate.

Because of these observations it was thought that succinic ions *per se* influenced the absorption of iron. This hypothesis was tested and turned out to be correct as shown in the present paper. The present paper also includes experiments to locate and to analyse the effect of succinic ions on the absorption of iron.

### METHODS AND MATERIAL

The same experimental design was used in the present study as previously described, employing two radioiron isotopes, and making each subject his own control<sup>2</sup>. The details of the experimental procedure and the material in different parts of the present study are described together with the results in the separate sections.

The methods for preparing solutions administered orally was the same as previously described if not otherwise stated. Determinations of  $Fe^{55}$  and  $Fe^{59}$  were also made according to a method earlier published<sup>3</sup>.

## RESULTS

The results of the present study are presented in six separate sections. The first one contains a study of the effect of different amounts of succinic acid on iron absorption. The following five sections contain experiments intended to analyze the mechanism of action of succinic acid.

### I. Effect of succinic acid orally on iron absorption

Thirty milligrams of elemental iron (as ferrous sulphate) were given in a 25 ml solution also containing 10 mg ascorbic

acid and 4 g sucrose. The solutions were given for 10 days in the morning after an overnight fast. Iron was labelled with  $^{55}\text{Fe}$  and  $^{59}\text{Fe}$  on alternate days. Every second day when iron was labelled with one of the isotopes, the solution also contained succinic acid (pro analysis, Merck, Darmstadt) in amounts from 30 to 500 mg.

This study included 81 subjects (33 healthy volunteers (N) and 48 healthy non-anaemic blood donors (BD) who had served as blood donors for varying time and who had never received any iron supplementation). It is divided into the letters F

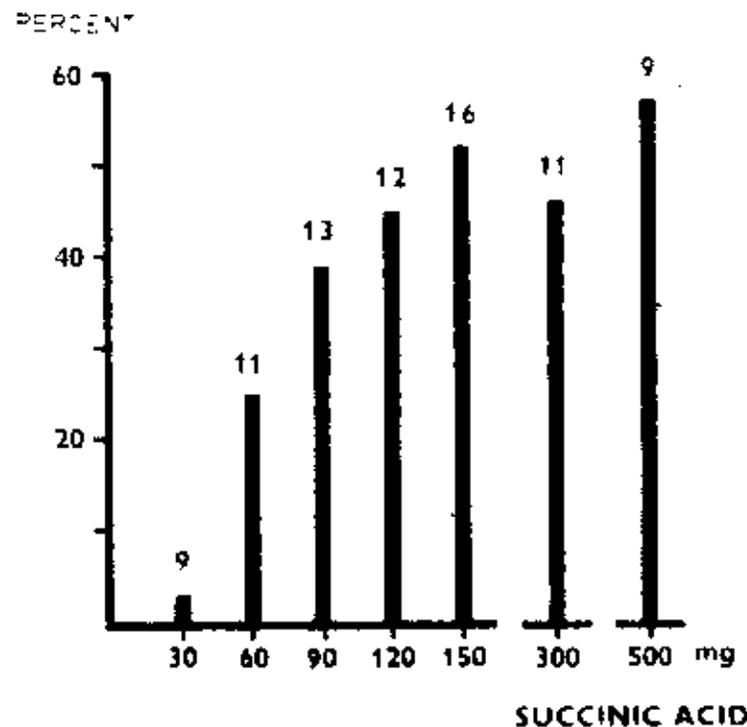


Fig. 1. Effect of succinic acid on iron absorption. The values shown are the mean values obtained from the number of subjects (n) given above the bars.

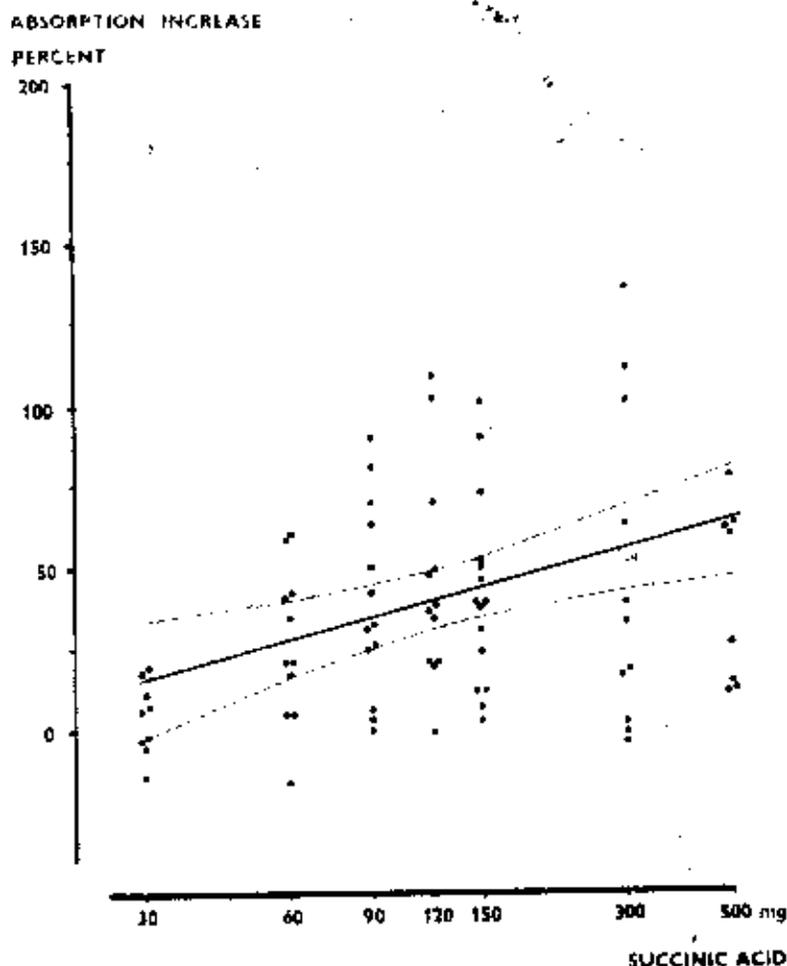


Fig. 2. Relationship between amount of succinic acid (logarithmic scale) given together with 30 mg. of iron and absorption increase.

The regression line is drawn as a solid line and the 95% confidence band for this line is marked by dashed lines. (The ordinate, absorption increase, is  $(y - B) \div 100$ ).

and M denote female and male subjects respectively. In 49 subjects the iron solutions containing succinic acid were administered with  $Fe^{59}$  and in 32 subjects with  $Fe^{55}$ . In 33 subjects the solutions containing succinic acid were given on odd days. The blood sample for analyses of  $Fe^{59}$  and

$Fe^{55}$  was drawn 2 weeks after the last oral iron dose as in previous studies<sup>2</sup>.

The results are given in table 1. The figures given as "Absorption" are not the true absorption figures as discussed in a previous paper<sup>2</sup>. The figures mean per cent of administered iron in the estimated

Age group (years)	SEX SUBJECT	ABSORPTION (percent)		ABSORPTION RATIO		Mean value and standard error of mean
		without antibiotic	with antibiotic	Individual value	with/without	
30	1-M-BD	5.2	5.0	0.96		
	2-F-BD	9.1	7.1	1.17		
	3-M-BD	7.0	7.7	1.10		
	4-M-BD	8.3	7.1	0.85		
	5-M-BD	10.7	11.1	1.05		1.05 ± 0.14
	6-M-BD	15.0	16.6	1.10		
	7-M-N	18.0	17.0	0.94		
	8-M-N	18.0	21.2	1.18		
	9-M-BD	27.3	26.3	0.96		
	10-M-N	5.1	6.1	1.04		
60	11-M-N	6.1	4.1	0.67		
	12-M-BD	7.9	9.1	1.16		
	13-M-BD	12.5	14.8	1.20		
	14-M-BD	13.8	13.5	1.00		
	15-M-BD	18.0	23.8	1.32		1.27 ± 0.07
	16-M-BD	19.7	22.8	1.15		
	17-M-BD	19.8	31.3	1.58		
	18-M-BD	21.0	32.0	1.52		
	19-M-BD	23.1	27.0	1.17		
	20-M-BD	25.2	41.3	1.63		
90	21-M-N	3.2	4.2	1.31		
	22-M-BD	3.5	8.2	2.34		
	23-M-N	5.1	6.7	1.31		
	24-M-N	5.8	3.5	0.60		
	25-M-BD	7.1	9.8	1.39		
	26-M-BD	7.8	12.6	1.62		
	27-M-BD	10.0	36.0	3.60		1.78 ± 0.08
	28-M-BD	10.2	12.0	1.18		
	29-M-BD	11.0	18.7	1.69		
	30-M-BD	11.9	21.6	1.81		
120	31-M-BD	13.9	19.0	1.36		
	32-M-BD	15.0	23.0	1.53		
	33-M-BD	26.8	27.3	1.02		
	34-M-N	2.5	4.7	1.88		
	35-M-N	5.0	7.3	1.46		
	36-M-N	5.1	10.7	2.09		
	37-M-N	6.1	7.2	1.19		1.43 ± 0.10
	38-M-BD	8.0	10.8	1.35		
	39-M-BD	8.0	11.7	1.47		
	40-M-BD	8.0	8.0	1.00		

Table 1 (Continued)

ABSORPTION<sup>a</sup>  
(per cent)

ABSORPTION RATIO  
with/without succinic acid

Percentage of  
succinic acid  
from

SUBJECT

without

with

Individual  
value

Mean value  
and  
standard error  
of mean

succinic acid

120

41-M-BD  
42-M-BD  
43-F-BD  
44-M-BD  
45-M-BD

8.5  
15.6  
21.6  
22.7  
28.2

17.1  
18.5  
29.7  
26.5  
37.6

2.04  
1.19  
1.37  
1.20  
1.33

150

46-M-N  
47-M-BD  
48-F-N  
49-M-BD  
50-M-BD  
51-M-BD  
52-M-BD  
53-M-BD  
54-M-BD  
55-M-BD  
56-M-BD  
57-M-BD  
58-M-BD  
59-M-BD  
60-M-BD  
61-M-BD

2.2  
3.4  
4.6  
5.4  
6.3  
7.7  
8.7  
8.5  
13.0  
16.8  
18.8  
19.6  
21.7  
25.8  
28.2

7.2  
6.4  
4.9  
9.4  
8.9  
15.3  
12.9  
9.0  
11.5  
19.7  
23.2  
19.2  
25.9  
31.4  
33.4  
31.2

3.28  
1.89  
1.06  
1.72  
1.37  
2.09  
1.49  
1.11  
1.38  
1.51  
1.38  
1.02  
1.23  
1.45  
1.30  
1.11

1.52 ± 0.13

300

62-M-BD  
63-M-BD  
64-M-BD  
65-M-BD  
66-M-BD  
67-M-BD  
68-M-BD  
69-M-BD  
70-M-BD  
71-M-BD  
72-M-BD

4.6  
5.7  
5.9  
9.0  
9.8  
11.3  
13.5  
15.2  
15.8  
16.1  
24.6

9.3  
9.3  
8.4  
21.1  
20.6  
11.7  
17.5  
17.7  
15.5  
18.9  
23.5

2.00  
1.62  
1.43  
2.35  
2.10  
1.04  
1.32  
1.16  
0.98  
1.17  
0.95

1.66 ± 0.15

500

73-M-BD  
74-M-BD  
75-M-BD  
76-M-BD  
77-M-BD  
78-M-BD  
79-M-BD  
80-M-BD  
81-M-BD

5.6  
9.7  
10.3  
10.9  
11.4  
11.8  
12.7  
20.7  
26.8

17.2  
16.8  
16.7  
12.1  
10.7  
18.8  
14.6  
27.8  
26.1

2.91  
1.61  
1.62  
1.11  
1.11  
1.59  
1.16  
1.35  
1.25

1.57 ± 0.19

red cell mass 2 weeks after the last oral iron dose. The systematic errors affecting these figures did not influence the absorption ratio figures. The figures for "Absorption" are only given to characterize the subjects and to facilitate comparisons between individuals.

A significant effect of succinic acid was observed when 60 mg or more were added to the solutions. The mean values graphed in figure 1 indicate that with increasing amounts of succinic acid more iron was absorbed.

The following functional relationship was found within the domain studied:

$$y = 0.4 + 0.2x - 0.00$$

where

y was the absorption ratio, and  
x was the dose of succinic acid in milligrams.

The regression coefficient 0.4 was statistically significant, different from zero ( $t = 3.16$ , of 7%). The test standard deviation was 0.40 and the correlation coefficient  $r$  was 0.30.

The observed data and the regression line are shown in figure 2.

## II. Effect of succinic acid intravenously on iron absorption

In an attempt to locate the effect of succinic acid on iron absorption the acid was given intravenously, instead of orally, together with the oral iron doses on the alternate days.

In 5 normal subjects a ferrous sulphate solution containing 30 mg of elemental iron, labelled with  $Fe^{55}$  and  $Fe^{59}$  on alternate days was given orally for 10 days as

TABLE II  
*Iron absorption from 30 mg of iron as ferrous sulphate with and without 150 mg of succinic acid administered intravenously*

SUBJECT	ABSORPTION (per cent)		ABSORP. RATIO with orally succinic acid
	with succinic acid	without succinic acid	
S2-F-N	3.1	4.7	0.66
S3-F-N	4.7	7	1.34
S4-F-N	11.1	5.4	1.97
S5-M-N	11.1	18.5	1.66
S6-M-N	13.5	18.7	1.37
Mean value 1.42			

described in section I. On alternate days, when the iron was labelled with one of the isotopes, 150 mg of succinic acid was given slowly intravenously, starting 5 minutes before the administration of the oral iron dose.

The pH of the solution given intravenously, was adjusted to 7 with sodium hydroxide and the solution contained 30 mg succinic acid and 6 mg sodium chloride per ml.

The results are shown in table II. In 4 of the 5 subjects a significantly higher absorption of iron was observed when succinic acid was administered intravenously.

## III. Effect of succinic acid orally on iron turnover

It has been observed that there is a close relationship between iron absorption and iron turnover. Any factor increasing the

turnover of iron, e.g. increased erythropoiesis, may not increase the absorption of iron.

In order to be able to analyze further the effect of succinic acid on iron absorption, it is thus necessary to know its effect on iron turnover.

In two healthy volunteers a tracer dose of  $^{59}\text{Fe}$  ( $3-4 \mu\text{Ci } ^{59}\text{Fe}$ ) was given intravenously after an overnight fast. The details of the method were the same as those described by HALLBERG and SÖLVESTRÖM. Blood samples were drawn at intervals of about 15 minutes. After one hour 200 mg of succinic acid was given orally in a 25 ml solution.

The results were identical in both subjects. As shown in figure 3 there was no effect on the iron turnover rate. Four plasma iron determinations were made during the study in each individual. Since there was no change in the plasma iron level during the study, it can be concluded that oral administration of succinic acid does not influence plasma iron turnover.

#### IV. Effect of some related organic acids on iron absorption

In theory one possible mechanism of action of orally administered succinic acid on iron absorption might be a buffering action on the gastrointestinal content. With this in mind the effects of some related acids were studied. Most of the acids studied were among those which are integral parts of intracellular metabolic processes. The reason for this selection will be discussed later.

The general experimental design was the same as in previous sections. A solution of ferrous sulphate containing 30 mg of elemental iron, labelled with  $^{59}\text{Fe}$  and  $^{55}\text{Fe}$  on alternate days, was given every morning for 10 days. On alternate days, when the iron was labelled with one of the isotopes one millimol of acid was given in the solution (e.g. 146 mg  $\alpha$ -ketoglutaric acid).

Thirtyfive subjects were included in this study. Twenty of these subjects were blood donors.

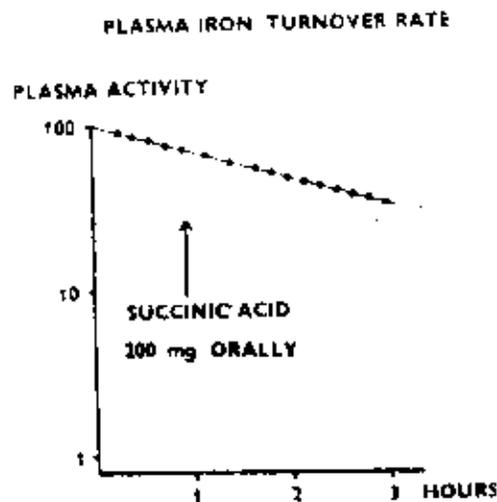


Fig. 3. Plasma iron turnover rate, before and after 200 mg of succinic acid orally.

TABLE III

*Iron absorption from 50 mg of iron as ferrous sulphate with and without various organic acids in amounts of one millimol.*

ORGANIC ACID	SUBJECT	"ABSORPTION" (per cent)		ABSORPTION RATIO	
		without	with	with	without
		organic acid		organic acid	organic acid
				Individual value	Mean value
α-Ketoglutaric acid (145 mg)	95-M-BD	5.7	6.0	1.06	1.11
	96-M-BD	16.7	11.2	1.50	
	97-F-BD	12.6	13.6	1.07	
	98-F-BD	19.1	21.3	1.12	
	99-F-BD	24.1	30.1	1.25	
Fumaric acid (116 mg)	100-M-N	3.5	3.7	1.06	1.07
	101-M-BD	4.0	5.5	1.39	
	102-M-N	4.5	5.1	1.17	
	103-M-BD	8.5	7.8	1.09	
	104-M-BD	7.2	7.6	1.06	
	105-M-N	8.0	7.6	0.94	
	106-M-BD	19.4	17.7	0.91	
	107-M-BD	22.8	24.7	1.07	
	108-F-BD	22.7	23.5	1.03	
109-M-BD	23.7	26.3	1.10		
L-Malic acid (134 mg)	111-M-N	4.5	4.3	0.95	0.89
	112-M-N	7.0	7.0	1.00	
	113-M-N	7.4	5.8	0.77	
D-Malic acid (134 mg)	114-M-N	2.7	1.8	0.66	0.78
	115-F-N	7.0	3.7	0.52	
	116-M-BD	13.5	16.0	1.18	
DL-Isocitric acid (102 mg)	117-M-N	7.0	6.7	0.96	0.87
	118-F-N	7.3	6.3	0.86	
	119-F-N	9.4	7.6	0.80	
Oxalacetic acid (132 mg)	120-M-N	3.1	2.0	0.64	0.80
	121-M-BD	29.6	27.4	0.92	
	122-M-BD	31.0	28.0	0.89	
	123-M-BD	34.9	31.3	0.89	
Citric acid (102 mg)	124-M-BD	40.3	36.0	0.89	0.82
	125-M-N	4.1	1.9	0.46	
	126-F-N	13.2	8.7	0.65	
	127-F-N	18.1	10.1	0.55	
	128-M-BD	25.2	20.3	0.81	
129-M-BD	35.7	27.5	0.76		

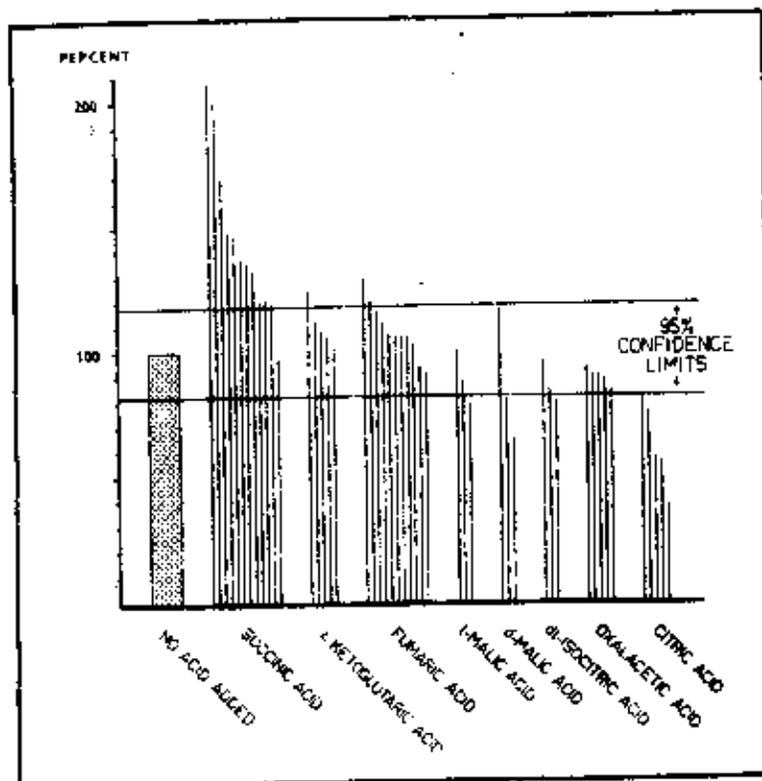


Fig. 4. Effect of some organic acids on iron absorption. Each acid is given in an amount of one millimol.

The results are given in table III and graphed in figure 4.

It is evident from table III that none of the acids studied promoted the absorption of iron. On the contrary administration of citric acid reduced the absorption of iron probably because complex ferrous-citrate ions were formed.

The difference between these acids and succinic acid (one millimol being about 120 mg) is clearly shown in figure 4. The 95 per cent confidence limits of individual values drawn in the figure were obtained in a previous study.<sup>2</sup>

#### V. Effect of succinic acid on intestinal motility

Changes in intestinal motility may change the absorption from the gastrointestinal tract. Because of this the effect of succinic acid was tested on intestinal motility.

This investigation was carried out with a method devised by Kewenter and Koek.<sup>3</sup> (For details see reference).

Three patients without current gastrointestinal disorders were investigated, one of them had been subjected to a Bilroth II resection some years previously.

A soft plastic catheter was introduced through the nose into the alimentary tract in the afternoon. After an overnight fast the position of the catheter tip was determined by roentgen examination to be situated about 10 cm below pylorus. The patient was lying horizontally on the back during the intestinal motility examination, and the basal activity of the intestine was recorded for 30 minutes. A solution of 150 mg of succinic acid, dissolved in 10 ml of water, was then administered through the catheter. The intestinal activity was then recorded for 15 minutes.

No change in intestinal motility was observed in any of the three subjects investigated after administration of the succinic acid.<sup>1)</sup>

<sup>1)</sup> Thanks are due to Doctors JAN KEWENTER and NILS G. KECK of the Department of Surgery I, University of Göteborg, who performed the motility tests.

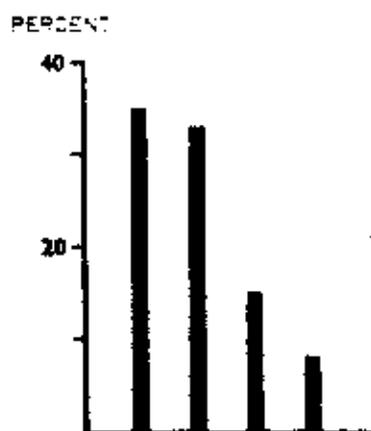


Fig. 5. Independent effect of succinic and ascorbic acid on iron absorption. Effect of ascorbic acid (200 mg) in the presence of succinic acid (150 mg).

## VI. Independent effect of succinic and ascorbic acids

In a previous paper it was reported that the addition of sufficient amounts of ascorbic acid to oral iron doses significantly increased the absorption of iron. It was concluded that this effect was mainly related to the reducing action of ascorbic acid in the gastrointestinal lumen since no effect on iron absorption was observed when the acid was given intravenously.

The probable site of action of L-succinic acid is within the mucosal cells as will be discussed later. Reasoning from the above conclusion regarding the different sites and mechanisms of action of succinic and ascorbic acids, it was thought that the acids may potentiate each other's effects.

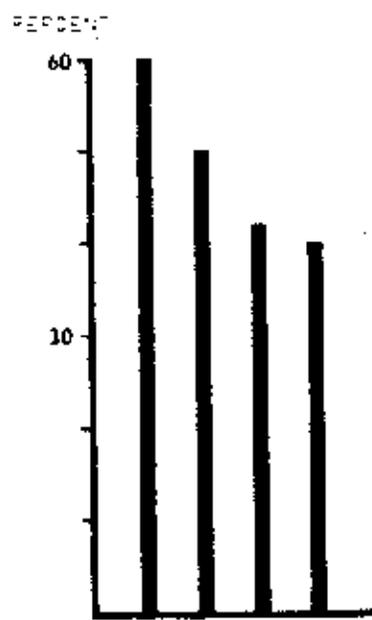


Fig. 6. Independent effect of succinic and ascorbic acid on iron absorption. Effect of succinic acid (150 mg) in the presence of ascorbic acid (100 mg).

TABLE IV

Iron absorption from 30 mg of iron as ferrous sulphate and 150 mg succinic acid with and without 200 mg of ascorbic acid.

SUBJECT	"ABSORPTION" (per cent)		ABSORP. TION RATIO with/without ascorbic acid
	without	with	
	ascorbic acid		
87-M-BD	12.1	16.4	1.35
88-M-BD	21.7	28.7	1.33
89-M-BD	24.4	26.4	1.09
90-M-BD	33.1	38.1	1.15
Mean value: 1.23			

This suggestion was tested using the same experimental design as employed in previous sections.

In the first study 30 mg of elemental iron (as ferrous sulphate) was given together with 150 mg of succinic acid in a solution every morning for 10 days. This amount of succinic acid was found to have an optimal absorption promoting effect. (Section I in the present paper.) On alternate days, when the iron was labelled with one of the isotopes, 200 mg of ascorbic acid was also given. In this way the effect of ascorbic acid could be tested when the absorption of iron was probably optimally increased by succinic acid. The results are shown in table IV and fig. 5.

TABLE V

Iron absorption from 30 mg of iron as ferrous sulphate and 200 mg ascorbic acid with and without 150 mg of succinic acid.

SUBJECT	"ABSORPTION" (per cent)		ABSORP. TION RATIO with/without succinic acid
	without	with	
	succinic acid		
91-M-BD	18.4	24.2	1.32
92-M-BD	22.0	32.0	1.50
93-M-BD	26.0	43.0	1.60
94-M-BD	30.3	39.4	1.30
Mean value: 1.43			

More iron was absorbed in all four subjects, when ascorbic acid was given. However, a statistically significant increase was obtained only in two of the four subjects.

In the second study the iron was given together with 200 mg of ascorbic acid on all 10 days. On alternate days 150 mg of succinic acid were given.

The results are given in table V and figure 6. A significant increase in the absorption of iron was observed in all four subjects. This increase was of the same magnitude as that observed when succinic acid alone was given as an absorption promoter (section I).

## DISCUSSION

The starting point for the present study was the unexplained observation that more iron was absorbed from a solution of ferrous succinate than from a solution of ferrous sulphate. The results in the first section of the present paper showed that the addition of succinic acid to a solution of ferrous sulphate increased the absorption of iron. These results indicate that succinic ions *per se* promote the absorption of iron, because the increase was related to the amount of succinic acid added, even when more than equivalent amounts (in relation to ferrous iron) were given. The practical importance of this marked promoting effect of succinic acid on iron absorption was not studied in the present paper. However, preliminary studies indicate that the use of succinic acid in oral iron preparations may have great practical advantages in iron therapy<sup>7</sup>.

In contradistinction to solutions, no increased absorption of iron was observed from tablets containing ferrous succinate in relation to tablets containing ferrous sulphate as reported in a previous paper<sup>8</sup>. As shown in table VI the solubility and rate of dissolution of ferrous succinate are considerably less than those of ferrous sulphate.

At the lowest pH usually existing in the stomach (ca. pH 1), both the solubility and the rate of dissolution of ferrous succinate are less than one tenth of the corresponding values for ferrous sulphate. At the higher pH of the upper part of the small intestine (pH ca. 5-6) the corresponding ratios are only 1:20. Table VI shows the amounts (in mg of elemental iron per ml) of ferrous succinate and ferrous sulphate which have gone into solution after different times of shaking 40 g ferrous sul-

TABLE VI

Rate of dissolution of ferrous sulphate and ferrous succinate at different pH levels expressed as a percentage of the amount which dissolved in water. See text.

IRON COMPOUND	Time (minutes)	pH		
		1 (0.1 N HCl)	5.5 (1.20 M bicarbonates-HCl buffers)	7 (water)
Ferrous sulphate (311.0)	2	0.054	0.057	0.053
	5	0.063	0.057	0.058
	10	0.092	0.071	0.095
Ferrous succinate (311.0)	2	0.001	0.002	0.002
	5	0.005	0.004	0.004
	10	0.008	0.004	0.004
	20	0.009	0.004	0.004

phate ( $7H_2O$ ) and 20 g ferrous succinate ( $3H_2O$ ) respectively in 50 ml solvent at  $37^\circ C$  at various pH levels.

Because the conditions for the absorption of iron are more favourable in the upper part of the gastrointestinal tract (e.g. lower pH, greater area for absorption), the time factor will be of importance for the absorption of iron. The time for the disintegration of the tablets and the dissolution of the iron compound can thus be expected to influence the amount of iron absorbed. The great difference in rate of dissolution of ferrous succinate and ferrous sulphate will thus be a probable explanation for the absence of the expected increased absorption of iron from ferrous succinate tablets.

The main part of the present paper consisted of studies designed to analyze the mechanism of action of succinic acid on iron absorption.

Roughly, the absorption of iron will be increased:

- (a) if the concentration of iron ions in the gastrointestinal lumen is increased (or more exactly if the product of the concentration and area of absorption is increased).
- (b) if the transfer of iron across the mucosal cells is stimulated or
- (c) if the elimination of absorbed iron from plasma to other sites of the body is enhanced.

The first alternative explanation for the action of succinic acid — increasing the concentration of iron ions in the gastrointestinal tract — was studied in various ways.

The absorption promoting effect of

succinic acid can not be a simple acid effect, because other acids related to succinic acid did not increase the absorption of iron (section IV). Neither is it probable that succinic acid acts as a reducing agent in the gastrointestinal lumen, inasmuch as the effect of succinic acid was not less when almost optimal amounts of ascorbic acid as a reducing agent were also given together with the iron (section VI).

The result in section II that the absorption of iron was increased also when succinic acid was administered intravenously indicate that the absorption promoting effect of succinic acid cannot be due to an action on the gastrointestinal content (the iron ion concentration). When given intravenously the concentration of succinic acid in the gastrointestinal content must be much lower than when the acid is given orally. In section I it was found that no effect of succinic acid was observed when doses lower than 60 mg were given orally. Only 150 mg succinic acid was injected intravenously and an absorption promoting effect was evident in most cases. An increased area of absorption is not a probable explanation for the effect of succinic acid, because no change in intestinal motility was observed as a result of oral administration of 150 mg succinic acid (section V). It can thus be concluded, that succinic acid does not exert its action through an increased iron ion concentration in the gastrointestinal content or through distribution of iron over a greater gastrointestinal surface.

The two main remaining alternatives attempting to explain the promoting effect of succinic acid on iron absorption are therefore (a) an increased transfer

across the mucosal cells and (3) an increased elimination of iron from plasma. The latter was studied in section III, and no effect of succinic acid on iron turnover could be observed.

By a process of elimination the probable explanation for the action of succinic acid on iron absorption, will then be a direct stimulation of the transfer of iron through the mucosal cells.

This interpretation of the present data fits in with recent observations suggesting that the absorption of iron is an active process dependent upon oxidative metabolism and the generation of phosphate-bound energy<sup>8</sup>.

Succinic acid is an integral part of the citric acid cycle. Addition of succinic acid can thus be expected to increase the energy available for the intracellular mucosal transfer of iron. However, the observation in section IV that other acids comprised in the Krebs' cycle did not

increase the absorption of iron, makes it probable that succinic acid exerts its action in some other step in the intracellular metabolism. Succinic acid is for instance also linked with the cytochrome system. There may also be other steps in which the amount (or concentration) of succinic acid has a determining influence on the rate of cellular metabolism. However, a further analysis necessitates the use of other methods than those employed in the present investigation.

The fact that succinic acid increases the active transport of iron across the mucosal cells suggests that the absorption of other substances may also be increased by succinic acid. It is also possible that succinic acid is a rate limiting factor for the active transfer of some substances through other cells besides those of the gastrointestinal mucosa because the energy metabolism of all cells in the body follows the same general pathways.

## SUMMARY

Succinic acid was found to increase the absorption of iron. The increase was related to the amount of succinic acid added to the oral iron dose. (81 subjects).

The mechanism of action of succinic acid on iron absorption was studied in a series of experiments in 48 subjects.

It was concluded that the promoting effect on iron absorption was due to a direct action on the transfer of iron across the mucosal cells, for the following reasons:

(1) succinic acid did not affect iron turnover or intestinal motility;

(2) intravenously administered succinic acid also increased the absorption of iron;

(3) other organic acids related to succinic acid (when given in equivalent amounts -- one millimol) did not increase iron absorption.

It is suggested that succinic acid exerts its action by increasing the intracellular mucosal metabolism. It is possible that the absorption of other substances may also be promoted by succinic acid.

## REFERENCES

1. BRISE, H., and HALLBERG, L.: Absorbability of different iron compounds. *Acta Med. Scand. Suppl.* 376, 171:23-37, 1962.
2. BRISE, H., and HALLBERG, L.: A method for comparative studies of iron absorption in man using two radioiron isotopes. *Acta Med. Scand. Suppl.* 376, 171:7-22, 1962.
3. HALLBERG, L., and BRISE, H.: Determination of  $Fe^{55}$  and  $Fe^{59}$  in blood. *Int. J. Appl. Rad. Isotopes* 9:109-105, 1960.
4. BOTSWELL, T.H., PIRAZIO-BIBOLI, G., and FINCH, C.A.: Iron absorption. I. Factors influencing absorption. *J. Lab. & Clin. Med.* 51:24-36, 1958.
5. HALLBERG, L., and SÖLVELL, L.: Absorption of a single dose of iron in man. *Acta Med. Scand. Suppl.* 358:19-42, 1960.
6. HALLBERG, L., and SÖLVELL, L.: Determination of the absorption rate of iron in man. *Acta Med. Scand. Suppl.* 353:3-17, 1960.
7. HALLBERG, L., and BRISE, H.: Förök med ny oral järnterapi. *Nord. Med.* In press.
8. KIEWENTER, J., and KOCK, N.G.: Motility of the human small intestine. A method for continuous recording of intraluminal pressure variations. *Acta Chir. Scand.* 119:430-438, 1960.
9. DOWDLE, E.B., SCHACHTER, D., and SCHENKER, H.: Active transport of  $Fe^{59}$  in everted segments of rat duodenum. *Am. J. Physiol.* 195:600-613, 1960.

## INJECTIONS OF CERTAIN PLANT GROWTH SUBSTANCES IN RATS AND CHICK EMBRYOS

W. S. DYE, JR., M. D. OVERHOLSER, AND C. G. VINSON

*Departments of Anatomy and Horticulture, University of Missouri, Columbia, Missouri*  
(Received for publication on May 31, 1943)

### A. INTRODUCTION

The purpose of the present experiment was to study the effects of certain plant growth substances on animals. Studies were made on growth effects and estrogenic properties of these substances.

It was shown by Boysen-Jensen (1911) and Paal (1919) that by placing the cut tip of the plant stem asymmetrically on the stump the growth of the lower zones was accelerated by a substance coming from the tip. They also demonstrated that this substance was a diffusible agent since it could be transmitted across gelatin. Substances (auxin *a* and *b*) having similar properties as the active principle in the coleoptile tips were isolated and crystallized from urine by Kögl and Haagen-Smit in 1931.

Kögl, *et al* (1934) discovered that indole-acetic acid has the property of promoting growth in stems and illustrates lack of specificity as to chemical structure and related activities in plants. Thimann (1935) showed that indole-acetic acid was produced by microorganisms, as *Rhizopus suinis*, in a peptone medium rich in tryptophane.

Many compounds have been investigated and are summarized by Zimmerman and Wilcoxon (1935), Zimmerman and Hitchcock (1937), and Went and Thimann (1937). Indole-propionic acid's activity was studied by Hitchcock (1935) and found to have the same ability as indole-3-acetic acid in producing bending, proliferation, and rooting responses in plants. In the case of naphthalene-acetamide and naphthalene-acetic acid, Mitchell and Stewart (1939) found the former was associated with secondary thickening, and the latter with cellular proliferation. Maleic acid, a dicarboxylic acid, was found to be active in inducing renewed cell division and cell extension in certain plants (English, Bonner, and Haagen-Smit, 1939).

In order to study the relationships between plant growth substances and their effects on organisms, many methods of attack have been employed.

Ball (1938) studied the effects of 3-indole-acetic acid on *Escherichia coli* and found that cell division of the organism was more than doubled in the media containing the chemical as compared with the controls. Continuing these studies on bacteria, Beckwith and Geary (1940) found that indole-3-acetic acid not only increased the growth of *Escherichia coli*, but also *Eberthella typhosa*. Working with protozoa (*Euglena gracilis*), Elliott (1938) observed a marked acceleration of growth using 3-indole-acetic acid, 3-indole-propionic acid and 3-indole-butyric acid. Certain other species were inhibited, however. Du Pan, *et al* (1935) found that hormones of orchid pollen introduced under the ectoderm of certain Batrachian embryos "during the blastula stage," accelerated cell division.

Recently, Narat and Chobot (1939) have contributed very important observations on the effects of certain plant growth substances on general growth, as well as local cell growth in rats. They used potassium naphthalene acetate and potassium indole butyrate. Growth in rats was not accelerated with injections of either chemical. The effect on local cell growth was pronounced in that both chemicals shortened the healing time of artificially produced burns 35 per cent.

The effects of plant growth substances on tumors have also been investigated. Robinson and Taylor (1941) found that indole-3-acetic acid inhibited normal respiration of both sarcomas and carcinomas in mice, being effective in the undissociated acid form. The effects of potassium alpha-naphthalene acetate on mouse and rat carcinoma were studied by Suminari (1941). He observed inhibition of these carcinomas using this compound. Kögl, Haagen-Smit, and Tonnies (1933) have shown, however, that certain indole compounds occur in carcinomatous tissue in concentrations twice that of normal tissue.

Another interesting aspect of the relation of plant growth substances to animal physiology is the enhancement of the effect of pituitary gonadotropic hormone by certain plant growth hormones as observed by Breneman (1939). He found that the effectiveness of pituitary gonadotropic extract was increased in the immature rat after admixture of indole-butyric acid and alpha-naphthalene-acetic

acid. The action of the plant growth hormone when given separately was questionable.

## B. MATERIALS AND METHODS

The experimental work was divided into three series. The substances tested in each series were as follows: (a) Alpha-naphthalene-acetic acid, (b) Amide of alpha-naphthalene-acetic acid, (c) Beta-indole-acetic acid, (d) Beta-indole-propionic acid, (e) Maleic acid, and (f) Succinic acid.

### 1. Series I

This series consisted of 180 rats, 90 being injected and 90 being controls. Each litter was divided into two groups, one group being injected, the other serving as a control. The animals were weighed and measured at the age of 7 days, and thereafter once a week up to the age of 56 days, then on the 60th day. The time of hair appearance, tooth eruption, eye opening, and vaginal opening were recorded for each rat. Weights were made accurately to 0.1 gm. The rats were measured by vernier calipers, measuring from the tip of the nose to the base of the tail. Variations possible with this method of measuring were noted to be in the range of 3 mm. Tooth eruption was recorded as the time of appearance of an incisor.

Six groups of 15 rats each were injected with each of the substances listed. Each rat was started at 7 days of age on a dosage of 0.5 mgm. (in sesame oil) and injected subcutaneously each day. This dosage was increased until they were receiving 2.0 mgm. daily by four weeks of age, and was given daily until the rats reached 60 days of age. The control rats received sesame oil only. Preliminary work using larger doses of 5.0 and 10.0 mgm. over a period of three weeks or more produced toxic symptoms in the younger rats. Some of the rats died and others showed retardation of growth and patchy distribution of hair.

### 2. Series II

The total number of rats used was 40, 30 being injected and 10 being controls. These rats were all females and approximately two months old. This series was used to test possible estrogenic properties of the six substances listed.

Daily vaginal smears were made on the 40 rats for a period of two

weeks. At the end of the two weeks, 30 of the rats were ovariectomized. A post-operative period of seven days was allowed to elapse before injections were started. Vaginal smears were continued to confirm the expected diestrus smear following ovariectomy.

Six groups of five spayed rats each were injected subcutaneously with 5.0 mgm. daily of each of the six substances (in sesame oil) for three weeks. Daily vaginal smears were made. At the end of the three weeks injection period, all 40 animals were sacrificed and microscopic sections were made of the uterine horn, cervix, and vagina.

### 3. *Series III*

This series consisted of 480 chick eggs, 240 being injected, and 240 serving as controls. Fertile eggs were obtained from local hatcheries and incubated in an electric incubator at a temperature between 100-105° F. Solutions for injections were prepared by dissolving 1.0 mgm. of each of the substances per 1.0 cc. of phosphate buffer solution (pH 7.4). The resultant pH was 7.0 approximately. These solutions were sterilized by boiling. Injection was carried out on the 10th day of incubation as follows: A hole was made over the air sac by means of a small electric drill, and then with a sterile tuberculin syringe and needle, 0.05 cc. of the solution was injected into the air sac, the hole then being sealed with cellulose tape. Six groups of 40 each were injected on the 10th day of incubation with each of the substances used. Phosphate buffer solution alone was injected into 120 eggs on the same day of incubation. The other 120 eggs were left intact. Weights and observations on development were recorded at hatching time.

## C. RESULTS

### 1. *Series I—Rats*

The average weight, length, and rate of development of the injected rats compared closely to those for the controls (Tables 1, 2, and 3), and showed no significant variations. In regard to the rate of development, there were no significant differences between injected and control animals, except possibly in the opening of the vagina in the animals injected with succinic acid (Table 3). The average age of opening in the injected animals was 57.2 days as compared to 49.4

TABLE 1  
THE EFFECT OF ALPHA-NAPHTHALENE-ACETIC ACID (SOL. 1), AMIDE OF ALPHA-NAPHTHALENE-ACETIC ACID (SOL. 2), BETA-INDOLE-ACETIC ACID (SOL. 3), BETA-INDOLE-PROPIONIC ACID (SOL. 4), MALEIC ACID (SOL. 5), AND SUCCINIC ACID (SOL. 6) ON THE BODY WEIGHT OF RATS

Average body wt. in gms. Under each average wt. the range in wts is given. ( ) = No. rats. Injections—daily from 7-60 days of age. Dosage—increased from 0.5 mgm. to 2.0 mgm. during the first 4 weeks of treatment.

Age (days)	Controls	Sol. 1	Sol. 2	Sol. 3	Sol. 4	Sol. 5	Sol. 6
7	9.8 (90) 7.0-13.6	10.9 (15) 10.4-11.3	7.2 (15) 7.0-7.3	11.5 (15) 10.0-12.3	9.0 (15) 7.2-11.2	7.5 (15) 7.0-8.5	10.6 (15) 10.1-11.4
14	19.9 (88) 11.8-25.3	20.6 (14) 20.3-21.4	17.4 (15) 16.9-18.0	22.5 (15) 20.3-23.6	15.9 (15) 11.8-24.0	19.5 (15) 18.0-24.0	20.4 (15) 19.0-22.4
21	25.9 (88) 16-38.5	31.3 (14) 30.5-31.1	27.5 (14) 26.2-29.0	33.1 (15) 30.0-35.5	21.0 (14) 13.0-37.6	32.9 (15) 27.5-36.0	33.2 (15) 30.0-36.4
28	45.5 (86) 32.8-59.6	47.9 (14) 44.2-49.6	40.7 (13) 39.0-44.1	51.9 (15) 47.8-43.6	34.6 (13) 29.3-40.1	45.0 (15) 41.2-47.0	48.1 (15) 45.4-51.0
35	71.7 (86) 45.7-85.3	66.9 (14) 58.4-71.9	59.0 (13) 56.7-62.6	71.8 (15) 66.2-74.1	65.3 (13) 52.5-78.1	60.0 (15) 56.0-65.0	65.2 (15) 63.0-67.0
42	83.4 (86) 66.5-100.0	91.2 (14) 81.3-97.3	73.3 (13) 71.0-75.0	78.7 (15) 72.3-82.3	72.4 (13) 68.1-74.0	77.0 (14) 72.2-83.1	85.2 (14) 68.2-90.1
49	98.3 (86) 79.8-115	108.9 (14) 97.2-115.2	83.8 (13) 85.2-93.1	100.2 (15) 90.9-110.8	89.4 (13) 82-96.4	80.0 (14) 73.4-85.0	99.2 (14) 92.1-106
60	116.5 (86) 90.5-136.3	123.4 (14) 104.8-133.5	112.8 (13) 106.4-116.5	121.3 (15) 111.0-135.0	113.4 (13) 102-119	101.5 (14) 98.4-105	114.1 (14) 102-120

TABLE 2  
THE EFFECT OF ALPHA-NAPHTHALENE-ACETIC ACID (SOL. 1), AMIDE OF ALPHA-NAPHTHALENE-ACETIC ACID (SOL. 2), BETA-INDOLE-ACETIC ACID (SOL. 3), BETA-INDOLE-PROPIONIC ACID (SOL. 4), MALIC ACID (SOL. 5), AND SUCCINIC ACID (SOL. 6) ON THE BODY LENGTH OF RATS

Average body length in mm. Under each average length the range in lengths is given. ( ) = No. rats. Injections—daily from 7-60 days of age. Dosage—increased from 0.5 m.m. to 2.0 m.m. during the first 4 weeks of treatment.

Age (days)	Controls	Sol. 1	Sol. 2	Sol. 3	Sol. 4	Sol. 5	Sol. 6
7	60.9 (90) 56.0-70.3	61.0 (15) 60-64	53.0 (15) 52.5-53.3	62.6 (15) 60.0-64.4	61.3 (15) 56-78	58.0 (15) 56-61	59.0 (15) 55.1-65
14	80.0 (88) 66.0-93.0	79.3 (14) 78.3-80	72.2 (15) 70-74	80.7 (15) 78-82	79.2 (15) 58-78	77.8 (15) 74-81	78.2 (15) 76.1-82
21	97.2 (88) 81-109	101.2 (14) 100.5-101.6	93.6 (14) 89-97	101 (15) 94-105	83.0 (14) 68-105	98 (15) 91-106.1	99.1 (15) 92-107
28	119.1 (86) 99-130	125.3 (14) 120-129	112.0 (13) 111-113	118-(15) 113-123	100.0 (13) 90-110	112.0 (15) 107-115	119.0 (15) 105-124
35	136.2 (86) 116-148	130.6 (14) 125-135	125.0 (13) 128-127	131.7 (15) 126-134	127.0 (13) 119-135	120.0 (15) 114-126	128.1 (15) 122-134
42	142.2 (86) 132-153	144.3 (14) 142-146	133.6 (13) 132-135	139.5 (15) 135-143	134.1 (13) 129-141	136.0 (14) 130-141.2	141.0 (14) 133-144
49	149 (86) 139-161	162.3 (14) 144-157	145.3 (13) 145-146	150.2 (15) 146-155	144.2 (13) 139-152	140.0 (14) 133-145	148.1 (14) 141-154
60	160.1 (86) 142-172	165 (14) 153-170	155.0 (13) 150-168	156.7 (15) 146-165	156.0 (13) 144-161	155.0 (14) 150-163	156.0 (14) 151-159

TABLE 3  
THE EFFECT OF ALPHA-NAPHTHALENE-ACETIC ACID (SOL. 1), AMIDE OF ALPHA-NAPHTHALENE-ACETIC ACID (SOL. 2), BETA-INDOLE-ACETIC ACID (SOL. 3), BETA-INDOLE-PROPIONIC ACID (SOL. 4), MALEIC ACID (SOL. 5), AND SUCCINIC ACID (SOL. 6) ON THE RATE OF DEVELOPMENT OF RATS

( ) = No. of rats. Injections and dosage same as Table 1 and Table 2.

	Hair appeared	Teeth erupted	Eyes opened	Vagina opened
Controls	4 days (90)	9 to 10 days (90)	17 days (90)	49.4 days (av.) 45-56 (range) (40)
Sol. 1	6 days (14)	9 to 10 days (14)	17 days (14)	50.1 days (av.) 46-57 (range) (6)
Sol. 2	6 days (13)	9 to 10 days (13)	17 days (13)	48.4 days (av.) 47-58 (range) (7)
Sol. 3	6 days (13)	9 to 10 days (13)	17 days (13)	54.1 days (av.) 49-58 (range) (7)
Sol. 4	6 days (13)	9 to 10 days (13)	17 days (13)	51.2 days (av.) 48-54 (range) (8)
Sol. 5	6 days (14)	9 to 10 days (14)	17 days (14)	52.0 days (av.) 47-55 (range) (6)
Sol. 6	6 days (14)	9 to 10 days (14)	17 days (14)	57.2 days (av.) 50-61 (range) (4)

for the controls. However, only four animals were used in the injected group.

### 2. Series II--Rats

Daily vaginal smears showed no change from the diestrus smear of ovariectomized rats as compared to the typical 4-day cyclic changes in the vaginal smears of the controls. Microscopic sections of the uterine horn, cervix, and vagina of each of the rats showed no significant changes. In the ovariectomized animals, the vaginal epithelium was atrophic and the walls and lumen were infiltrated with leucocytes. This is in contrast to a control rat killed while in estrus. Here the vaginal epithelium was greatly thickened and cornified. The uterus of the ovariectomized rat was small, anemic, contained atrophied glands, and its wall was infiltrated with leucocytes. The uterus of the rat in estrus was hyperemic, increased in size, and its glands were hypertrophic and hyperplastic.

TABLE 4

THE EFFECT OF ALPHA-NAPHTHALENE-ACETIC ACID (SOL. 1), AMIDE OF ALPHA-NAPHTHALENE-ACETIC ACID (SOL. 2) BETA-INDOLE-ACETIC ACID (SOL. 3), BETA-INDOLE-PROPIONIC ACID (SOL. 4), MALEIC ACID (SOL. 5), AND SUCCINIC ACID (SOL. 6) ON THE BODY WEIGHT OF CHICK EMBRYOS

Dosage = 0.05 cc. of a 1:1000 solution (in phosphate buffer solution) injected into the air sac on the 10th day of incubation. Average body wt. at hatching in gms. Under each average wt. the range in wts. is given. ( ) = No. of chicks.

Controls	Buffer Sol. only	Sol. 1	Sol. 2	Sol. 3	Sol. 4	Sol. 5	Sol. 6
42.3 (120)	44.3 (120)	40.2 (40)	43.5 (40)	40.9 (40)	43.0 (40)	44.0 (40)	39.3 (40)
38.0-44.2	41.6-47.0	38-41.2	40-46.2	35.5-44	37-44.9	41.2-46	36-41.2

### 3. Series III—Chick Eggs

The average weights of the chicks from the injected eggs compared closely with the controls with the possible exception of the chicks from the eggs injected with solution No. 6—succinic acid (Table 4). Here the average weight of the chicks was 39.5 gms. as compared with 42.3 gms. for the intact controls, and 44.3 gms. for the controls injected with phosphate buffer solution alone. No abnormalities of development were noted at hatching time. One-fourth of the chicks were saved, and all developed without incident to maturity.

### D. CONCLUSIONS

Alpha-naphthalene-acetic acid, amide of alpha-naphthalene-acetic acid, beta-indole-acetic acid, beta-indole-propionic acid, maleic acid, and succinic acid produced no significant effects on growth and rate of development in albino rats and chick embryos, nor did they exhibit any estrogenic properties in the doses used.

### E. SUMMARY

1. The effects of six plant growth substances on growth and development were studied in 180 albino rats. Ninety of the rats were injected (6 groups of 15 each) with the substances. The other 90 served as controls.
2. The rats were injected with a maximum of 2.0 mg. daily of each of the substances, and all were weighed and measured each week. No significant differences in the rate of growth and development between the injected and control animals were found.
3. Injections of 5.0 mgm. daily for three weeks into 30 ovariectomized rats did not change their typical diestrus vaginal smear.
4. Injections of 0.05 cc. of a 1:1000 solution of each of the six substances were made into 240 chick eggs on the 10th day of incubation. Controls consisted of 120 intact eggs and 120 eggs injected with phosphate buffer solution. No significant differences in weight or rate of development between the chicks of the injected and control eggs were noted at hatching.

## REFERENCES

1. BAAL, E. 1935. Heteroauxin (3-indole-acetic acid) and growth of *Escherichia coli*. *J. Bact.*, **20**, 559-568.
  2. BECKWITH, T. D., & SEARY, E. M. 1940. Effect of indole-3-acetic acid on multiplication of *Escherichia coli* and *Eberthella typhosa*. *J. Infect. Dis.*, **66**, 78-79.
  3. BÉTHÉLENOT, A. 1936. Sur la présence d'Acide Indol-3-acétique dans le Sang. *Compt. Rend. Soc. Biol.*, **128**, 847-849.
  4. BÖYSEN-JENSEN, P. 1919. Über die Leitung des phototropischen Reizes in Avena-Kolbenpflanzen. *Ber. der Deut. Botan. Gesell.*, **28**, 118-120.
  5. BRUNSMAN, W. B. 1936. Augmentation of pituitary gonadotropic hormone by chlorophyll, plant growth hormones, and hemin. *Endocrinology*, **24**, 484-494.
  6. DASTOORIAN, M., & VENKATESAN, T. R. 1941. Amide synthesis in plants: I. Substrates used in plants. *Proc. Indian Acad. Sci.*, **12B**, 345-369. (Cited from *Chem. Abstr.*, **36**, 8021.)
  7. DE PAZ, H. M., & RAMAKRISHNAN, M. 1935. Recherches sur l'influence des hormones de cytoplasmes végétales sur le développement du germe des Extraxens. *Compt. Rend. Soc. Biol.*, **128**, 1236.
  8. ELASSOFF, A. M. 1938. The influence of certain plant hormones on the growth of protozoa. *Phys. Zool.*, **11**, 31-39.
  9. ENGLISH, J. JR., BOEKER, J., & HAAGEN-SMIT, A. J. 1939. Wound hormones: IV. Structure and synthesis of a traumatin. *J. Amer. Chem. Soc.*, **61**, 3434-3436.
  10. HITCHCOCK, A. E. 1935. Indole-3-n-Propionic acid as a growth hormone and the quantitative measurement of plant response. *Contrib. Boyce Thompson Inst.*, **7**, 87-95.
  11. KÖHL, F., ERKLESEN, H., & HAAGEN-SMIT, A. J. 1934. Über die Isolierung der Auxine a und b aus pflanzlichen Materialien: IX. Mitteilung. *Z. f. Physiol. Chem.*, **228**, 215-229.
  12. KÖHL, F., & HAAGEN-SMIT, A. J. 1931. Über die Chemie des Wuchsstoffe. *Proc. Kon. Akad. Amsterdam*, **36**, 1411-1416. (Cited from *Chem. Abstr.*, **26**, 2755.)
  13. KÖHL, F., HAAGEN-SMIT, A. J., & ERKLESEN, H. 1934. Über ein neues Auxin "Hetero-auxin" aus Harn. *Z. f. Phytol. Chem.*, **228**, 90-103.
  14. KÖHL, F., HAAGEN-SMIT, A. J., & TÖNNIS, B. 1933. Über das Vorkommen von Auxinen und von Wuchstrostoffen der Bios-Gruppe in Carcinomen. *Z. f. Physiol. Chem.*, **220**, 162-172.
  15. MIRENKA, J. W., & STEWART, W. S. 1939. Comparison of growth responses induced in plants by naphthalene-acetamide and naphthalene-acetic acid. *Botan. Gaz.*, **52**, 416-427.
  16. McNAIR, J. B. 1936. The sequence and climatic distribution of some plant acids. *Am. J. Bot.*, **23**, 629-634.
  17. NARAY, J. K., & CHUDOV, G. 1939. Studies on the growth stimulating effect of potassium naphthalene acetate and potassium indole butyrate. *Surg., Gynec., & Obs.*, **68**, 63-66.
  18. PAAL, A. 1919. Über phototropische Reizleitung. *Jahrb. Wiss. Bot.*, **58**, 406-458. (Cited by Went and Thimann, 1937.)
  19. ROBINSON, T. W., & TAYLOR, A. B. 1941. The effect of indole-3-acetic acid on tumor respiration. *Am. J. Physiol.*, **133**, 429.
  20. SUMINARI, T. 1941. The influence of potassium alpha-naphthalene acetate on the growth of the mouse and rat carcinoma. *Mitt. med. Akad. Kyoto*, **32**, 176-188. (Cited from *Chem. Abstr.*, **36**, 7019.)
- W. S. DYE, JR., M. D. OVERHOLSER, AND C. G. VINSON
21. THIMANN, K. V. 1935. The plant growth hormone produced by *Rhizopus stolonis*. *J. Biol. Chem.*, **100**, 279-291.
  22. WENT, F. W., & THIMANN, K. V. 1937. *Phytohormones*. New York: Macmillan. (Pp. 17, 137-140.)
  23. ZIMMERMAN, P. W., & HITCHCOCK, A. R. 1937. Comparative effectiveness of acids, esters, and salts, as growth substances and methods of evaluating them. *Contrib. Boyce Thompson Inst.*, **9**, 337.
  24. ZIMMERMAN, P. W., & WILCOXON, P. 1935. Several chemical growth substances which cause initiation of roots and other responses in plants. *Contrib. Boyce Thompson Inst.*, **7**, 209-229.

EFFECT OF 1,3-BUTANEDIOL AND OTHER BUTYRIC ACID CONGENERS  
ON BRAIN CATECHOLAMINES

G.L. Gessa, P.F. Spano, L. Vargin, F. Crabai, A. Tagliamontè  
and L. Nannelli

Institute of Pharmacology, University of Cagliari (Italy)

(Received 15 September 1967; in final form 22 December 1967)

Gamma-hydroxybutyrate ( $\gamma$ -OH) endogenous metabolite mammalian brain (1) possesses sedative, hypnotic and anesthetic properties (2), therefore it has been introduced in clinic as a depressant and anesthetic agent (3). We have shown that  $\gamma$ -OH induces a selective increase in brain dopamine (DA) in different animal species (4).

After injecting  $\gamma$ -OH, DA rises faster than and reaches higher levels than after the administration of monoamineoxidase inhibitors (MAOI). DA accumulation is temporally correlated with the anesthetic action of this compound. The mechanism by which  $\gamma$ -OH produces its effect on brain DA is not yet clarified: it does not depend on an inhibition either of MAO or of catechol-O-methyl transferase (5). Finally, we have demonstrated that the effect of  $\gamma$ -OH on brain DA is not mediated through its intermediate,  $\gamma$ -butyrolactone ( $\gamma$ -BL) (6).

In the experiments reported in this paper, we have screened a large number of butyric acid congeners in hopes of clarifying the relationships of chemical structure, anaesthetic property and capacity to modify DA.

The compounds studied were:

- a)  $\alpha$ ;  $\beta$  and  $\gamma$ -hydroxybutyrate;  $\alpha$ -hydroxy-isobutyrate;
- b) di-hydroxy alcohol congeners of  $\gamma$ -OH: 1,3-butanediol (1,3 BD) and 1,4-butanediol (1,4 BD).

- c) dicarboxylic acids: succinate and maleic acid;
- d) lactone congeners of  $\gamma$ -OH:  $\gamma$ -butyrolactone ( $\gamma$ -BL);  $\gamma$ -butyrolactone- $\gamma$ -carboxylic acid ( $\gamma$ -BL- $\gamma$ -carboxylic a.);  $\alpha$ -acetyl- $\alpha$ -methyl- $\gamma$ -butyrolactone;
- e) aminated and aminated-hydroxylated derivatives of butyric a.:  $\alpha$ ,  $\beta$  and  $\gamma$ -aminobutyric a.;  $\gamma$ -amino- $\beta$ -hydroxybutyric a.

We have found that all compounds endowed with sedative properties, and only those endowed with such action, raise selectively brain DA.

#### Material and methods.

Animals. We used male Long-Evans rats weighing 180 to 220 g, and rabbits of our colony weighing 1.5 to 2.5 Kg. The various drugs used were injected as indicated under the results. At various intervals after treatments the animals were sacrificed: rats by decapitation and rabbits with i.v. injection of air. The brains were removed for the fluorometric analysis of catecholamines and serotonin (5HT) or for enzyme activity determinations.

Amine assay. Brains from two animals were homogenized with 4 vol. of cold 0.4 N HCl; the homogenates centrifuged at 9,000 x g for 30'; 2 ml of the supernatant were used for the catecholamine analysis and 4 ml for the determination of 5HT. This was measured by the method of Snyder et Al. (7), modified as follows: 4 ml of the perchloric phase were alkalinized to pH 10 by the addition of 1.5 ml of N NaOH and 3 ml of 0.5 M borate buffer pH 10. 5HT was extracted with the butanol extraction described by Bondarsky et Al. (8) and 1 ml of the final acidic phase was neutralized with 1 ml of 0.1 M phosphate buffer pH 7.4, followed by the ninhydrin reaction as described by Snyder et Al. (7). Catecholamines were determined by the method described by Brodie et Al. (9).

Enzyme activity. MAO activity was measured with the method of Lovenberg et Al. (10) when Tryptamine was used as a substrate; when instead DA was used, RAD and nicotinamide were omitted and

of the incubating media.

### Results

Each compound listed in table 1 was injected i.p. into rats up to the maximum tolerated dose (i.e. a dose that kills 10% of the animals in 24 hours). The behavioural effects were scored and the effect on brain amines was checked. As expected the following compounds produced anaesthetic effect:  $\gamma$ -OH (2),  $\gamma$ -BL (11), 1,4-BD (12). Moreover, succinic a.,  $\gamma$ -BL- $\gamma$ -carboxylic a. and  $\alpha$ -OH-butyric a. produced sedation. All other compounds tested gave no sedative response.

It appears that those compounds endowed with sedative and/or anaesthetic action are also capable of increasing selectively brain DA; they are in decreasing order of potency:  $\gamma$ -OH, 1,4-BD,  $\gamma$ -BL, succinic a.,  $\gamma$ -BL- $\gamma$ -carboxylic a. and  $\alpha$ -OH-butyric a.

NA and 5HT levels are much less influenced. No effect on brain amines was noted with any of the other compounds lacking sedative action.

1,4 butanediol was studied in some detail because it possesses anaesthetic properties very similar to  $\gamma$ -OH (12) and influences brain DA to a similar degree. With the following series of experiments we have tried to establish:

- a) whether the sedative action of 1,4 BD is correlated to the change in brain DA;
- b) whether the increase in this amine is confined to the areas of the extrapyramidal system, where it is normally contained;
- c) the mechanism by which 1,4 BD produces its effect.

Subsequently, twenty rats were given 1 g/kg of 1,4-BD intraperitoneally. At various intervals 4 of these rats were sacrificed and their brain amines analysed.

TABLE 1

Influence on brain amines and on behaviour of different butyric acid congeners, in rats.

Compound	mg/Kg i.p.	Brain amines $\mu\text{g/g}$			Observations
		DA	NA	5HT	
-	-	1.18 $\pm$ 0.21	0.42 $\pm$ 0.03	0.63 $\pm$ 0.09	-
$\gamma$ -OH	1700	1.80 $\pm$ 0.19	0.64 $\pm$ 0.11	0.71 $\pm$ 0.12	Sleep
$\alpha$ -OH-butyric a.	2000	1.35 $\pm$ 0.23	0.47 $\pm$ 0.04	0.60 $\pm$ 0.13	Sedation
$\beta$ -OH-butyric a.	4000	1.20 $\pm$ 0.20	0.48 $\pm$ 0.07	0.57 $\pm$ 0.08	-
$\alpha$ -OH-isobutyric a.	500	1.18 $\pm$ 0.11	0.38 $\pm$ 0.11	0.49 $\pm$ 0.11	-
1,4-BD	1000	1.84 $\pm$ 0.18	0.55 $\pm$ 0.09	0.73 $\pm$ 0.11	Sleep
1,4-BD	750	1.50 $\pm$ 0.09	0.50 $\pm$ 0.11	0.74 $\pm$ 0.08	"
1,4-BD	500	1.47 $\pm$ 0.12	0.47 $\pm$ 0.08	0.79 $\pm$ 0.03	"
1,3-BD	2000	1.20 $\pm$ 0.12	0.47 $\pm$ 0.09	0.70 $\pm$ 0.15	-
Succinate	4000	1.51 $\pm$ 0.18	0.42 $\pm$ 0.11	0.59 $\pm$ 0.13	Sedation
Maleic a.	750	1.09 $\pm$ 0.16	0.40 $\pm$ 0.13	0.71 $\pm$ 0.11	-
$\gamma$ -BL	500	1.58 $\pm$ 0.08	0.57 $\pm$ 0.07	0.70 $\pm$ 0.13	Sleep
$\gamma$ -BL- $\gamma$ -carboxylic a.	500	1.40 $\pm$ 0.18	0.48 $\pm$ 0.12	0.68 $\pm$ 0.07	Sedation
$\alpha$ -acetyl- $\alpha$ -methyl- $\gamma$ -BL	1000	1.25 $\pm$ 0.11	0.46 $\pm$ 0.03	0.59 $\pm$ 0.08	-
$\alpha$ -NH <sub>2</sub> -butyric a.	2000	1.07 $\pm$ 0.16	0.46 $\pm$ 0.05	0.57 $\pm$ 0.11	-
$\beta$ -NH <sub>2</sub> -butyric a.	2000	1.20 $\pm$ 0.09	0.42 $\pm$ 0.05	0.63 $\pm$ 0.09	-
$\delta$ -NH <sub>2</sub> -butyric a.	2000	0.99 $\pm$ 0.31	0.38 $\pm$ 0.14	0.60 $\pm$ 0.03	-
$\delta$ -NH <sub>2</sub> - $\beta$ -OH-butyric a.	2000	0.98 $\pm$ 0.12	0.35 $\pm$ 0.18	0.55 $\pm$ 0.13	-

Of each compound a time-response curve was made. For brevity, in the table the data obtained 2 hours after treatment are reported. This time coincided roughly with the maximal effect on brain amines and behaviour.

The data presented in table 2 shows a direct correlation between the degree of central activity and the increase of brain DA produced by 1,4 BD.

The animals lost their righting reflex at about 30' after treatment when brain DA rose to about 13% above the normal. The animals awoke approximately 6-8 hours afterwards when brain DA had declined to about 9% above the normal levels.

TABLE 2

Time-response curve of 1,4-BD effect on brain DA, in rats.

1,4-BD 1 g/kg i.p. given at the fol- lowing times be- fore sacrifice	Brain catecholamines µg/g		Observations
	DA	NA	
0	1.16±0.21	0.42±0.03	
15'	1.18±0.12	0.42±0.08	sedated
30'	1.35±0.11	0.53±0.09	sedated-sleep loss r.r.
60'	1.44±0.21 §	0.55±0.06	sleep loss r.r. *
2 h	1.60±0.30 §	0.52±0.11	" " "
3 h	1.76±0.21 §	0.61±0.12	" " "
8 h	1.29±0.21	0.50±0.09	normals

§  $p < 0.01$  in respect to controls.

\* r.r. = righting reflex.

Experiments in rabbits. This species was more convenient than rats for studying not only the problem of localization of the newly formed DA, but also that of the temporal correlation between the DA accumulation and the sedative effect of 1,4-BD.

Four groups of four rabbits each were injected very slowly i.v. with 1, 2, 3 and 4 grams/kg of 1,4-butanediol dissolved in distilled H<sub>2</sub>O 5 ml/kg.

Two of the rabbits injected with the highest dose had convulsions and one died in 2 min. The other three survived. The

other animals displayed no observable behavioural changes for 1 h 30' to 2 h 20'. Afterwards, they exhibited sedation (following 1  $\mu$ /Kg) or sleep with loss of righting reflex (following 2,3 and 4  $\mu$ /Kg).

The delay between the injection and the sedative effect is much longer in rabbits than in rats; in both species it is shorter for the higher doses. Rabbits tolerate much higher doses than rats.

TABLE 3

Dose-response curve of 1,4-BD effect on brain DA, in rabbits. The compound was injected 4 hours before sacrifice.

1,4-BD $\mu$ /Kg i.v.	Brain catecholamines $\mu$ g/g			
	Caudate DA	Putamen-Pallidum DA	Brain-stem NA	5HT
-	8.10 $\pm$ 0.91	1.11 $\pm$ 0.11	0.46 $\pm$ 0.01	0.60 $\pm$ 0.03
0.6	11.2 $\pm$ 1.21 §	1.20 $\pm$ 0.15 §	0.50 $\pm$ 0.09	0.58 $\pm$ 0.06
1.2	12.50 $\pm$ 1.11*	1.30 $\pm$ 0.23*	0.51 $\pm$ 0.11	0.63 $\pm$ 0.09
2	14.64 $\pm$ 2.16*	1.41 $\pm$ 0.41*	0.59 $\pm$ 0.13	0.68 $\pm$ 0.08
3	14.08 $\pm$ 1.16*	1.49 $\pm$ 0.38*	0.41 $\pm$ 0.11	0.71 $\pm$ 0.13
4	15.00 $\pm$ 2.04*	1.39 $\pm$ 0.14*	0.47 $\pm$ 0.21	0.70 $\pm$ 0.23

§ p = 0.05 in respect to controls.

\* p < 0.01 in respect to controls.

The fluorescence readings for the cerebellum and the cortex of the treated animals did not differ from readings of controls. The same applies to the serotonin values of the limbic system.

As shown in table 3, 1,4-BD also produced in rabbits a selective, dose-dependent, increase in brain DA. This was localized in the striatum and in the pallidum.

Presently, there are insufficient data to reach a definite conclusion, however the results reported in table 4 indicate that the increase of DA parallels the anaesthetic effect of 1,4-BD.

TABLE 4

Time-response curve of 1,4-BD effect on brain DA, in rabbits.

1,4-BD 2 g/kg i.v. at the following intervals before sacrifice	Caudate nucleus DA $\mu\text{g/g}$	Observations
0	8.10 $\pm$ 0.91	-
30'	8.67 $\pm$ 1.12	normals
1 h 30'	9.34 $\pm$ 1.04 §	sedation
5 h	14.84 $\pm$ 1.11 *	sleep loss r.r.
15 h	6.94 $\pm$ 2.21	normals

§ p = 0.05 in respect to controls.

\* p < 0.01 in respect to controls.

In fact, DA rises to about 15% above the normal in one and a half hours, when the animals are deeply sedated; reaches a peak of 90 % above the normal in 4-5 hours when the animals have lost their righting reflex and remains at that level for the rest of the time the animals are anaesthetized. The DA levels decline toward the normal at the 8<sup>th</sup> - 10<sup>th</sup> hour, when the animals are awake.

Although it was improbable that a selective increase in brain DA unaccompanied by a parallel increase in serotonin and norepinephrine could result from MAO inhibition, nevertheless, the effect of 1,4-BD on brain monoamineoxidase activity both in vivo and in vitro was checked.

The results of these experiments showed that 1,4-BD,

added in vitro does not inhibit the enzymatic deamination either of tryptamine or of DA. Moreover, brain-homogenates of rats treated with 1,4-BD deaminate tryptamine and DA to the same degree as those of control rats.

#### Discussion.

We found that all butyric acid derivatives endowed with depressant action on the CNS, and only those endowed with such action, are able to increase selectively brain DA. In fact, besides  $\gamma$ -OH (4) and  $\gamma$ -BL (6), other butyric acid congeners were found to increase selectively brain DA: 1,4-BD, and to a lesser extent,  $\alpha$ -OH-butyric acid,  $\gamma$ -BL- $\gamma$ -carboxylic acid and succinate. Therefore, there is a remarkable correlation between the sedative effect and DA accumulation produced by these compounds. However, whether the two events are causally related remains to be demonstrated.

From the point of view of the structure activity relationship our results indicate that the molecular structures which regulate the sedative activity are also responsible for the capacity of these compounds to modify brain DA.

The results with 1,4-BD are of particular interest. This compound produces sleep after a latency period longer than that following  $\gamma$ -OH: in rats after about 30' in rabbits after several hours. The latency at the onset of the pharmacological and biochemical effects after 1,4-BD strongly suggests that the effects of this compound are mediated through an active metabolite and that a different speed in biotransformations is present in different animal species.

The data provided by the time-course response add further evidence that the increase in brain DA and the pharmacological effects produced by 1,4-BD are directly related; it is difficult to reconcile these findings to what is known about the central actions of DA (see 13).

The experiments performed up to now do permit us to clarify the mechanism of action of 1,4-BD. This compound raises the same problems encountered with  $\gamma$ -OH (5).

In fact, theoretically it is difficult to conceive: an accumulation of brain DA: a) without a parallel increase of serotonin and NE, b) accompanied by sedation instead of CNS stimulation. This problem becomes more puzzling because 1,4-BD does not inhibit MAO.

The depression of CNS could be explained by assuming that  $\gamma$ -OH blocks dopamine - sensitive structures of the brain preventing the endogenous DA from acting on its specific receptors. The increased concentrations of DA in the brain might originate from a compensatory activation of the specific neurons, secondary to the functional deficiency of DA.

We are currently carrying out a study of DA metabolites after  $\gamma$ -OH and 1,4-BD in order to shed some light on this puzzling problem.

#### Summary

Many butyric acid congeners were screened for their behavioural effect and for their influence on brain amines. Among the compounds examined, all butyric acid derivatives endowed with depressant action on the CNS, and only those endowed with such action, were capable of increasing selectively brain DA. Brain NE and serotonin were not influenced. Besides  $\gamma$ -OH and  $\gamma$ -BL, the following compounds were found to be active: 1,4-BD,  $\alpha$ -OH-butyric acid,  $\gamma$ -BL- $\gamma$ -carboxylic acid and succinate.

1,4-BD was studied in detail in rats and in rabbits. The effect of this compound shows close similarities to that of  $\gamma$ -OH. There is a direct correlation between the degree of sedation produced by this compound and the accumulation of DA in the brain. This is particularly evident in rabbits: the anaesthetic effect occurs after a delay of about two hours, and the

rise in brain DA. The DA levels return to normal when the animals are awake.

The newly-formed DA is localized in the pallidum and in the striatum. The mechanism by which the above compounds, and specifically 1,4-BD, produce the change in brain DA is not clear: 1,4-ED, like  $\gamma$ -OH, does not inhibit MAO either in vivo or in vitro.

The fact that 1,4-BD produces its effect after a long delay suggests that it acts through an active metabolite.

Acknowledgement -  $\gamma$ -OH was supplied by Farmosmerica, Roma.

This work was supported by a C.N.R. Grant No. 115.1545.0. 1207.

#### References

1. S.P. BESSMAN and W.N. FISHBEIN, *Nature* **200**, 1207 (1963).
2. H. LABORIT, J.M. JOUANY, J. GERARD and F. FABIANI, *Agresseologie* **1**, 397 (1960).
3. H. LABORIT, A. KIND and G. DE L. REGIL, *Presse Méd.* **60**, 1216 (1961).
4. G.L. GESSA, L. VARGIU, F. CRABAI, G.C. BOERO, F. CABONI, R. CANBA, *Life Sciences* **5**, 1921 (1966).
5. G.L. GESSA, F. CRABAI, L. VARGIU and P.F. SPANO, *J. Neurochemistry*, in press.
6. G.L. GESSA, L. VARGIU, F. CRABAI, G. BEZZI and R. CANBA, *Boll. Soc. It. Biol. Sper.* **43**, 287 (1967).
7. S.H. SNYDER, J. AXELROD, W. ZWEIG, *Biochem. Pharm.* **14**, 831 (1965).
8. D.F. BOGDANSKY, A. PLETSCHER, B.B. BRODIE and S. UDENFRIED, *J. Pharm. Exp. Therap.* **117**, 82 (1956).
9. B.B. BRODIE, A. DLABACK and E. COSTA, *J. Pharm. Exp. Therap.* **152**, 340 (1966).
10. W. LOVENBERG, R.J. LEVINE and A. SJOERDSTRA, *J. Pharm. Exp. Therap.* **135**, 7 (1962).
11. S.P. BESSMAN and S.L. SKOLNIK, *Science* **143**, 1045 (1964).
12. H. SPRINCE, J.A. JOSEPHS, Jr. and C.R. WILPIZESKI, *Life Sciences* **5**, 2041 (1966).
13. O. HORNYKIEWICZ, *Pharm. Rev.* **18**, 925 (1966).

From the Private Research Institute of Prague H/1482  
(Director: Dr. Siegwart Hermann, Privat-Dozent of the  
German University in Prague)

EFFECT AND FATE OF ACIDS IN THE ORGANISM

Part I.: Determination of the Hydrogen Ion Concentration  
in Circulating Rabbit Blood after Intravenous  
Injection of Free Acids

by

Siegwart Hermann, Richard Neiger and Margot Zentner

With 5 Figures

(Entered on December 15, 1937)

We have established in earlier investigations<sup>1</sup> that after the intravenous injection of gluconic acid, the hydrogen ion concentration is increased. On the basis of various effects after the oral administration of gluconic acid, we could also draw the conclusion that an increase in the hydrogen ion concentration takes place in the organism. Thus calcification phenomena which occurred after the administration of vitasterin could be hindered and even partly reversed by the use of kombuchol, which contains 30% free gluconic acid.<sup>2</sup> The change in the calcium quotient<sup>3</sup>, that is the increase in dissociated calcium in the blood, can also be attributed to the change in the hydrogen ion concentration. Gluconic acid belongs to the acids which are not easily combustible, and thus it is easy to see, that it can shift the pH in the organism to the acid side. However, there are also acids which reveal the opposite behavior, e.g. acetic acid, which for various reasons is counted among the easily combustible acids. In our investigations, we made some findings which could not always be brought into accord with previous conceptions. For this reason, we decided to examine systematically a larger number of acids. The present work is concerned exclusively with the changes in the hydrogen ion concentration in the blood following the introduction of free acids (not salts). In choosing the method of measuring the hydrogen ion concentration, we decided upon the intravital method of von Brehmer<sup>4</sup>, since we have garnered the impression from the works of Bicheler, Zucker and Capaldi<sup>5</sup>, and Wünsche, as well as from our own comparative measurements, that this method yields very useful results for comparative determinations.

<sup>1</sup> Hermann, Siegwart: *Nahrung-Schmidheberg's Arch.* 151, 117-120, 1934.  
— <sup>2</sup> Hermann, Siegwart: *Klin. Woch.* 1939, Nr. 38, S. 1732-33.  
— <sup>3</sup> Hermann, Siegwart u. Margot Zentner: *Nahrung-Schmidheberg's Arch.* 153 (1934). — <sup>4</sup> v. Brehmer: Sonderdruck aus dem *Paracelsus Inst. Sch.* 10 des Vorles. Deutsche Volkshilfskunde, 3.

<sup>5</sup> Zucker u. Capaldi: *Pathologische Rundschau* VI, 1937. — <sup>6</sup> Wünsche, *Opus.* *Klin. Woch.* 1936, Nr. 31, S. 1102.

## Method

Rabbits of an average weight of 2000 g were placed on an oats and water diet before the commencement of the experiment. For the execution of the experiment, it is important that the animals never come into contact with metal parts. In cases of necessity, clamp devices can be wound with insulating tape. The rabbit is stretched out on a wooden board, the right front paw placed through an opening in the board and attached. The paw, which is cleaned of hair and fat, is dipped in a vessel of saturated potassium chloride solution. This vessel is attached to the measuring vessel of the ionometer by means of a tube filled with potassium chloride agar. The antimony-hemovein needle is introduced into the helix vein of the rabbit and the circulation is thus closed. The usefulness of the occasionally used hemovein needle is tested in solutions with a known pH, and also already results from the testing of the normal pH values of the rabbit. Sometimes there is a sudden drop of the pH. If this is only due to a clot formation, faultless values can once again be obtained as soon as the clot is removed. Otherwise, the hemovein needle should be replaced by another. The rectal temperature of the rabbit was considered by means of the correction of the pH values. Those values which remained constant for  $\frac{1}{2}$  hours before the injection were considered the normal pH values. The injection of the acids was made with a cock syringe into the Vena jugularis dextra. In the course of three minutes 10 ccm of the acid in question were injected, thereupon the pH of the circulating blood measured, another injection made, etc. In the following we present the results of our measurements after the injection of acids. The results are shown in graphs as well. The observation time was approximately 6-8 hours. Although for each acid 4-6 animals were used, we are publishing, for the sake of brevity, only one of the curves obtained for each, since they all essentially agree.

### Results of the pH-measurements after intravenous introduction of free acids into circulating blood

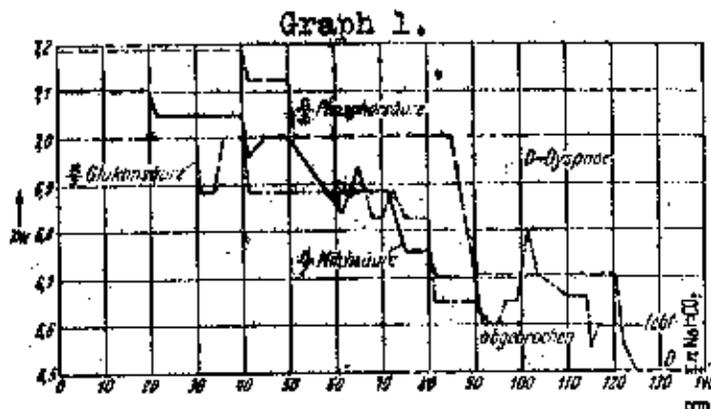
#### 1. Phosphoric acid

We injected n/2 phosphoric acid of a pH of 1.45. It is lipid-soluble. The lethal dose is 62.30 ccm n/2 acid, or 1 g  $H_3PO_4$  per kg rabbit. The pH lowering is on the average 0.58. Death follows suddenly after the appearance of dyspnea and cramps of the extensor musculature. Attempts to save the animals by means of intravenous injections of sodium carbonate immediately after the appearance of the first intoxication symptoms failed. The influence of the acid on the circulating blood in the individual phases can be seen in graph 1.

#### 2. Gluconic acid

The pH is lowered an average of 0.46 by n/2 gluconic acid. The pH of the n/2 acid is 2.05. Gluconic acid is lipid-insoluble. Large amounts of gluconic acid are tolerated both orally and parenterally. In our experiments, the lethal doses was not even reached. The animals received 6 g or 61 ccm n/2 gluconic acid

per kg intravenously. However, dyspnea appeared after these amounts, though it was relieved by means of injections of two or three times 10 ccm of an n/sodium carbonate solution. The blood pH also returned to its normal values. After removal of the syringe, the animals lived several days (graph 1).



Key:

1. = gluconic acid 2. = phosphoric acid 3. = dyspnea  
4. = lactic acid 5. = interrupted

### 3. Lactic acid

n/2 lactic acid, which has a pH of 1.90, clearly acidifies the blood. It is tolerated by rabbits in relatively large quantities. It is lipid-soluble. The toxic dose is 1.76 g per kg of animal, the lethal dose 2.01 g or 80 ccm n/2 acid per kg (graph 1).

### 4. Tartaric acid

We injected n/2 acid of a pH of 1.8. The average lowering of the pH after the intravenous injection was 0.4. Tartaric acid is lipid-soluble. After oral intake it is supposed to be incombustible. The lethal dose was 1.1 g or 30 ccm n/2 tartaric acid per kg of rabbit weight. Before death there are extensor cramps (graph 2).

### 5. Hydrochloric acid

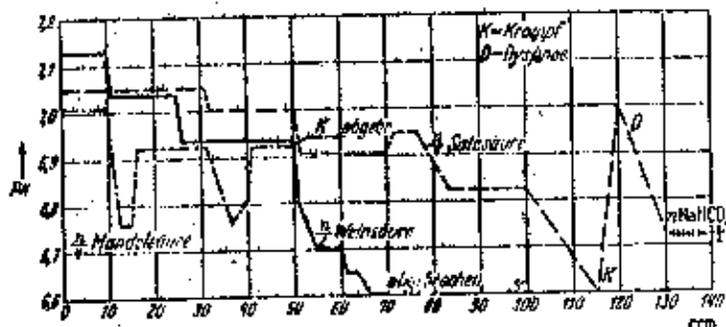
We injected n/4 hydrochloric acid. Its pH is lower than 1.0. It is lipid-soluble and incombustible. The largest amount which can be injected, per kg rabbit weight, before death occurs, is 0.6 g or 54.2 ccm n/4 hydrochloric acid. The pH is lowered an average of 0.35, the hydrogen ion concentration is correspondingly raised. Death occurs with the same symptoms as with phosphoric acid. Injections of sodium carbonate as an anti-toxin are unsuccessful (graph 2).

### 6. Mandelic acid

We injected n/4 solutions of a pH of 2.05. The average decrease of the pH was 0.25. Mandelic acid is lipid-soluble and incombustible in the organism. Its intense toxicity should

be pointed out. The lethal dose is 1.65 g or 15.5 ccm of an n/4 solution per kg of rabbit weight. n/2 solutions, because of the high toxicity, could not even be injected. The course of the pH influencing is seen in graph 2.

Graph 2



Key;

- 1.= cramps 2.= dyspnea  
 3.= hydrochloric acid 4.= mandelic acid 5.= tartaric acid  
 6.= interrupted.

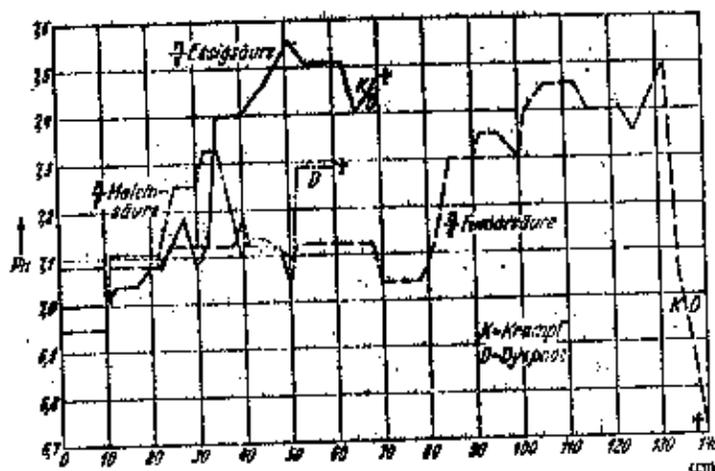
#### 7. Acetic acid

After the intravenous injection of n/4 acetic acid, a clear alkalinisation of the blood can be seen. The blood pH is raised an average of 0.5. This increase is attributable to sodium acetate formation. The pH of an n/2 sodium acetate solution is 7.9. After the injection of the 50 ccm of n/4 acetic acid there was a sharp drop of the blood pH. The animals then underwent the appearance of dyspnea and cramps, and quickly died. At this point, the acetic acid had unbound the available alkali reserves, so that an overflow of the free acetic acid rapidly leads to death. The spontaneously released urine is bloody. The measurements of the urine pH during the experiment showed a slight increase from 5.3 to 5.45. Acetic acid is lipid-soluble; the pH of the n/4 acid is 2.70. The lethal dose is 0.5 g  $\text{CH}_3\text{COOH}$  or 33.5 ccm of an n/4 solution per kg of animal weight (graph 3).

#### 8. Maleic acid

We injected n/2 solutions with a pH of 1.50; n/4 acid with a pH of 1.60 and n/8 acid with a pH of 1.70. The n/2 acid is extraordinarily toxic. After the injection of an n/2 acid, the pH rapidly falls, then to rise slightly again. At this point, death occurs. After the injection of the n/4 acid, the pH rises considerably, then immediately returns to the initial value and rises sharply. Only shortly before death does there occur, amid the appearance of dyspnea and cramps, a drop of the the blood pH, almost to the norm. After the injection of the n/8 acid, there is marked alkalinization. Maleic acid is lipid-soluble. The lethal dose is 0.19 g maleic acid for the n/8 solution, or 26 ccm per kg animal weight (graph 3.).

Graph 3



Key:

1.= acetic acid 2.= maleic acid 3.= fumaric acid  
4.= cramps 5.= dyspnea

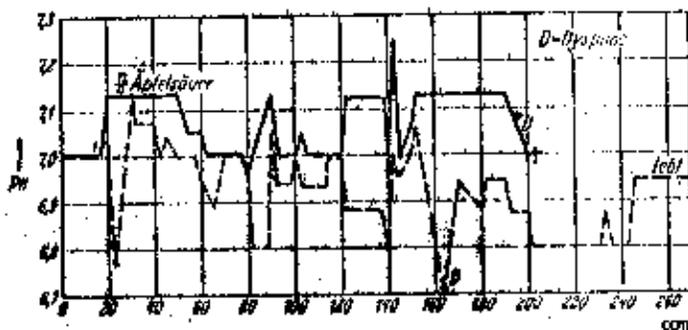
#### 9. Fumaric acid

Fumaric acid is structural isomer with maleic acid. If we observe the third curve of graph 3, showing the course of the injections of a n/8 acid, we notice the difference in contrast to maleic acid. 70 ccm of fumaric acid can be injected, without any pH change taking place. Only later does the pH rise somewhat sharply, to drop rapidly below the norm before death. n/8 fumaric acid has a pH of 2.25. It is lipid-soluble. The lethal dose of n/8 fumaric acid is 0.47 g per kg of animal weight, thus more than double that of maleic acid.

#### 10. Malic acid

n/4 malic acid, which has a pH of 2.20, shows a changing course in its curves during injections in four different experiments. Two experiments showed an increase, two a decrease of the pH. Perhaps this is connected with the metamorphic capacity of this acid in the organism. It is also possible, that malic acid forms, as two basic acids, both acid and neutral salts in the blood, so that one or the other determine the reaction. Malic acid is not very lipid-soluble. The lethal dose of an n/4 solution is 2.40 g or 106 ccm per kg of animal weight (graph 4).

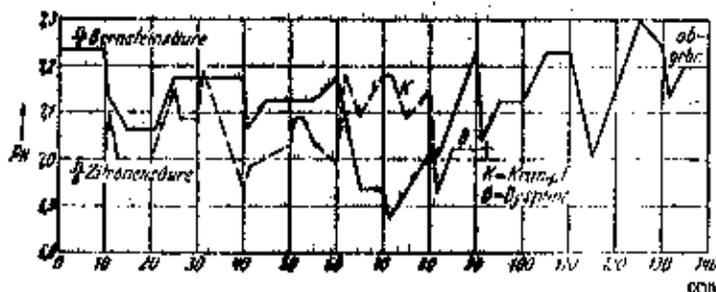
Graph 4



### 11. Succinic acid

We emphasize the great difference in the tolerability according to whether n/4 or n/2 acid was injected. Over 200 ccm of the n/4 acid were tolerated, while a maximum of 50 ccm of the n/2 acid could be injected. The course of the curves reminds us of malic acid (the chemical relationship is a very close one) insofar as here as well there is a strong zig-zag course. The pH values hover about the initial value. In the case of succinic acid, as in that of malic acid, partly acid and partly neutral salts can be formed, so that there is no clear pH alteration. Succinic acid is lipid-insoluble. Of the n/2 acid 0.54 g or 18.1 ccm per kg of animal weight were tolerated, of the n/4 1.63 g or 88.0 ccm (graph 5).

Graph 5



Key:

- 1. = succinic acid
- 2. = citric acid

### 12. Citric acid

We injected n/4 and n/8 solutions. The pH of the n/4 acid is 2.15, that of the n/8 acid 2.3. It is not very lipid-soluble. According to Lengacker, it is combustible after oral administration. The lethal dose is 0.28 g or 17.10 ccm per kg for the n/4 acid, 0.22 g or 32.90 ccm per kg animal weight for the n/8 acid. From graph 5 we see the tendency toward temporary alkalisation. The pH is temporarily raised, the hydrogen ion concentration correspondingly lowered.

#### Summary

1. After the intravenous injection of various acids, the change of the hydrogen ion concentration in the circulating blood was determined.
2. Three basic types of acids can be differentiated: those which acidify the blood, those which alkalize it, and finally those which yield eruptions in the acid or alkali direction.
3. The following series is determined according to blood acidification capacity: phosphoric acid > gluconic acid > lactic acid > tartaric acid > hydrochloric acid > mandelic acid.

4. The following series is determined according to blood alkalisation capacity: acetic acid > maleic acid > fumaric acid.
5. Both acidification and alkalisation is revealed by malic acid; succinic acid shows eruptions toward the acid side, citric acid toward the alkaline.

\* Underhill, Grace & Zalesky: J. of Pharm. 43, 350 (1931).

\* Lungecker, Hedwig: Naunyn-Schneidebergs Arch. 171, 744 (1933).

## Succinate, glycerophosphate and ascorbate as sources of cellular energy and as antiteratogens

By WALTER LANDAUER<sup>1</sup> AND DINAH SOPHER<sup>1</sup>

*From the Department of Animal Genetics, University College London*

### SUMMARY

Our experimental findings show that succinate and ascorbate greatly reduce the teratogenic effects of 3-acetylpyridine, 6-aminonicotinamide and sulfanilamide. Glycerophosphate led to similar alleviating results when used in combination with 3-acetylpyridine and 6-aminonicotinamide, but not with sulfanilamide. With certain other teratogens the high-energy intermediates failed to alleviate; in some instances (acetazolamide, insulin) they even led to potentiation of teratogen-induced defects.

The results of our experiments demonstrate clearly that high-energy intermediates, by being fed into the respiratory chain of the mitochondria, can alleviate incidence and degree of expression of malformations produced by specific teratogens. In concert with earlier evidence on the nature of antiteratogenic compounds, it can further be concluded that the particular teratogens in question exert their effects by interfering with mitochondrial energy production in the tissues for which they have specific affinity.

### INTRODUCTION

As a key to teratogenic action a study of antiteratogens is probably at present most likely to yield fruitful results if there is proof or presumptive knowledge that a specific teratogen interferes with the utilization of Krebs cycle intermediates or of steps in hydrogen transfer and phosphorylation and if the corresponding antiteratogens correct this interference. This presumption is derived from a long series of experiments in which we found that the following compounds, when used as supplements, are effective in preventing, wholly or in part, the teratogenic consequences of a variety of substances injected into the yolk sac of developing chicken eggs on the fourth or fifth day of incubation, namely, glucose, pyruvate, tryptophan,  $\alpha$ -ketoglutaric acid, 3-hydroxyanthranilic acid, nicotinamide and ADP (Landauer, 1948, 1949, 1953*a, b*, 1957; Landauer & Rhodes, 1952; Landauer & Wakasugi, 1967, 1968; Zwilling & DeBelf, 1950); and from the virtually certain conclusion that the common denominator, presumably the only common one, in which these compounds share their antiteratogenic activity is related to the synthesis of either nicotinamide adenine dinucleotide (NAD) or adenosine triphosphate (ATP) and the subsequent

<sup>1</sup> *Authors' address:* Department of Animal Genetics, University College London, 4 Stephenson Way, London, N.W.1, U.K.

production of energy. The question arose, therefore, if this means that the principal, if not only, role played by antiteratogens is that of providing affected tissues with an extra burst of cellular energy, thereby compensating for damage done by the teratogens. In what follows we shall report on experiments which were designed to test the likelihood of this being true by supplementing teratogen treatment with succinate, glycerophosphate and ascorbate. These compounds were chosen because, as will be discussed further on, they are known to be high-energy sources which can be fed into the respiratory chain of mitochondria and are likely to promote the energy-dependent steps of protein and nucleic acid synthesis.

#### MATERIALS AND METHODS

The eggs used in our present work came from an interline cross of White Leghorn fow bred by Sykes International Ltd. in Warrminster, Wiltshire. All tests were done after 96 h of incubation. At the end of 18 days of incubation the embryos were removed from the shells and inspected for gross abnormalities. The methods of incubation and the experimental procedures were the same as those recorded earlier (Landauer & Wakasugi, 1967). The number of fertile eggs used; post-operative mortality; number of survivors of the eighteenth day of incubation and incidence of any gross defects found among the survivors are recorded in the tables.

The compounds used in our tests and their sources were the following: 3-acetylpyridine, sulfanilamide and DL-2-glycerophosphate hexahydrate disodium salt, Sigma Chemical Co.; L-ascorbic acid, iso-ascorbic acid sodium salt and propylene glycol, British Drug Houses Ltd.; 6-aminonicotinamide, Mann Research Laboratories; insulin, Burroughs Wellcome and Co.; sodium acetazolamide (Diamox), Lederle Laboratories; sodium succinate hexahydrate, Hopkin and Williams Ltd.

The compounds to be tested were dissolved in demineralized water with 0.3% phenol added, except for sulfanilamide and L-ascorbic acid for which propylene glycol (with added phenol) was used as solvent, and insulin which was used as furnished by Burroughs Wellcome and Co. The aqueous solution of sodium ascorbate was adjusted, with a 10% aqueous solution of L-ascorbic acid, to a pH of about 5. All phenol-containing solutions were left prior to use for 4 days at room temperature. The injection of test compounds was always done into the yolk sac and the volume was generally 0.05 ml, though in some instances 0.1 ml. Substances to be used as supplements were either dissolved together with the teratogen in question or as separate solutions; in the latter instance supplement and test injections were made immediately one after the other, the sequence being indicated in the tables. The dosages of teratogens were arrived at by preliminary toxicity tests; the amounts of supplements to be used were those that could in each instance readily be dissolved in 0.05 or 0.1 ml of solvent. Available evidence (e.g. data of Tables 9 and 10) indicates that it

made no significant difference in our results whether teratogen and supplement were used by separate injections or in the same vehicle. There was no evidence of interaction between those compounds which were dissolved together and, within the dosage ranges of our tests, no changes of solubility.

The developmental defects to which the teratogens used in our tests give rise have been described in the following papers: insulin (Landauer, 1947), sulfanilamide (Ancel, 1945; Zwilling & DeBell, 1950), 3-acetylpyridine and 6-aminonicotinamide (Landauer, 1957), acetazolamide (Landauer & Wakasugi, 1967). Details will not be reviewed in the present report.

## RESULTS

### *Tests with sodium succinate*

Table 1 presents the results of a test in which chicken embryos were treated with 3-acetylpyridine alone or in combination with sodium succinate. In our original report on the teratogenic effects of 3-acetylpyridine (Landauer, 1957)

Table 1. *The effect of treatment at 96 h of incubation with 0.6 mg/egg (4.95  $\mu$ ) 3-acetylpyridine either alone or in combination with 7.5 mg/egg (27.76  $\mu$ M) sodium succinate.*

(Supplement and teratogen injected together in the same vehicle.)

	3-Acetylpyridine	3-Acetylpyridine supplemented with sodium succinate
Treated ...	196	199
Mortality to 18 days (%)	54.1	24.6
Survivors	90	150
Normal appearance (%)	13.3	44.0
Muscular hypoplasia (%)		
Extrema	57.8	24.7
Slight	12.2	22.0
Suggestive (thin tarsometatarsus)	16.7	9.3
Total	86.7	56.0
Short upper beak (%)	16.7	2.0
Crooked neck (%)	2.2	0.7
Miscellaneous defects (%)	0	0.7

we called attention to the fact that, in addition to the major abnormality of muscular hypoplasia, chiefly of the leg muscles, there is also thinning of the long bones. This latter defect, especially thinning of the tarsometatarsal bone, was in some instances clearly discernible when there was doubt whether the leg musculature was normal or not. Hence the condition of the tarsometatarsus served in a small proportion of cases as the means of diagnosis. The data of

Table 1 indicate a highly significant reduction in toxicity, as expressed by embryo mortality, when 3-acetylpyridine was given in combination with sodium succinate ( $P \ll 0.0001$ ). Among survivors of the eighteenth day of incubation succinate supplementation led to a great increase in incidence of normal-

Table 2. The effect of treatment at 96 h of incubation with 5  $\gamma$ /egg (0.035  $\mu$ M) 6-aminonicotinamide either alone or in combination with 7.5 mg/egg (27.76  $\mu$ M) sodium succinate

(Succinylent and teratogen injected together in the same vehicle.)

	6-Aminonicotinamide	6-Aminonicotinamide supplemented with sodium succinate
Treated . . .	150	141
Mortality to 18 days (%)	15.3	11.3
Survivors	127	125
Normal appearance (%)	3.9	31.2
Micromelia (%)	96.0	68.8
Abnormal beak (%)	55.1	32.8
Syndactylism (%)	26.0	12.8

Table 3. The effect of treatment at 96 h of incubation with 1.3 mg/egg (7.55  $\mu$ M) sulfanilamide either alone or preceded by injection of 7.5 mg/egg (27.76  $\mu$ M) sodium succinate

	Sulfanilamide	Sulfanilamide and sodium succinate
Treated . . .	183	117
Mortality to 18 days (%)	21.9	31.6
Survivors	143	80
Normal appearance (%)	32.2	58.8
Micromelia (%)		
Extreme	35.7	22.5
Slight	31.5	18.8
Total	67.1	41.3
Abnormal beak (%)	23.8	13.8
Miscellaneous defects (%)	0	2.1

appearing embryos and a lowering in frequency of over-all muscular hypoplasia, of shortening of the upper beak and of abnormality of the cervical vertebrae. The differences between the two groups are highly significant for muscular hypoplasia and abnormality of the beak ( $P \ll 0.0001$ ), and this remained true when, to guard against any possible misclassification, account was taken of only the more extreme expressions of muscular hypoplasia.

Table 2 summarizes our data for 6-aminonicotinamide with or without succinate supplementation. In the presence of succinate the embryo mortality

was slightly, but not significantly, lowered. The incidence of normal-appearing embryos was greatly increased by succinate supplementation, and the frequency of micromelia, of abnormality of the beak and of syndactylism was much reduced. The difference between the two groups was significant ( $P < 0.01$ ) in the incidence of syndactylism; the frequencies of micromelia and beak defect, as well as that of gross normality, differed highly significantly ( $P < 0.0001$ ).

The results of a test with sulfanilamide and the effect of succinate on its teratogenicity are given in Table 3. As compared with sulfanilamide alone, embryo mortality was somewhat increased in the presence of sodium succinate, but the difference lacked statistical significance ( $P > 0.05$ ). Among survivors of the eighteenth day of incubation highly significant differences occurred between the two groups in 'normality' and in incidence of micromelia ( $P < 0.001$ ). Lest it be thought that differential mortality accounted for these results, the significance was also calculated on the assumption that in the group treated with sulfanilamide alone, all dead embryos had been non-micromelic, and that all dead embryos had been micromelic in the succinate-supplemented group. The results still demonstrated beyond doubt that the presence of succinate lowered the incidence of micromelia ( $P < 0.01$ ). The percentage reduction in incidence of beak defects after succinate supplementation was even greater than the of micromelia (53.4 versus 38.5%), but the difference did not reach statistical significance.

In earlier experiments (Landauer & Rhodes, 1952) it had been shown that the combination of insulin and sodium succinate caused a high degree of potentiation of all insulin-induced malformations, and this in spite of the fact that treatment with sodium succinate alone was quite non-teratogenic.

#### *Tests with DL- $\alpha$ -glycerophosphate hexahydrate disodium salt*

We conducted two tests in which the interaction, if any, of 3-acetylpyridine and glycerophosphate was to be examined. Since the two experiments produced closely similar results we combined the data and these are shown in Table 4. It will be seen that the toxicity of 3-acetylpyridine, as expressed in embryo mortality, was greatly reduced in the presence of glycerophosphate and that the incidence of muscular hypoplasia, abnormality of the upper beak and defective formation of the cervical vertebrae was greatly lowered, with a corresponding increase in the frequency of grossly normal embryos. All these differences in toxicity and teratogenicity are statistically highly significant ( $P \leq 0.0001$ ).

The data for similar tests with 6-aminonicotinamide are presented in Table 5. The presence of glycerophosphate in combination with 6-aminonicotinamide led to a significant rise in embryo mortality. Among embryos which survived the eighteenth day of incubation the incidence of micromelia and of abnormal formation of the beak was much reduced. It can be shown that as far as micromelia is concerned, which was quite obviously in these tests the principal effect of 6-aminonicotinamide, the lowering of incidence due to the presence of

glycerophosphate remains highly significant ( $P < 0.0001$ ) even on the unwarranted assumption that all undiagnosed embryos dying in the unsupplemented group had been normal and all those dying in the supplemented group had been abnormal! The same holds for frequency of over-all abnormality (or

Table 4. *The effect of treatment at 96 h of incubation with 0.6 mg/egg (4.95  $\mu$ M) 3-acetylpyridine either alone or in combination with 10 mg/egg (30.86  $\mu$ M) DL- $\alpha$ -glycerophosphate hexahydrate disodium salt (Supplement and teratogen injected together in the same vehicle.)*

	3-Acetylpyridine	3-Acetylpyridine supplemented with glycerophosphate
Treated ...	236	229
Mortality to 18 days (%)	44.5	19.7
Survivors	133	183
Normal appearance (%)	15.8	80.3
Muscular hypoplasia (%)	82.0	19.1
Short upper beak (%)	13.5	0.5
Crooked neck (%)	8.3	0

Table 5. *The effect of treatment at 96 h of incubation with 5  $\gamma$ /egg (0.035  $\mu$ M) 6-aminonicotinamide either alone or in combination with 10 or 15 mg/egg (30.86 or 46.29  $\mu$ M) DL- $\alpha$ -glycerophosphate hexahydrate disodium salt (Supplement and teratogen injected in the same vehicle.)*

	6-Aminonicotinamide	6-Aminonicotinamide supplemented with glycerophosphate
Treated ...	251	235
Mortality to 18 days (%)	9.6	19.6
Survivors	227	189
Normal appearance (%)	11.9	41.8
Microphthalmia (%)	88.1	58.2
Abnormal beak (%)	27.8	14.8
Syndactylism	4.8	6.9
Miscellaneous defects (%)	1.3	0.5

of grossly normal appearance) in the two groups. Syndactylism occurred with slightly higher incidence in the presence of glycerophosphate, but the frequencies were low and the differences not statistically significant.

Our data demonstrate clearly that in the presence of glycerophosphate the teratogenic effects of the two niacin antagonists 3-acetylpyridine and 6-aminonicotinamide are greatly reduced. In contrast, no such beneficial results were

observed when, in work not reported in detail, glycerophosphate was used as supplement to treatment with either sulfanilamide (1.3 mg/egg), acetazolamide (1.3 mg/egg) or inulin (8  $\mu$ g).

*Tests with sodium ascorbate or ascorbic acid*

The effects of sodium ascorbate or ascorbic acid as supplement to treatment with 3-acetylpyridine, 6-aminocaproinamide and sulfanilamide are illustrated by the data in Tables 6-10. With 3-acetylpyridine we conducted two tests. In the

Table 6. *The results of treatment at 96 h of incubation with 0.5 mg/egg (4.13  $\mu$ M) 3-acetylpyridine either alone or combined with 10 mg/egg (50.57  $\mu$ M) sodium ascorbate, buffered with ascorbic acid*

(Supplement and teratogen injected in the same vehicle.)

	3-Acetylpyridine	3-Acetylpyridine supplemented with ascorbate
Treated . . . . .	75	67
Mortality to 18 days (%)	24.0	12.0
Survivors	57	59
Normal appearance (%)	43.9	88.1
Muscular hypoplasia (%)	56.1	10.2
Short upper beak (%)	5.3	1.7
Abnormal neck (%)	1.8	0
Miscellaneous defects (%)	0	1.7

Table 7. *The results of treatment at 96 h of incubation with 0.5 mg/egg (4.13  $\mu$ M) 3-acetylpyridine either alone or combined with 10 mg/egg (56.78  $\mu$ M) ascorbic acid*

(Supplement and teratogen injected together in propylene glycol.)

	3-Acetylpyridine	3-Acetylpyridine supplemented with ascorbic acid
Treated . . . . .	163	151
Mortality to 18 days (%)	13.5	15.9
Survivors	141	127
Normal appearance (%)	79.4	98.4
Muscular hypoplasia (%)	19.8	1.6
Miscellaneous defects (%)	0.7	0

first of these the embryos were exposed either to an aqueous solution of 3-acetylpyridine (0.5 mg/egg) or to the same amount of the teratogen in an aqueous solution of sodium ascorbate (10 mg/egg), buffered with ascorbic acid (Table 6). In the presence of ascorbate embryo mortality was reduced and a much higher proportion of embryos escaped teratogenic effects, the incidence of muscular

hypoplasia and of shortening of the upper beak being greatly reduced. The differences in frequency of normal embryos and of specimens with muscular hypoplasia were highly significant between the two groups ( $P < 0.0001$ ). In the second test we used propylene glycol as solvent either for 3-acetylpyridine

Table 8. *The effects of treatment at 96 h of incubation with 5  $\gamma$ /egg (0.035  $\mu$ M) 6-aminonicotinamide either alone or supplemented with 10 mg/egg (50.47  $\mu$ M) sodium ascorbate, buffered with ascorbic acid (Supplement and teratogen injected together in the same vehicle.)*

	6-Aminonicotinamide	6-Aminonicotinamide supplemented with ascorbate
Treated . . .	167	165
Mortality to 18 days (%)	14.4	10.3
Survivors	143	150
Normal appearance (%)	7.0	35.3
Micromelia (%)	92.3	64.0
Abnormal beak (%)	65.7	23.3
Syndaactylism (%)	22.4	11.4
Miscellaneous defects (%)	0	0.7

Table 9. *The effects of treatment at 96 h of incubation with 1.3 mg/egg (7.55  $\mu$ M) sulfanilamide either alone or supplemented with 10 mg/egg (50.47  $\mu$ M) sodium ascorbate, buffered with ascorbic acid*

(Sulfanilamide injected in propylene glycol, followed by aqueous solution of ascorbate.)

	Sulfanilamide	Sulfanilamide supplemented with ascorbate
Treated . . .	159	158
Mortality to 18 days (%)	22.6	29.1
Survivors	123	114
Normal appearance (%)	55.3	84.2
Micromelia (%)	43.9	15.8
Abnormal beak (%)	26.0	10.5
Miscellaneous defects (%)	0.8	0

alone or for its combination with ascorbic acid (Table 7). In propylene glycol as vehicle 3-acetylpyridine was less toxic and much less teratogenic than in aqueous solution. The incidence of muscular hypoplasia was greatly reduced and we did not find any cases of shortened upper beak or abnormality of the neck. With ascorbic acid as supplement the frequency of grossly normal embryos was much increased, with a corresponding drop in occurrence of muscular hypoplasia ( $P < 0.0001$ ).

Our results with 6-aminonicotinamide are presented in Table 8. Supplementing 6-aminonicotinamide with sodium ascorbate reduced embryo mortality to a statistically non-significant extent, definitely raised the incidence of grossly normal-appearing embryos and greatly lowered the occurrence of micromelia, beak defects and syndactylism. All these differences in teratogenic activity were statistically highly significant ( $P \ll 0.0001$ ).

Table 10. *The effect of treatment at 96 h of incubation with 1 mg/egg (5.81  $\mu$ M) sulfanilamide either alone or supplemented with 10 mg/egg (56.78  $\mu$ M) ascorbic acid*

(Supplement and teratogen injected together in propylene glycol.)

	Sulfanilamide	Sulfanilamide supplemented with ascorbic acid
Treated ...	153	159
Mortality to 18 days (%)	13.1	23.3
Survivors	133	122
Normal appearance (%)	63.2	90.2
Micromelia (%)	35.3	9.8
Abnormal beak (%)	18.0	6.6
Miscellaneous defects (%)	0.8	0

We made two experiments on the effect which supplementation with sodium ascorbate or ascorbic acid has on the teratogenicity of sulfanilamide. In the first of these tests (Table 9) we wished to compare the effect on embryos of 1.3 mg/egg sulfanilamide dissolved in propylene glycol with or without the subsequent additional injection of buffered sodium ascorbate. The results show that the presence of ascorbate increased embryo mortality slightly, but not significantly; the differences, on the other hand, of embryos which were grossly normal and of those with micromelia and beak defects were proof of a highly significant beneficial effect of supplementation with ascorbate, with probability of  $\ll 0.0001$  for frequencies of normal and micromelic embryos and of 0.01 for defects of the beak. In a second test we reduced slightly the amount of injected sulfanilamide and observed the effects alone or with the addition of ascorbic acid rather than sodium ascorbate (Table 10). Embryo mortality was somewhat increased in the presence of ascorbic acid over that following sulfanilamide alone ( $P 0.02$ ); however, the incidence of normality and micromelia ( $P \ll 0.0001$ ) and of malformations of the beak ( $P \ll 0.01$ ) demonstrate that ascorbic acid and sodium ascorbate are similar in antiteratogenic activity.

In contrast to the beneficial effect which ascorbate has on the teratogenic activity of 3-acetylpyridine, 6-aminonicotinamide and sulfanilamide, we found that the combination of ascorbate with either acetazolamide or insulin was quite detrimental in its results on survival and development. This is illustrated

by the results of supplementing insulin with sodium ascorbate (Table 11). The data of this table do not, in fact, illustrate the full extent to which the insulin-induced aberrations of development were exaggerated, the defectiveness of the skeleton of limbs and beak being quantitatively much more extreme in the ascorbate-supplemented group than in that treated with insulin alone. The combination of nectazolamide and ascorbate led to similar potentiation.

Table 11. *The effect of treatment at 96 h of incubation with 4 units insulin either alone or supplemented with 10 mg/egg (56.78  $\mu$ M) sodium ascorbate, buffered with ascorbic acid*

(Injection of insulin followed by aqueous solution of ascorbate.)

	Insulin	Insulin supplemented with ascorbate
Treated...	162	161
Mortality to 18 days (%)	33.3	62.7
Survivors	108	60
Normal appearance (%)	71.3	25.0
Micromelia (%)	24.1	73.3
Short upper beak (%)	13.0	50.0

A few remarks should finally be added which apply to all reported data. (1) Preliminary tests made earlier and control experiments done now have shown that neither succinate, nor glycerophosphate, nor sodium ascorbate or ascorbic acid when injected separately at 96 h of incubation, and at the dosages used for supplementation, produce toxic or teratogenic effects in chicken embryos. (2) In the tests in which teratogens were used either with or without metabolic supplement, we found that embryo mortality and teratogenicity were often, but by no means regularly, correlated. (3) All three types of supplements used in our tests, i.e. succinate, glycerophosphate and ascorbate, when effective at all, led to quantitatively similar beneficial results in all parts affected by the teratogens (general body growth, musculature, facial or appendicular skeleton). (4) The effects of succinate, glycerophosphate and ascorbate were limited to the dosages recorded in the text and to treatment at 96 h of incubation. Other dosages and stages of development might well have produced even more favorable results.

#### DISCUSSION

We shall open the discussion of our results with a quotation from the review on 'Biological oxidoreductions' by Ernster & Lee (1964): 'It is now firmly established that mitochondria and submitochondrial particles from a variety of tissues catalyse an energy-dependent reversal of electron transport through the respiratory chain. The energy for the reaction can be supplied either by

added ATP or by endogenous high-energy intermediates generated by the respiratory chain. In the former case, the reaction involves an actual reversal of oxidative phosphorylation, and there is now evidence that electron transport through all carriers of the respiratory chain, from  $\text{NAD}^+$  to cytochrome oxidase, can be reversed in this way. When endogenous high-energy intermediates generated by the respiratory chain serve as the source of energy, the reversal of electron transport can proceed directly at the expense of the intermediates, without the involvement of the phosphorylating system. The two types of energy-dependent reversal of electron transport and their relation to oxidative phosphorylation are illustrated in Scheme 1 (Fig. 1). Ernster & Lee then show in their review that both types of energy-dependent reversal of electron transport in the respiratory chain have been studied by a number of investigators and under a great variety of conditions.

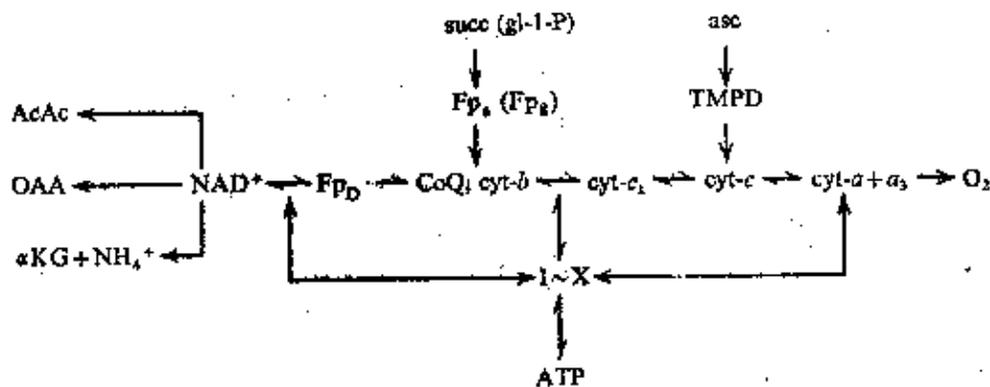


Fig. 1. Energy-dependent reversal of electron transport in the respiratory chain, and its relation to oxidative phosphorylation (courtesy Ernster & Lee, 'Biological oxidoreductions' and *Annual Reviews, Inc.*, Palo Alto, California). AcAc, acetoacetate; asc, ascorbate; ATP, adenosine triphosphate; CoQ, coenzyme Q (ubiquinone); cyt, cytochrome; Fp<sub>D</sub>, NADH dehydrogenase; Fp<sub>s</sub>, glycerol-1-phosphate dehydrogenase; Fp<sub>s</sub>, succinate dehydrogenase; gl-1-P, glycerol-1-phosphate; I and X denote hypothetical energy-transfer carriers; α-KG, α-ketoglutarate; NAD<sup>+</sup>, nicotinamide adenine dinucleotide phosphate oxidized; OAA, oxaloacetate; succ, succinate; TMPD, *NNN'*-tetramethyl-*p*-phenylenediamine.

The high-energy intermediates shown in the scheme of Ernster & Lee are succinate, glycerophosphate and ascorbate. The importance of succinic acid and of glycerophosphic acid in processes of cellular oxidation had first been clearly enunciated by Meyerhof (1919). The mechanism of energy-dependent reversal of electron transport via succinate-linked reduction of  $\text{NAD}^+$  and involving succinic dehydrogenase and the respiratory chain was well established by Chance & Hollunger (1957). Extensive documentation is also available for the conclusion that the glycerophosphate shuttle carries electrons from extra-mitochondrial DPNH to the intra-mitochondrial electron transport chain (Boxer & Devlin, 1961). The prominent role which ascorbic acid plays in elec-

iron transport was clearly brought out by Staudinger, Krisch & Leonhäuser (1961) and has since found ample verification.

The important basis for our present experimentation was the recognition that the high-energy intermediates succinate, glycerophosphate and ascorbate need not originate endogenously, but can play their role when derived from exogenous sources (review by Ernster & Lee, 1964; Ernster, 1965). This being so, it became our primary purpose to determine if these compounds can serve as exogenous supplements for relieving the noxious effects of specific teratogens when administered to developing chicken embryos. Our data show quite unambiguously that they can, indeed, be used in this manner. We could at the same time demonstrate clearly that their antiteratogenic effectiveness applies only to certain teratogens and not to others. This teratogen-specificity did not come as a surprise. Much of our earlier work had provided similar evidence, e.g. that succinate, far from being helpful, exaggerates insulin-induced effects in incidence and extent (Landauer & Rhodes, 1952) or that nicotinamide, which is so clearly antiteratogenic with insulin and many other teratogens, was found ineffective as supplement to either acetazolamide, dichlorphenamide or methazolamide, whereas ATP was helpful with either of the three (Landauer & Wakasugi, 1967, 1968). To these specific responses we shall return below.

It would seem well at this point to list compounds which in our experience have had alleviating properties in combination with teratogens that were used by us for treatment of chicken embryos after four days of incubation. They are the following: L-tryptophan hydrochloride (with 3-acetylpyridine and 6-aminonicotinamide, Landauer, 1957);  $\alpha$ -ketoglutaric acid (with insulin, Landauer, 1948; Landauer & Rhodes, 1952); 3-hydroxyanthranilic acid (with 3-acetylpyridine and 6-aminonicotinamide, Landauer, 1957); pyruvate (with nicotine sulphate, Landauer, 1960*b*); nicotinamide (with insulin, Landauer, 1948; Landauer & Rhodes, 1952; with eserine, Landauer 1949; with sulfanilamide, Zwilling & DeBell, 1950; with pilocarpine, Landauer, 1953*b*; with 3-acetylpyridine and 6-aminonicotinamide, Landauer, 1957); ADP (with acetazolamide, dichlorphenamide and methazolamide, Landauer & Wakasugi, 1967, 1968); glucose (with pilocarpine, Landauer, 1956; with nicotine sulphate, Landauer, 1960*b*; with thallium acetate, Landauer, 1960*a*); riboflavin and riboflavin-5-phosphate (with boric acid, Landauer, 1952; Landauer & Clark, 1964). The metabolic functions which all of these compounds, used as supplements, have in common lie in their value for the production of cellular energy via phosphorylation and hydrogen transport (see Landauer & Wakasugi, 1968). It seems likely that one can add sorbitol (used with boric acid, Landauer, 1953*a*; with pilocarpine, Landauer, 1956; with thallium acetate, Landauer, 1960*a*), as well as sorbose, arabinol, imidazole and histidine (with thallium acetate, Landauer, 1960*a*), to this list since strong evidence has been presented by Boyer and his associates (Boyer, 1963; Boyer *et al.*, 1963) in support of the conclusion that imidazole and phosphohistidine participate as intermediates in oxidative phos-

phorylation and since the same may well be true for the other polyhydroxy compounds used in our experiments. It is not surprising, therefore, that succinate, glycerophosphate and ascorbate should fall in line with the foregoing compounds in having antiteratogenic value.

Considerable evidence exists for the conclusion that interference with mitochondrial functions and with the activity of other cytoplasmic organelles is a major cause of teratogenesis. Conclusions to that effect were first arrived at by Braun (1954, 1964, 1966) on the basis of his work with Janus green B as it affected chicken embryos. Additional information has come from investigations of Kaplan & Johnson (1968), Aksu, Mackler, Shepard & Lemire (1968), Pollard & Fraser (1968), Verrusio, Pollard & Fraser (1968), Verrusio & Watkins (1969) and Chepenik, Johnson & Kaplan (1969). Details need not be discussed here. An interesting exposition of the basic problems was presented by Herken (1966).

Conclusive evidence for the importance of cellular energy in the origin of malformations can also be found in the role which general body growth plays in aberrations from normal morphogenesis. Such information is available from studies on hypoxia (Büchler, 1948, 1955; Rübssaamen, 1952; Degenhardt, 1954; Grabowski, 1970; and others), on hypocaloric diets (Runner & Miller, 1956; Kalter, 1960; Miller, 1962; Yasuda, Nanjo & Suzuki, 1966; Rogóyski, 1967; and others) and, of course, on the effects of antimetabolites. All students of experimental teratology are aware of the important relationship existing between general body growth and the occurrence of specific defects. Some metric data on dose and stage dependence are available for insulin and pilocarpine (Landauer, 1953c). Evidence suggesting that antimetabolites or other chemical teratogens interfere with growth rather than with differentiation became particularly impressive when it was found that repair of teratogenic damage with supplements of nicotinamide (or either its precursors or derivatives) was still possible an appreciable time after treatment with particular teratogens such as sulfanilamide (Zwilling & DeBell, 1950) or bidrin (Roger, 1967; Roger, Lipshall & Casida, 1969).

The metabolic intermediates used in our present experiments, namely succinate, glycerophosphate and ascorbate, as well as the compounds which in our earlier work had been found to have alleviating or antiteratogenic properties, clearly have much in common as far as our interests are concerned. They also differ, however, in important ways. There is first of all the fact that in combination with the particular teratogens with which we tested them, only some of the supplements were helpful, while others were inert or even produced an exaggeration of teratogenic activity. It has been pointed out, for instance, that, used as supplement, ascorbate greatly reduced the teratogenic effects of 3-acetylpyridine, 6-aminonicotinamide and sulfanilamide, but strikingly potentiated incidence and gross expression of insulin-induced malformations, whereas nicotinamide, on the other hand, abolished or reduced the teratogenic activity of all these

compounds. It is clear, therefore, that there are separate ways by which teratogens interfere with energy production and by which the effects of teratogens can be mitigated. The details of such differences remain unknown.

Secondly, it seems clear from the results of our present experiments that teratogenic damage may occur at different points along one and the same pathway. For, our results have shown that in all instances in which succinate reduced the noxious effects of a teratogen, ascorbate had a quantitatively similar effect. This is of great interest since it is known that these two intermediates, whether contributed by endogenous or introduced from exogenous sources, become active at different sites of the respiratory chain. It seems very unlikely that three different teratogens (namely 3-acetylpyridine, 6-aminonicotinamide and sulfamylamide) should similarly impede the metabolic events occurring at both sites. Whatever their specific interference with mitochondrial activity, our results seem to demonstrate clearly that the role of the intermediates, when used as supplement to the teratogens, is that of stimulus for cellular energy production, thereby increasing the chances for protection of the embryo against teratogen-induced damage or of its repair.

#### RÉSUMÉ

##### *Succinate, glycerophosphate et ascorbate, sources d'énergie cellulaire et substances antiteratogènes*

Nos recherches expérimentales montrent que le succinate et l'ascorbate réduisent considérablement les effets tératogènes de la 3-acétylpyridine, la 6-aminonicotinamide et la sulfamylamide. Le glycerophosphate a donné des résultats similaires lorsqu'il a été utilisé en association avec la 3-acétylpyridine et la 6-aminonicotinamide, mais non avec la sulfamylamide. Avec certains autres agents tératogènes ces intermédiaires à potentiel énergétique élevé n'ont pas manifesté d'action protectrice; dans quelques cas (acetazolamide, insuline) ils ont même eu pour résultat de renforcer les perturbations induites par les agents tératogènes.

Les résultats de nos expériences montrent clairement que des intermédiaires à potentiel énergétique élevé, introduits dans le chaîne respiratoire des mitochondries, peuvent diminuer l'incidence et le degré d'expression des malformations produites par des agents tératogènes spécifiques. En accord avec des informations antérieures sur la nature des composés antiteratogènes, on peut conclure que les substances tératogènes en question agissent par interférence avec la production d'énergie mitochondriale dans les tissus pour lesquels elles ont une affinité spécifique.

Our work was generously supported by the Association for the Aid of Crippled Children in New York. We are greatly indebted to the Director of the Association, Dr Robert J. Slater, for sympathetic interest. Our initial tests with 3-acetylpyridine were done under a grant from the Muscular Dystrophy Associations of America in New York, for which we wish to express our gratitude. We are very grateful to Professor Salome Waelsch for reading the manuscript and advising on it. We also wish to express appreciation to Mr Roger F. R. Mills, Production Director, and to Mr D. Lyon, Production Controller, of Sykes International Ltd. in Warrminster, Wiltshire, for unfailing help and courtesy in providing the hatching eggs for our work and to Mr A. J. Lee for the drawing of Fig. 1.

## REFERENCES

- AKSO, O., MACLELL, B., SHEPARD, T. H. & LEHR, R. J. (1968). Studies of the development of congenital anomalies in embryos of riboflavin-deficient, galactoflavin fed rats. II. Role of the terminal electron transport systems. *Teratology* **1**, 92-102.
- AKOBI, P. (1945). L'achondroplasie. Sa réalisation expérimentale - sa pathogénie. *Annls Embryol.* **6**, 1-24.
- BONER, G. F. & DEVLIN, T. M. (1961). Pathways of intracellular hydrogen transport. *Science, N.Y.* **134**, 1495-1501.
- BOYER, P. D. (1963). Phosphohistidine. *Science, N.Y.* **141**, 1147-1153.
- BOYER, P. D., HULTQUIST, D. E., PETER, J. B., KREB, G., MITCHELL, R. A., DELUCA, M., HINSON, J. W., BUTLER, L. G. & MOYER, R. W. (1963). Role of the phosphorylated imidazole group in phosphorylation and energy transfer. *Fedn Proc. Fedn Am. Soc. exp. Biol.* **22**, 1080-1087.
- BRAUN, S. (1954). Janus green B teratological action in embryonated hen's eggs and embryogenetic and carcinogenic bearings of its mechanism of action. *Acta morph. hung.* **4**, 61-79.
- BRAUN, S. (1964). Current problems of experimental teratogenesis. *Acta morph. hung.* **13**, Suppl., 1-14.
- BRAUN, S. (1966). The role of mitochondria in the early morphogenesis of chick embryos. *Acta morph. hung.* **14**, 54-58.
- BÜCHNER, F. (1948). Experimentelle Entwicklungsstörungen durch allgemeinen Sauerstoffmangel. *Klin. Wschr.* **26**, 38-42.
- BÜCHNER, F. (1955). Von den Ursachen der Missbildungen und Missbildungskrankheiten. *Münch. med. Wschr.* **97**, 1673-1677.
- CHANCE, B. & HOLLINGER, G. (1957). Succinate-linked pyridine nucleotide reduction in mitochondria. *Fedn Proc. Fedn Am. Soc. exp. Biol.* **16**, 163.
- CHEPENIK, K. P., JOHNSON, E. M. & KAPLAN, S. (1969). Energy metabolism in normal and abnormal rat embryos (Abstract). *Teratology* **2**, 259.
- DEGENHARDT, K.-H. (1954). Durch O<sub>2</sub>-Mangel induzierte Fehlbildungen der Axialgradienten bei Kunin-hen. *Z. Naturf.* **9B**, 530-536.
- ERNSTER, L. (1965). Control of cell metabolism at the mitochondrial level. *Fedn Proc. Fedn Am. Soc. exp. Biol.* **24**, 1222-1236.
- ERNSTER, L. & LEE, CH.-P. (1964). Biological oxidoreductions. *A. Rev. Biochem.* **33**, 729-788.
- GRABOWSKI, C. F. (1970). Embryonic oxygen deficiency—a physiological approach to analysis of teratological mechanisms. *Adv. Teratol.* **4**, 125-167.
- HERKIN, H. (1966). Die Wirkung von Antimetaboliten auf den Zellstoffwechsel des Warmblüters. *Internist* **7**, 375-391.
- KALTER, H. (1960). Teratogenic action of a hypercaloric diet and small doses of cortisone. *Proc. Soc. exp. Biol. Med.* **104**, 518-526.
- KAPLAN, S. & JOHNSON, E. M. (1968). Oxygen consumption in normal and trypan blue-treated chick embryos. *Teratology* **1**, 369-374.
- LANDAUER, W. (1947). Insulin-induced abnormalities of beak, extremities and eyes in chickens. *J. exp. Zool.* **105**, 145-172.
- LANDAUER, W. (1948). The effect of nicotinamide and  $\alpha$ -ketoglutaric acid on the teratogenic action of insulin. *J. exp. Zool.* **109**, 283-290.
- LANDAUER, W. (1949). Le problème de l'efficacité dans les expériences de tératogénèse biochimique. *Archs Anat. microsc. Morph. exp.* **38**, 184-189.
- LANDAUER, W. (1952). Malformations of chicken embryos produced by boric acid and the probable role of riboflavin in their origin. *J. exp. Zool.* **120**, 469-508.
- LANDAUER, W. (1953a). Complex formation and chemical specificity of boric acid in production of chicken embryo malformations. *Proc. Soc. exp. Biol. Med.* **82**, 633-656.
- LANDAUER, W. (1953b). On teratogenic effects of piperazine in chick development. *J. exp. Zool.* **122**, 469-483.

- LANDAUER, W. (1953c). The effect of time of injection and of dosage on absolute and relative length of lower, (thoracicus and mesothoracicus) in chicken embryos treated with insulin or pilocarpine. *Growth* 17, 87-109.
- LANDAUER, W. (1956). The teratogenic activity of pilocarpine, pilocarpidine and their isomers, with special reference to the importance of steric configuration. *J. exp. Zool.* 132, 39-50.
- LANDAUER, W. (1957). Nicotin antagonists and chick development. *J. exp. Zool.* 136, 509-530.
- LANDAUER, W. (1960a). Experiments concerning the teratogenic nature of thalium: polyhydroxy compounds, histidine and thiazole as supplements. *J. exp. Zool.* 143, 101-105.
- LANDAUER, W. (1960b). Nicotine-induced malformations of chicken embryos and their bearing on the phenocopy problem. *J. exp. Zool.* 143, 107-122.
- LANDAUER, W. & CLAWL, E. M. (1949). On the role of riboflavin in the teratogenic activity of boric acid. *J. exp. Zool.* 119, 307-312.
- LANDAUER, W. & RHODES, M. B. (1952). Further observations on the teratogenic nature of insulin and its modification by supplementary treatment. *J. exp. Zool.* 109, 221-251.
- LANDAUER, W. & WATANABE, N. (1967). Problems of acetazolamide and 6-aminonicotinamide as teratogens. *J. exp. Zool.* 204, 309-316.
- LANDAUER, W. & WATANABE, N. (1968). Teratological studies with sulphonamides and their implications. *J. Embryol. exp. Morph.* 26, 261-284.
- MEYERHOFF, O. (1919). Über die Atmung der Fröschmuskulatur. *Arch. ges. Physiol.* 175, 20-37.
- MILLER, J. R. (1962). A strain difference in response to teratogenic effect of maternal fasting in the house mouse. *Can. J. Genet. Cytol.* 4, 69-75.
- POLLARD, D. R. & FRASER, F. C. (1968). Further studies on a cytoplasmically transmitted difference in response to the teratogen 6-aminonicotinamide. *Teratology* 1, 335-338.
- ROBER, J. C. (1967). *Mode of Teratogenic Action of Organophosphate Esters in Hen Eggs*. Ph.D. thesis, University of California, Berkeley.
- ROGER, J. C., URRAGL, B. D. & CROWL, J. E. (1969). Structure-activity and metabolism studies for organophosphate teratogens and their alleviating agents in developing hen eggs with special emphasis on diazinon. *Modern. Pharmac.* 18, 373-392.
- ROGOVSKII, A. (1967). The effect of fasting combined with hydrocortisone acetate treatment on the formation of developmental disturbances of the fetus in pregnant mice. *Sov. Med. J.* 6, 1646-1649. (Also *S. Med. Morph.* 25, 313-319.)
- RÖHMANN, H. (1952). Über die teratogenetische Wirkung des O<sub>2</sub>-Mangels in der Fröschentwicklung. *Beitr. zool. Anat.* 112, 386-379.
- RUNNER, M. N. & MILLER, J. R. (1956). Congenital deformity in the mouse as a consequence of fasting. *Anal. Rec.* 124, 417-428.
- STAUDINGER, H., KRAUCH, R. & LEONHÄUSER, S. (1961). Role of ascorbic acid in microsomal electron transport and the possible relationship to hydroxylation reactions. *Ann. N.Y. Acad. Sci.* 92, 193-207.
- VERBURGH, A. C., POLLARD, D. R. & FRASER, F. C. (1968). A cytoplasmically transmitted, diet-dependent difference in response to the teratogenic effects of 6-aminonicotinamide. *Science*, N.Y. 160, 206-207.
- VERBURGH, A. C. & WATKINS, C. A. (1969). Effects of 6-aminonicotinamide on the mitochondria of C57BL/6J and A/J mice (Abstract). *Teratology* 2, 271-272.
- YASUDA, M., NANO, H. & SUZUKI, M. (1968). The effect of fasting upon the development of embryos in elderly pregnant mice. *Acta anat. nippon.* 41, 43-48.
- ZWILLING, E. & DUBELL, J. F. (1959). Micromelia and growth retardation as independent effects of sulfanilamide in chick embryos. *J. exp. Zool.* 115, 59-81.

9875

**Conversion of Succinic Acid to Glucose in the Phloridized Dog**

**RAYON M. MACKAY AND RICHARD H. JONES**

*From the College of Medicine, Case, La Jolla, California*

Koranyi and Szent-Györgyi have reported<sup>1</sup> that succinic acid will decrease the glucose in diabetes. Although other investigators<sup>2,3</sup> have been unable to confirm this observation it has renewed interest in the behavior of succinic acid in metabolism. Ringer, Frankel and Jones in a widely quoted study<sup>4</sup> found that extra glucose was consumed when sodium succinate was fed to the phloridized dog. It seemed desirable to reexamine this point for various reasons. Our experiments are summarized in Table I. All percentages are included with the exception that 5 gm. of NaCl were administered daily by stomach tube in order to obtain good urine volumes. The bladder was emptied by catheter at the end of each 24-hour period of urine collection. Urine ketones were determined

<sup>1</sup> Koranyi, A., and Szent-Györgyi, A. V., *Bioch. and Med. Res.*, 1937, 69, 1000.

<sup>2</sup> Szent-Györgyi, A. V., and Koranyi, A., *ibid.*, 1937, 69, 1011.

<sup>3</sup> Dooling, R. M., and Jones, R. H., *Lancet*, 1937, 285, 700.

<sup>4</sup> Ringer, A. J., Frankel, R. M., and Jones, R. H., *J. Biol. Chem.*, 1935, 14, 529.

TABLE I.

Day	Urine Excretion					Extra dextrose mm.	Succinic acid fed mm.	% succinic acid $\rightarrow$ glucose
	Urine Vol. ml.	Total acetone bodies gm.	Nitrogen gm.	Dextrose gm.	D:N			
Exp. 1.—Mongrel terrier, male, weight 21.7 kg. Fasted for 4 days before and throughout experiment. Given 1 gm. phloridzin in oil twice daily for 3 days before and throughout experiment.								
1	1220	5.24	6.48	21.9	3.36			
2	1630	2.54	11.24	25.4	3.16			
3	1295	6.15	10.11	34.6	3.41			
4	1740	0.93	8.51	39.7	4.53	20.8	42.4	98.0
5	1020	6.14	9.50	41.2	4.33	16.7	42.4	79.8
6	9020	4.97	8.17	27.2	3.24			
7	1360	5.08	7.90	24.7	3.27			
Exp. 2.—Spaniel, male, weight 19 kg. Fasted for 3 days before and throughout experiment. Given 1 gm. phloridzin in oil once daily for 3 days before and throughout experiment.								
1	1640	1.60	18.00	63.9	3.54			
2	940	2.71	14.41	61.6	3.59			
3	360	2.46	17.28	67.0	3.29			
4	980	1.32	10.30	67.2	5.34	56.4	424.0	17.2
5	650	0.60	6.52	48.0	7.33	66.8	424.0	21.5
6	620	2.03	9.68	34.6	3.57			
7	390	0.04	11.60	40.8	3.52			
Experiments of Ringer, Frankel and Jones ( <i>J. Biol. Chem.</i> , 1913, 24, 529).								
Succinic acid given per os						26.0	100.0	62.0
" " " subcutaneously						47.7	100.0	68.4

by Van Slyke's method, total nitrogen by the macro-Kjeldahl method and sugar by Benedict's method. The succinic acid was fed in the free state and there was no diarrhea.

In estimating dextrose formation from a fed compound by means of the D:N ratio in the urine of the phloridzinized dog it is necessary to base your conclusions upon the highest ratio which is obtained in various experiments. On this basis our results (Exp. 1, 4th day) confirm the best observation of Ringer, *et al.*,<sup>6</sup> and indicate that in the phloridzinized organism succinic acid may be entirely converted to dextrose (2 mols. succinic acid = 1 mol. dextrose). However, an explanation of the relatively low percentages of conversion of succinic acid to dextrose in our Exp. 2 when large doses were fed, is necessary. This is exactly what happens when large doses of sugar are fed to the phloridzinized organism. There is no intrinsic impairment of the ability to oxidize carbohydrate in the phloridzinized dog.<sup>6</sup> The unnatural gradient of tissue sugar  $\rightarrow$  blood sugar  $\rightarrow$  urine simply reduces or prevents its oxidation. If

<sup>6</sup> Deuel, H. J., Jr., *J. Biol. Chem.*, 1920, 80, 77.

enough carbohydrate or, as in this case, dextrose former is given, the gradient tissue sugar  $\rightarrow$  blood sugar, becomes reversed enough to approach normal and permit an increase in dextrose oxidation. The latter is evident in Exp. 2 in the antiketogenic and nitrogen sparing effects of the succinic acid.

*Summary.* In the phloridzinized dog the changes in the D:N ratio indicate that small doses of succinic acid are entirely converted to dextrose (2 mols. succinic acid = 1 mol. glucose). When larger doses are given a smaller percentage is excreted in the urine as sugar but anti-ketogenic and nitrogen sparing activity indicate its conversion to dextrose before being burned.

THE ANTIKETOGENIC ACTIVITY OF SUCCINIC ACID

By EATON M. MACKEY, JAMES W. SHERRILL, AND RICHARD H. BARNES

(From The Scripps Metabolic Clinic, La Jolla)

(Received for publication November 22, 1938)

Korányi and Szent-Györgyi have reported (1, 2) that relatively small doses of succinic acid would reduce or abolish the ketosis in diabetes. We have been unable to demonstrate this action of succinic acid. In Table I are presented three typical examples of the result of administering succinic acid to diabetic patients. All of these patients had been on their régime for some time and their diets were constant. They were given the free acid. The total ketone bodies in the

TABLE I  
The influence of succinic acid on ketosis in diabetes upon a fixed régime  
Urine excretion per day

Day	Total ketone bodies grams	Nitrogen grams	Glucose grams	Succinic acid per day grams
<i>Experiment 1*</i>				
1	3.21		10.2	
2	4.50		13.4	
3	3.96		7.0	
4	4.22		7.9	
5	5.28		9.3	10
6	4.12		12.5	10
7	5.30		22.3	10
8	4.20		18.4	10
9	5.40		21.3	10
10	2.10		3.2	
11	1.40		5.4	
12	0.09		1.1	
<i>Experiment 2†</i>				
1	1.02		3.2	
2	0.88		4.4	
3	0.74		4.8	5
4	1.10		4.6	5
5	0.68		3.2	5
6	1.30		5.8	5
7	2.80		20.2	30
8	2.10		4.3	
9	1.20		2.2	

\* *Experiment 1.* Female, 48 years of age, weighing 54.5 kgm. and receiving 24 units of insulin each day. The daily diet was composed of protein, 85 grams; fat, 145 grams; and carbohydrate, 120 grams. The dose of insulin was increased to 34 units on the tenth day.

† *Experiment 2.* Male, 26 years of age, weighing 75.6 kgm. and receiving 30 units of insulin each day. The daily diet was composed of protein, 120 grams; fat, 170 grams; and carbohydrate, 200 grams. On the eighth and ninth days, 42 units of insulin were given.

TABLE I—Continued

Day	Total ketone bodies grams	Nitrogen grams	Glucose grams	Succinic acid per day grams
<i>Experiment 3‡</i>				
1	2.81	8.9	8.2	
2	3.08	7.1	5.7	
3	0.68	6.5	8.6	
4	1.34	10.9	3.0	
5	2.56	13.5	33.9	50
6	2.37	9.7	23.6	15
7	1.71	8.5	5.6	
8	2.53	8.4	11.7	
9	2.07	9.9	13.0	

‡ *Experiment 3.* Female, 17 years of age, weighing 41.3 kgm. and receiving 28 units of insulin per day. The daily diet was composed of protein, 55 grams; fat, 140 grams; and carbohydrate, 150 grams.

urine were determined by Van Slyke's method (3), glucose according to Benedict (4), and total nitrogen by the macro-Kjeldahl (5). Small doses (Table I, Experiment 1) of succinic acid had no demonstrable effect upon the ketosis as measured by the ketonuria. This confirms the observations of several English investigators (6, 7). Larger doses of succinic acid had quite the opposite effect to the action claimed by Korányi and Szent-Györgyi. The ketonuria was actually increased (Table I, Experiments 2 and 3). In these cases the compound behaved in the same manner as might have been expected of glucose. This is hardly surprising for succinic acid is converted to glucose in the phloridzinized dog (8, 9).

In the fasting normal human being the glucose effect of succinic acid is even more obvious (Table II). Contrary to its behavior in the diabetic, succinic acid is nitrogen sparing and has marked antiketogenic activity such as might be expected from an equivalent amount of glucose in a subject of this kind. The antiketogenic activity of succinic acid in the normal fasting organism may also be demonstrated in fasting rats in which a ketonuria has been induced by feeding the sodium salt of  $\beta$ -hydroxybutyric acid (Table III). If

TABLE II  
The influence of succinic acid upon the ketosis of a fasting normal subject\*

Day	1	2	3	4	5	6
Total urine nitrogen, grams	5.13	7.00	6.44	5.12	4.03	7.91
Total ketone bodies, grams	2.17	5.87	5.96	2.23	0.45	6.70
Succinic acid fed, grams				50	50	

\* This individual was a female who was normal in every respect except for being slightly (5 to 10 kgm.) overweight. She was 38 years old and weighed 71 kgm. A general diet preceded the fast. While fasting, three cups of coffee without additions were allowed each day. The succinic acid was fed in dilute solution.

TABLE III  
Antiketogenic action of succinic acid in fasting rats\*

Group	Body weight	Body surface	Total nitrogen excreted per rat per day				Total ketone bodies excreted per rat per day					
			Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4		
A	grams	sq. dm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.
B	261	4.63	47	56	43	51	50	72	72	74	74	11
	256	4.56	41	45	35	39	15	13	13	13	11	

\* There were 10 male rats in each group which were fasted for a day before the experiment and throughout the experimental period. During the latter they were all given 1 cc. per sq. dm. of body surface of a 6.3 per cent solution of racemic sodium  $\beta$ -hydroxybutyrate twice each day. The solution given to Group B contained 5.9 per cent of succinic acid in addition.

sufficient of the succinic acid is administered the ketonuria will completely disappear as may be seen in another experiment.

It is reasonable to assume that succinic acid is antiketogenic in the normal organism because it is converted to glucose. The relative activity of these compounds in this regard should throw some light upon this point. An excellent ketosis may be produced in rats if they are fasted after a period upon a diet low in choline and protein which results in a fatty liver (10, 11, 12). When a high fluid intake is provided so as to insure large urine volumes the ketonuria becomes a good measure of the degree of ketosis. The ketosis increases to a maximum on about the third day of fasting and then tapers off. In comparing the antiketogenic action of the two compounds two millimols of succinic acid were fed for comparison with each millimol of glucose. This is the theoretical relation between these substances if three carbon atoms of succinic acid go to glucose (8), and under optimal conditions one millimol of

glucose may be obtained from each two millimols of succinic acid fed to the phloridzinized dog (8, 9). Our results in Table IV show that succinic acid has essentially the same antiketogenic activity as an equivalent amount of glucose.

Substances which exhibit antiketogenic activity are generally, if not always, good glycogen formers (13, 14). Succinic acid is no exception. The results in Table V demonstrate that this compound is practically as good a glycogen former as is glucose.

#### DISCUSSION

We are unable to explain why we and others (6, 7) have been unable to corroborate in any degree the findings of Korányi and Szent-Györgyi concerning the desirable therapeutic effect of succinic acid in human diabetes in that the ketosis may be reduced or controlled by this compound. It is probable that their patients had not been adequately controlled before the study and were exhibiting the benefit of other therapeutic measures which had been showing their effects slowly when the succinic acid happened to be given. There is also, of course, the very remote possibility that if diabetics differ in the cause of their disturbed metabolism one type of case might be benefited by succinic acid, and it happened that the four patients treated by these authors were of this type.

Although not absolute proof, the fact that the antiketogenic activity of succinic acid is proportional to the glucose it may form is strong evidence that the antiketogenic action of succinic acid in the intact organism is simply a result of its conversion to glucose and is not due to any catalytic action on metabolism such as Szent-Györgyi and coworkers (15) and others (16) believe possible.

Our conclusion that in the normal organism succinic acid is as good an antiketogenic agent as an equivalent quantity of glucose is opposed to the conclusions of Deuel *et al.* (17) regarding the activity of this substance. A perusal of their data shows why they failed to obtain the same results. They fed sodium succinate. The succinic acid was oxidized leaving a substantial amount of alkali. Alkali is very ketogenic (18, 19) so that while their glucose-fed group had only the formation of ketones by the fasting fatty

TABLE IV  
The relative antiketogenic activity of succinic acid and glucose in fasting rats

Group	Sex	Body weight grams	Body surface <sup>a</sup> sq. cm.	Excretion per sq. cm. of body surface per day								Dose per sq. cm. of body surface per day		
				Total ketone bodies				Total nitrogen				NaHCO <sub>3</sub> mm.	Glucose mm.	Succinic acid mm.
				Day 1 mgm.	Day 2 mgm.	Day 3 mgm.	Day 4 mgm.	Day 1 mgm.	Day 2 mgm.	Day 3 mgm.	Day 4 mgm.			
<i>Experiment 1†</i>														
A	♂	201	390	1	4	54	13					2.0		
B	♂	188	373	2	41	77	29					2.0		
B	♀	207	397	0	2	21	14					2.0	0.25	
B	♀	161	336	1	10	52	27					2.0	0.25	
C	♀	211	403	0	0	0	0					2.0	0.50	
C	♀	161	336	1	1	7	0					2.0	0.50	
D	♀	211	403	1	5	31	10					2.0		0.5
D	♀	188	373	2	9	37	34					2.0		0.5
E	♀	222	416	0	0	0	0					2.0		1.0
E	♀	161	336	1	1	3	0					2.0		1.0
<i>Experiment 2‡</i>														
A	♀	167	344	46	53	36	61	22	17	16	17	2.0		
B	♀	165	340	27	19	15	16	18	14	13	18	2.0		
B	♀	165	340	5	1	0	0	15	13	14	16	2.0	0.33	
C	♀	167	344	33	26	19	22	15	15	15	15	2.0		0.5
E	♀	166	342	9	8	1	0	16	14	14	15	2.0		1.0
<i>Experiment 3§</i>														
A	♀	161	336	19	20	28	15							
A	♀	165	340	46	30	36	5							
B	♀	171	350	0	2	0	0						0.25	
B	♀	168	345	0	6	9	4						0.25	
B	♀	161	336	0	0	0	0						0.75	
C	♀	159	333	0	0	0	0						0.75	
C	♀	186	370	0	2	2	0							0.5
D	♀	157	330	0	8	6	1							0.5
D	♀	183	366	0	0	0	0							1.5
E	♀	165	340	0	0	0	0							1.5
<i>Experiment 4  </i>														
A	♀	151	322	4	10	8	6	27	24	23	19	2.5		
A	♀	138	304	3	15	10	6	31	29	27	22	2.5		
B	♀	157	330	0	0	1	0	22	27	26	18	2.5	0.25	
B	♀	144	310	0	1	0	0	23	24	26	16	2.5	0.25	
B	♀	150	321	0	2	0	0	33	29	26	21	2.5		0.5
C	♀	137	302	1	0	0	0	29	27	24	22	2.5		0.5

\* In all of these experiments "Body surface" was calculated from the formula (20) that we have generally used. The methods of urine collection and administering the various solutions have already been described (22, 23).

† Rats which had been on the low protein fatty liver producing diet (12) for 17 days were used. Urine collections were made and the various solutions administered beginning on the first day of fasting. There were 6 rats in each group, the averages for which are given here. The solutions were administered in two doses each day, the succinic acid in 2.95 and 5.90 per cent, the glucose in 2.25 and 4.50 per cent, and the NaHCO<sub>3</sub> in 8.4 per cent solution.

‡ The rats had been on the special diet for 15 days. Collections were made on and after the second day of fasting. Due to an error in calculation (the incorrect assumption being made that all four carbon atoms of succinic acid form glucose) the glucose fed rats received more glucose than the equivalent of the succinic acid fed, and the antiketogenic action was consequently greater. The concentration in the solutions of succinic acid was 2.95 and 5.90 per cent, of glucose 3.0 and 6.0 per cent, and of NaHCO<sub>3</sub> 8.4 per cent. The results are averages for 5 rats in each group.

§ In this experiment there were 5 rats in each group and they had been on the fatty liver producing diet for 17 days. Urine collections were commenced on the first day of fasting. The succinic acid solutions were not neutralized, nor was NaHCO<sub>3</sub> fed to any of the other groups. All of the rats were given 1 cc. per sq. cm. of body surface of 1 per cent NaCl twice each day and other substances administered were incorporated in this glucose, in 2.25 and 7.75 per cent and succinic acid in 2.95 and 9.84 per cent solution.

|| These rats were fasted directly from the stock diet, collections being commenced on the second day of fasting. The ketonuria is due solely to the alkalosis produced by the NaHCO<sub>3</sub>. This was fed in 10.5 per cent, the glucose in 2.25 per cent and the succinic acid in 2.95 per cent solutions. The results are averages for 6 rats in each group.

TABLE V  
 Glycogenic activity of succinic acid and glucose in the albino rat\*

Group	Body weight	Body surface	Liver weight	Liver glycogen				Amount fed per sq. cm. of body surface		
				Minimum	Maximum	Average	Average	NaHCO <sub>3</sub>	Glucose	Succinic acid
				grams	grams	per cent	per cent	per cent	mgm. per sq. cm. of body surface	mm.
A	150	3.20	4.12	0.2	0.8	0.6	8	5.0		
B	149	3.18	4.92	7.1	15.4	9.6	148	5.0	1.25	
C	150	3.21	4.82	6.3	12.9	8.8	138	5.0		2.50

\* Each group was composed of 12 male rats. They were fasted for 24 hours and then given by stomach tube every 4 hours 1 cc. per sq. cm. of body surface of the various solutions over a period of 20 hours, when they were anesthetized with nembutal and the liver glycogen determined (21). All of the solutions contained 8.4 per cent NaHCO<sub>3</sub>. In Group B there was 4.5 per cent glucose and in Group C 5.9 per cent succinic acid in addition.

liver rats to overcome, the group fed sodium succinate had in addition the ketogenic influence of the alkali to overcome. Experiment 4 in Table IV shows the effect on ketosis of alkali in the form of a dose of sodium bicarbonate comparable to the alkali involved in these experiments. When fasting from the stock diet without alkali administration there is no measurable ketonuria. Whenever we fed sodium succinate we naturally added an equivalent amount of sodium bicarbonate to the control and glucose-fed groups so that the observations in a given experiment would be comparable.

#### SUMMARY

Succinic acid has no antiketogenic activity in the human diabetic. In a normal fasting person it is as antiketogenic as an equivalent amount of glucose, to which succinic acid is converted in the phloridzinized and probably also in the normal organism.

The ketosis of fasting rats which previously had been receiving a fatty liver producing diet is reduced in the same degree by glucose as by an equivalent amount of succinic acid. These compounds are also almost equally good glycogen formers.

#### BIBLIOGRAPHY

- Korányi, A., and v. Szent-Györgyi, A., Curing acidosis of diabetics by means of succinic acid treatment. *Orvosi hetil.*, 1937, 81, 615. Cited from *Chem. Abstr.*, 1937, 31, 6335.
- Korányi, Andreas and v. Szent-Györgyi, Albert, Ueber die Bernsteinsäurebehandlung diabetischer Azidose. *Deutsche med. Wchnschr.*, 1937, 63, 1029.
- Van Slyke, D. D., Studies in acidosis. VII. The determination of  $\beta$ -hydroxybutyric acid, acetoacetic acid, and acetone in urine. *J. Biol. Chem.*, 1917, 32, 455.
- Benedict, S. R., The detection and estimation of glucose in urine. *J. A. M. A.*, 1911, 57, 1193.
- Peters, J. P., and Van Slyke, D. D., Quantitative Clinical Chemistry. Vol. II. Methods. Williams and Wilkins Co., Baltimore, 1932, p. 534.
- Lawrence, R. D., McCance, R. A., and Archer, N., Clinical memoranda: Succinic acid treatment of diabetic ketosis. *Brit. M. J.*, 1937, 2, 214.
- Dunlop, D. M., and Arnott, W. M., Effect of succinic acid on diabetic ketosis. *Lancet*, 1937, 2, 738.
- Ringer, A. I., Frankel, E. M., and Jonas, L., The chemistry of gluconogenesis. IV. The fate of succinic, malic, and malonic acids in the diabetic organism, with consideration of the intermediary metabolism of aspartic and glutamic acids, proline, lysine, arginine, and ornithine. *J. Biol. Chem.*, 1913, 14, 539.
- MacKay, E. M., and Barnes, R. H., Conversion of succinic acid to glucose in the phloridzinized dog. *Proc. Soc. Exper. Biol. and Med.*, 1938, 39, 417.
- Best, C. H., and Channon, H. J., The action of choline and other substances in the prevention and cure of fatty livers. *Biochem. J.*, 1935, 29, 2651.
- Channon, H. J., and Wilkinson, H., Protein and the dietary production of fatty livers. *Biochem. J.*, 1935, 29, 350.
- MacKay, E. M., The influence of a pancreas extract ("fat metabolizing hormone") upon fat deposition in the liver on a low protein diet. *Am. J. Physiol.*, 1937, 119, 783.
- Shapiro, I., Studies on ketosis. V. The comparative glycogenic and ketolytic action of glucose and some carbohydrate intermediates. *J. Biol. Chem.*, 1935, 108, 373.
- Mirsky, I. A., Heiman, J. D., and Broth-Kahn, R. H., The antiketogenic action of glucose in the absence of insulin. *Am. J. Physiol.*, 1937, 118, 270.

15. Anna, E., Banga, I., Blazsó, A., Bruckner, V., Laki, K., Straub, F. B., and Szent-Györgyi, A., Ueber die Bedeutung der Fumarsäure für die tierische Gewebsatmung. *Ztschr. f. physiol. Chem.*, 1936, **244**, 105.
16. Krebs, H. A., The intermediate metabolism of carbohydrates. *Lancet*, 1937, **2**, 736.
17. Deuel, H. J., Jr., Murray, S., and Hailman, L., A comparison of the ketolytic effect of succinic acid with glucose. *Proc. Soc. Exper. Biol. and Med.*, 1937, **37**, 413.
18. Beumer, H., and Soecknick, A., Ueber organische Acidose bei anorganischer Acidose und Alkalose. *Ztschr. f. Kinderh.*, 1924, **37**, 236.
19. Forster, O., and Lipschütz, H., Ueber Azetonurie und Alkalose. *Arch. f. exper. Path. u. Pharmacol.*, 1923, **97**, 379.
20. Catman, G. G., and Mitchell, H. H., Estimation of the surface area of the white rat. *Am. J. Physiol.*, 1926, **76**, 380.
21. Good, C. A., Kramer, II., and Somogyi, M., The determination of glycogen. *J. Biol. Chem.*, 1933, **100**, 465.
22. MacKay, E. M., and Barnes, R. H., Influence of adrenalectomy on ketosis of fasting and on the action of the anterior pituitary ketogenic principle. *Am. J. Physiol.*, 1937, **118**, 184.
23. MacKay, E. M., and Barnes, R. H., Influence of adrenalectomy upon ketolytic activity. *Am. J. Physiol.*, 1938, **122**, 101.

THE NEPHROPATHIC ACTION OF THE DICARBOXYLIC  
ACIDS AND THEIR DERIVATIVES

I. TARTARIC, MALIC AND SUCCINIC ACIDS<sup>1</sup>

WILLIAM C. ROSE

*From the Laboratories of Physiological Chemistry of the University of Texas,  
Galveston, and the University of Illinois, Urbana*

Received for publication May 23, 1924

In 1907, Baer and Blum (1) tested the influence of a variety of organic acids upon the output of sugar and acetone bodies in diabetes. They found that, of the substances employed, sodium glutarate was unique in that its subcutaneous administration to phlorhizinized dogs led to marked decrease in, or entire disappearance of sugar from the urine, accompanied by a great fall in output of nitrogen and acetone bodies. They stated that "Die Wirkung der Säure erstreckt sich nicht auf die Ausscheidung von vorgebildetem Zucker, sondern auf die Bildung von Zucker aus anderem Material als Kohlenhydraten." In later communications (2), (3) they reported that several other acids likewise manifested the "glutaric acid effect," notably, tartaric, adipic, pimelic, suberic, and glutaconic acids. Several mono-, di-, and tri-hydroxy derivatives of glutaric acid also gave positive results. On the other hand, malonic, succinic, pyrotartaric, tartronic, malic,  $\alpha$ -hydroxy glutaric, and glutamic acids were without influence. Azelaic and sebacic acids did not affect the output of total nitrogen and sugar, but caused decreases in the elimination of acetone bodies. Mucic acid was said to give anomalous results because of its toxicity.

<sup>1</sup>Aided by a grant from the Research Fund of the American Association for the Advancement of Science. A preliminary report of this investigation appeared in the Proceedings of the American Society of Biological Chemists, Jour. Biol. Chem., 1922, 1, xxiii.

Soon after the work of Baer and Blum, Underhill (32), and Underhill, Wells and Goldschmidt (36) showed very convincingly that in the case of tartaric acid, the decreases in output of urinary ingredients are due to renal changes, and not, as suggested by Baer and Blum, to metabolic alterations. Underhill and his associates found that tartrates, when administered subcutaneously to either phlorhizinized or normal animals, produce an acute nephritis chiefly involving the convoluted tubules. Wells, who made the histological examinations, stated that following tartrate administration "the greater part of the epithelium of the convoluted tubules is entirely necrotic, and most of the tubules, almost all, in fact, are occluded by large hyaline and granular casts, frequently containing more or less hemoglobin . . . . The glomerules show almost no change beyond an occasional small hemorrhage" (32). Following the intravenous administration of sodium chloride-urea mixtures in tartrate-nephritic animals (37), the sodium chloride was completely recovered in 48 hours, while very little or none of the urea reappeared in the urine. In the light of these data, and of the histological findings, the authors conclude that under normal conditions chlorides and water pass through the glomerular mechanism, whereas urea becomes a urinary constituent by way of the convoluted tubules.

It is strange that practically none of the other acids reported as "active" by Baer and Blum have been examined in regard to their renal effects, despite the fact that the development of the modern methods of blood analysis has provided a very delicate means of detecting alterations in kidney function. Information concerning the nephropathic action of the dicarboxylic acids is desirable especially from two viewpoints. First, such studies would appear to offer an unusual opportunity of investigating the relationship of chemical constitution to physiological action. Second, a knowledge of the effects of the dicarboxylic acids upon kidney activity is essential before reliable studies can be made of the method or degree of oxidation of these acids in the body. Obviously, determinations of the sugar output in phlorhizinized animals, or of the percentage recovery in the urine of a compound whose fate in the body is being sought,

may lead to grossly erroneous deductions if the administered substance manifests nephropathic action. With these considerations in mind, and as a preliminary to studies of the mode of oxidation of dicarboxylic acids, we have undertaken a rather comprehensive investigation of their influence upon renal function.

In this communication we report the results of comparative studies with tartaric, malic and succinic acids. We have begun with tartaric acid because, (a) data obtained with an agent known to be nephropathic would serve as a basis for comparison with results secured in the use of other acids; and (b) a search of the literature has revealed the fact that information concerning the distribution of the blood ingredients following tartrate administration is exceedingly meager. Karsner and Denis (14) reported marked increases in the non-protein nitrogen and urea of the blood in cats, and Underhill and Blatherwick (33), and Potter and Bell (22) observed pronounced decreases or reductions to zero in the excretion of phenolsulphonaphthalein in rabbits following the subcutaneous administration of tartrates. More recently, Underhill and Wakenan (34) found decreases in the chloride concentration of the blood, and corresponding increases in the relative blood volumes in tartrate-nephritic animals.

Data based upon studies of the urine, and histological examinations of the kidneys, indicate that the characteristic renal lesions occur regardless of the mode of administration of the tartrate, provided the latter reaches the circulation (19). When given by mouth, much larger doses are required than when the substance is introduced parenterally (30), (19), (25). Probably this difference is due in part to the development of diarrhoea following oral administration, and the resulting rapid removal of the salt from the intestine (19). Perhaps the "selective action" of the intestinal cells may also play a part in preventing rapid tartrate absorption. In human subjects, Post (21) was unable to obtain evidence of albuminuria or cast formation after giving medicinal doses of Rochelle salts to normal individuals, nor could he demonstrate an aggravation of an existing nephritis following oral tartrate administration.

In animals, the character of the diet has been said to alter the degree of susceptibility. Thus Salant and his associates (25), (26), (27) report that carrot-fed rabbits are more resistant to tartrates than are similar animals living upon oats and hay. Probably this observation may be explained on the basis of the earlier findings of Underhill, Wells, and Goldschmidt (36), namely, that alkalis exert a beneficial action upon the excretory power of the tartrate-nephritic kidney. Carrots yield a decidedly basic ash while oats contain a preponderance of acid-forming elements.

All four of the tartaric acids are markedly toxic. Chabrié (6) regarded the levo variety as most toxic, and the inactive least, with the dextro and racemic forms occupying intermediate positions. More recent studies indicate that there are no decided differences in action of the four isomeric forms (cf. Salant and Smith (25), and Underhill, Wells, and Goldschmidt (36)).

Apparently tartrates are oxidized in the animal organism only with great difficulty, but Neuberg and Saneyoski (17), contrary to the opinion of Brion (5), were unable to demonstrate any differences in this regard between the dextro, levo, and racemic acids. Inasmuch as such studies were based upon the assumption that all unrecovered tartrate had been oxidized, the results are untrustworthy, since the tartrate-nephritic animal has doubtless lost the power of tartrate excretion (35).

The literature contains practically no information concerning the effects of malic and succinic acids upon renal activity. Wise (30) concluded from experiments upon rabbits and cats that the subcutaneous injection of sodium l-malate, in amounts not exceeding 1 gram per kilo, was not followed by nephritis. His deduction was based upon tests of the urines for albumin. Both the dextro and levo malic acids, as well as succinic acid, are said to be oxidized in the animal body (cf. Pohl (20), and Ohta (18)). Of the malic acids, the l-form is apparently more easily destroyed than is its isomer (30). Ringer, Frankel and Jonas (23) found that both succinic and malic acids yield extra glucose in phlorhizinized dogs. These authors do not state whether the malic acid employed was the levo or racemic form.

## EXPERIMENTAL

In each experiment one or more samples of blood were submitted to analysis before the administration of the acid, in order to determine the normal proportions of the blood ingredients. The acids were always exactly neutralized by the addition of the calculated quantity of sodium carbonate, and were administered subcutaneously. The blood was analyzed at stated intervals after the injection, and the renal function was further tested by the phenolsulphonaphthalein test of Geraghty and Rowntree (12). In the latter, the dye was administered intramuscularly, and the urines were collected in two periods of seventy and sixty minutes respectively by gentle abdominal pressure. Rabbits were employed as the experimental animals throughout. Unless otherwise stated, the blood samples were always obtained from the marginal ear veins. Each animal was allowed to fast for the duration of the experiment in order to avoid the possible influence of food consumption upon blood composition, and upon the toxicity of the acid in question.

The non-protein nitrogen, urea, and creatinine were determined by the Folin-Wu procedures (10), sugar by the modified method of the same authors (11), chlorides by the Whitehorn method (38), and cholesterol by the method of Autenrieth and Funk as described by Folin (9). In the single experiment in which the urines were analyzed, total nitrogen was estimated by the Kjeldahl-Gunning procedure after removal of protein, and chlorides by the Volhard-Arnold method.

A description of the acids employed is given below.

*d-tartaric acid 1.* A beautifully crystalline preparation secured from de Haën. Melting point, 165° to 166°C.; theoretical, 168° to 170°C.

*d-tartaric acid 11.* Prepared by recrystallizing acid 1 from hot water, filtering, and washing with alcohol and ether. Melting point, 168° to 170°C.; theoretical, 168° to 170°C.

*dl-malic acid 2.* A "chemically pure" preparation having a very slight gray color. Tests for the presence of oxalic acid were entirely negative. Melting point, 127° to 128°C.; theoretical, 130° to 131°C.

*l-malic acid 3.* A beautifully crystalline colorless preparation giving absolutely no test for oxalic acid. Melting point not determined.

*Succinic acid 4.* A snow-white product obtained by recrystallizing a commercial preparation. Gave no test for oxalic acid. Melting point, 185°C.; theoretical, 185°C.

*Succinic acid 14.* A white crystalline preparation. Was used without previous recrystallization. Gave absolutely no test for oxalic acid. Melting point, 185°C.; theoretical, 185°C.

TABLE I  
Control Experiment  
Rabbit 3, female, 2155 grams

DATE	PERCENTAGE BLEEDING	BLOOD						NOTES, ETC.
		Non-protein nitrogen	Urea N	Urea Nitro	Phos	NaCl	Uric acid	
May 19.....	50*	50.8	25.9	1.5	0.131	0.49	0.21	Water ad lib, but no food
May 20.....	50*		24.2			0.51		9:30 a. m., 9 cc. blood removed. 11:00 a. m., renal test
May 21.....								8:45 a. m., 11 cc. blood removed
May 22.....								
May 23.....	70†	46.9	23.4	1.5	0.134	0.50	0.27	10:00 a. m., 9 cc. blood removed. 10:30 a. m., renal test
May 24.....			25.1			0.54		9:45 a. m., 5 cc. blood removed
May 25.....	76‡	46.0	21.6	1.6	0.131	0.33	0.29	9:45 a. m., 8 cc. blood removed. 10:10 a. m., renal test. Experiment discontinued.

\* First period, 13 cc. urine containing 60 per cent dye; second period, 7 cc. urine containing 20 per cent dye; total, 80 per cent.

† First period, 7 cc. urine containing 40 per cent dye; second period, 4 cc. urine containing 30 per cent dye; total, 70 per cent.

‡ First period, 17 cc. urine containing 55 per cent dye; second period, 27 cc. urine containing 21 per cent dye; total, 76 per cent.

*Control experiments.* In order to determine the influence upon blood composition of the experimental procedures—inattention and the moderate hemorrhage incidental to the daily removal of samples of blood for analysis—several control experi-

ments were carried out. These were conducted in the same manner as the other experiments with the exception that no acid was administered. The results of two such controls are summarized in tables 1 and 2. Cholesterol and chlorides progressively increased, the latter especially in the experiment out-

TABLE 2  
*Control Experiment*

DATE	FURFURALIN SOLUTION PER CENT	BLOOD			NOTES, ETC.
		Non-protein nitrogen	Urea-nitrogen	NaCl	
		mgm.	mgm.	per cent.	
March 25.....					Animal placed in cage at 7:00 p.m. Water ad lib., but no food
March 26.....	78*	40.3	1.6	0.50	9:30 a.m., 8 cc. blood removed. 2:45 p.m., renal test
March 27.....		47.4	1.4	0.52	9:30 a.m., 7 cc. blood
March 28.....		46.9	1.5	0.54	9:30 a.m., 7 cc. blood
March 29.....	81†	51.4		0.57	9:30 a.m., 7 cc. blood. 9:30 a.m., renal test
March 30.....		93.5	1.6	0.62	1:00 p.m., 7 cc. blood
March 31.....	87‡	90.0	1.5	0.64	9:30 a.m., 8 cc. blood. 9:50 a.m., renal test. 4:00 p.m., animal quite weak. Body weight, 1340 grams. Began feeding oats and hay, and continued until April 5
April 5.....		41.7	1.4	0.53	9:30 a.m., 8 cc. blood. Body weight, 1465 grams. Experiment discontinued

\* First period, 6 cc. urine containing 44 per cent dye; second period, 4 cc. urine containing 34 per cent dye; total, 78 per cent.

† First period, 11 cc. urine containing 68 per cent dye; second period, 2 cc. urine containing 18 per cent dye; total, 81 per cent.

‡ First period, 12 cc. urine containing 69 per cent dye; second period, 10 cc. urine containing 18 per cent dye; total, 87 per cent.

lined in table 2. In rabbit 3 (table 1), the sugar and nitrogenous components of the blood remained quite constant. In most experiments, however, the non-protein nitrogen values showed pronounced increases after the first few days of the fasts. Typical

results of this kind are presented in table 2. As will be observed, the non-protein nitrogen rose to more than 90 mgm. on the fifth day. That this was not due to the development of a spontaneous nephritis is indicated by the phenolsulphonephthalein tests, the normal values for blood creatinine, and the return to normal of the non-protein nitrogen after feeding the animal for a few days. Similar results have been obtained in several other controls. Evidently the rise in non-protein nitrogen is associated with the increased protein catabolism induced by fasting. As might be expected, the period of time necessary for an increase to occur is variable, and depends upon the nutritive condition of the animal at the beginning of the experiment. In exceptional cases, a week or more may elapse, while in other animals three or four days may be sufficient to occasion distinct effects.

These facts must always be taken into account in interpreting experimental data obtained with moderately toxic or non-toxic substances. It is noteworthy that in all of our controls, the creatinine remained unaffected, as did the phenolsulphonephthalein elimination. Variations of 8 to 10 per cent in dye recovery are not to be regarded as indicative of alterations in renal permeability, inasmuch as the small volumes of urine secreted in rabbits during the sixty- and seventy-minute periods can not be quantitatively pressed from the bladders.

The normal values for non-protein nitrogen and urea nitrogen in rabbits are almost double the quantities of these components present in human blood. Moreover, there appears to be much wider individual variations in different rabbits, and in the same rabbit at different times, than are observed in men. It is thus necessary to determine the "normal" for each animal before administering the acid to be tested.

*Tartaric acid.* In tables 3 to 6 inclusive are presented data obtained after the subcutaneous administration of d-tartaric acid as its sodium salt. As will be observed, not only did the non-protein nitrogen and urea of the blood markedly increase, as was previously shown by Karsner and Denis (14), but the quantities of creatinine, sugar, and cholesterol also manifested enormous increments. Chlorides, on the contrary, generally

TABLE 3  
*d*-tartaric acid  
 Rabbit 2, female, 3200 grams

DATE	ACID ADMIN- ISTERED	URINARY RE- COVERED	URINE						OTHER, ETC.
			Non- protein nitro- gen	Free N	Creati- nine	Sugar	NaCl	Chloro- form	
			mgm.	mgm.	mgm.	per cent	per cent	per cent	
April 18.....		78*							No food; water ad lib. 3 p.m., renal test
April 19.....			33.3	14.3	1.6	0.138	0.53	0.16	10:15 a.m., 10 cc. blood removed
April 19.....	1.0								12:00 n., acid 1 injected subcutaneously as so- dium salt in 15 cc. water
April 20.....		0†	75.2	47.2	8.6	0.135	0.50	0.17	10:00 a.m., 7 cc. blood. 11:10 a.m., renal test
April 21.....			112.6	86.3	14.1	0.148	0.47	0.17	10:00 a.m., 7 cc. blood
April 22.....		1‡	126.4	97.6	13.5	0.146	0.41	0.20	10:00 a.m., 7 cc. blood. 10:20 a.m., renal test
April 23.....		1.8§	125.6	93.7	13.9	0.190	0.41	0.24	9:15 a.m., 8 cc. blood. 9:45 a.m., renal test
April 24.....									
April 25.....		4.5¶	162.6	169.8	13.1	0.267	0.44	0.30	9:15 a.m., 5 cc. blood. 10:20 a.m., renal test. Animal died at 2:00 p.m.

\* First period, 31 cc. urine containing 60 per cent dye; second period, 4 cc. urine containing 18 per cent dye; total, 78 per cent.

† First period, 19 cc. urine containing 0 per cent dye; second period 0 cc. urine containing 0 per cent dye; total, 0 per cent.

‡ First period, 35 cc. urine containing 0.5 per cent dye; second period, 10 cc. urine containing 0.5 per cent dye; total, 1 per cent.

§ First period, 8 cc. urine containing 0.8 per cent dye; second period, 7 cc. urine containing 1.0 per cent dye; total, 1.8 per cent.

¶ First period, 18 cc. urine containing 2 per cent dye; second period, 18 cc. urine containing 2.5 per cent dye; total, 4.5 per cent.

TARTARIC, MALIC AND SUCCINIC ACIDS

TABLE I  
*D-tartaric acid*  
 Rabbit 19, male, 2945 grams

DATE	ACID ADMIN- ISTRATION	POTASSI- UM RE- TENTION	Non- protein nitro- gen	URINE				NOTES, ETC.
				Urea N	Urea- time	Sugar	NaCl	
	grams	per cent	mgm.	mgm.	mgm.	per cent	per cent	
February 23.....								No food; water ad lib.
February 24.....		70*	36.1		1.6	0.108	0.52	9:00 a.m., 9 cc. blood removed. 12:30 p.m., renal test
February 25.....		70†						9:50 a.m., renal test
February 26.....	1.0							1:00 p.m., acid 1 injected subcutaneously as sodium salt in 16 cc. water
February 26.....								
February 27.....		2‡	141.5	100.5	10.8	0.125	0.45	9:00 a.m., 10 cc. blood. 10:20 a.m., renal test
February 28.....			171.5	114.3	13.3	0.138	0.47	9:00 a.m., 8 cc. blood
March 1.....		1§	170.5	115.4	10.5	0.171	0.52	9:00 a.m., 6 cc. blood. 10:40 a.m., renal test
March 2.....								
March 3.....			130.5	101.3	6.8	0.171	0.56	9:00 a.m., 7 cc. blood. Experiment discontinued

\* First period, 5 cc. urine containing 46 per cent dye; second period, 3 cc. urine containing 24 per cent dye; total, 70 per cent.

† First period, 4 cc. urine containing 55 per cent dye; second period, 4 cc. urine containing 21 per cent dye; total, 70 per cent.

‡ First period, 5 cc. urine containing 2 per cent dye; second period, 4 cc. urine containing 0 per cent dye; total, 2 per cent.

§ First period, 7 cc. urine containing 0.5 per cent dye; second period, 2 cc. urine containing 0.5 per cent dye; total, 1 per cent.

TABLE 6  
*d*-lactic acid  
 Rabbit 21, male, 2400 grams

DATE	ACID ADMINISTERED	WATER INTAKE	URINE VOLUME	URINARY DYE CONCENTRATION	BLOOD				NOTES, ETC.
					Non-protein nitrogen	Cr. concn	Sugar	NaCl	
	grams	cc.	cc.	per cent	mgm.	mgm.	per cent	mgm. c.c.f.	
March 16.....									No food; water ad lib.
March 17.....		64	41	71 <sup>*</sup>	40.8	1.5		0.50	9:15 a.m., 7 cc. blood removed. 9:50 a.m., renal test.
March 17.....	0.5								5:30 p.m., acid 1 injected subcutaneously as sodium salt in 15 cc. water
March 18.....		3	1	Trace <sup>†</sup>	71.0	3.1		0.48	9:15 a.m., 7 cc. blood. 9:20 a.m., renal test
March 19.....		22	3						
March 20.....		48	25		193.5	11.2		0.47	9:20 a.m., 8 cc. blood
March 21.....		32	72	Trace <sup>‡</sup>	221.5	13.2		0.46	9:30 a.m., 7 cc. blood. 10:00 a.m., renal test
March 22.....		20	149		265.5	16.9	0.167	0.44	9:30 a.m., 7 cc. blood
March 23.....		100	139	1 <sup>‡</sup>	307.7	16.5		0.46	9:30 a.m., 6 cc. blood. 9:40 a.m., renal test
March 24.....		147	176		283.0	12.5		0.49	9:40 a.m., 5 cc. blood
March 25.....				2 <sup>‡</sup>	272.7	10.8		0.49	9:40 a.m., 6 cc. blood. 11:00 a.m., renal test. 3:00 p.m., animal was very weak and was chloroformed

\* First period, 20 cc. urine containing 3 $\frac{1}{2}$  per cent dye; second period, 3 cc. urine containing 20 per cent dye; total, 71 per cent.

† First period, 0.5 cc. urine containing trace of dye; second period 0.5 cc. urine containing trace of dye; total, trace.

‡ First period, 4 cc. urine containing trace of dye; second period, 2 cc. urine containing trace of dye; total, trace.

§ First period, 6 cc. urine containing 0.5 per cent dye; second period, 5 cc. urine containing 0.5 per cent dye; total, 1 per cent.

¶ First period, 9 cc. urine containing 1 per cent dye; second period, 5 cc. urine containing 1 per cent dye; total, 2 per cent.

TABLE I  
*d*-tartaric acid  
 Rabbit 6, male, 1940 grams

DATE	ACID AD- MINIS- TERED	FURTHER SIN RE- COVERED	WATER INGEST.	URINE			BLOOD			NOTES, ETC.
				Vol- ume	Total N	NaCl	Non- protein nitro- gen	Creati- nine	NaCl	
	grams	per cent	cc.	cc.	mgm.	mgm.	mgm.	mgm.	per cent	
April 10.....										No food; water ad lib.
April 11.....		70*	127	126	707	109				9:15 a.m., renal test
April 12.....			226	215	759	80	43.2	1.4	0.52	9:30 a.m., 7 cc. blood removed
April 13.....	4.25									9:30 a.m., acid 11 injected as sodium salt in 15 cc. water
April 13.....			69	8	31	20				
April 14.....		0†	72	104	291	420	93.7	7.6	0.47	10:00 a.m., 7 cc. blood. 10:05 a.m., renal test
April 15.....			96	84	539	143	136.4	11.0	0.45	9:45 a.m., 7 cc. blood
April 16.....			67	102	859	68				
April 17.....		21‡	119	129	1017	63	130.4	6.6	0.48	9:30 a.m., 7 cc. blood. 10:00 a.m., renal test
April 18.....							88.0	3.9	0.50	9:45 a.m., 7 cc. blood
April 18.....	0.25		106	70	458	110				1:00 p.m., acid 11 injected as sodium salt in 12 cc. water
April 19.....		8§	115	128	808	167	95.5	6.7	0.45	9:45 a.m., 7 cc. blood. 11:10 a.m., renal test

April 20.....		138	132	763	68	81.6	5.5	0.46	10:00 a.m., 6 cc. blood
April 21.....		115	98	497	38				
April 22.....	42°					42.0	2.7	0.45	9:45 a.m., 6 cc. blood. 10:00 a.m., renal test. 1:00 p.m., experiment discontinued

\* First period, 2 cc. urine containing 45 per cent dye; second period, 3 cc. urine containing 52 per cent dye; total, 78 per cent.

† First period, 0.5 cc. urine containing 0 per cent dye; second period, 1.5 cc. urine containing 0 per cent dye; total, 0 per cent.

‡ First period, 7 cc. urine containing 11 per cent dye; second period, 5 cc. urine containing 10 per cent dye; total, 21 per cent.

§ First period, 15 cc. urine containing 4 per cent dye; second period, 8 cc. urine containing 4 per cent dye; total, 8 per cent.

° First period, 3 cc. urine containing 16 per cent dye; second period, 4 cc. urine containing 26 per cent dye; total, 42 per cent.

showed decreases in amount. On the last day of the experiment with rabbit 19 (table 4), the chlorides increased above the original level. This is doubtless to be accounted for by the fact that this animal had begun to recover from the effects of the tartrate. Similar results have been observed in mercuric chloride nephrosis by Killian (15). This author states that the blood chlorides decrease during the period of severe nitrogen retention, and return to normal with recovery of the functional capacity of the kidneys.

The effect upon the phenolsulphonphthalein elimination is manifested in each experiment. Following doses of 0.5 to 1 gram of the tartrate, the dye excretion fell practically to zero. In the experiments in which the animals manifested a tendency to recover from the tartrate (cf. tables 4 and 6), the creatinine was the first blood component to show a decrease in quantity. This is in accord with the well-known observation of Myers, Fine, and Lough (16) that creatinine is the easiest of the nitrogenous waste products for the kidneys to excrete. It is interesting to observe that whenever the creatinine decreased in amount the phenolsulphonphthalein elimination rose.

In the single experiment in which the urines were analyzed (table 6), the effect of the tartrate administration is clearly seen in the total nitrogen figures. The comparatively small dose of 0.25 gram of d-tartaric acid led to a fall in output of total nitrogen to 31 mgm. per day. With recovery, the nitrogen excretion promptly rose, but for several days did not keep pace with the tendency for the non-protein nitrogen of the blood to increase. Thus there was no marked diminution in the latter until the total nitrogen of the urine had reached the high value of 1.017 grams per day.

The experiment in table 6 is interesting from another point of view. As will be observed, the administration of a second dose of 0.25 gram of d-tartaric acid, following partial recovery from the first administration, failed to induce as marked effects as did the initial injection. We have obtained similar data in several other experiments. These results corroborate the findings of Potter and Bell (22), and Salant and Swanson (28) to the

TABLE 7  
*l-malic acid*  
 Rabbit 11, male 2650 grams

DATE	ACID ADMINISTERED	FURFURALIN RECOV- ERED	BLOOD		NOTES, ETC.
			N-nitro-protein ratio		
			mgm.	per cent	
November 22....	0.000*				No food; water ad lib.
November 23....		86†	35.9	0.53	11:00 a.m., 4 cc. blood. 1:50 p.m., renal test
November 23....	2.0				9:05 p.m., acid 3 injected subcutaneously as sodium salt in 20 cc. water
November 24....	2.0				10:00 a.m., acid 3 injected subcutaneously as sodium salt in 20 cc. water
November 24....		74‡	35.3	0.48	12:30 p.m., 4 cc. blood. 6:10 p.m., renal test
November 25....	1.7				1:00 p.m., acid 3 injected subcutaneously as sodium salt in 18 cc. water
November 25....			41.7	0.48	4:00 p.m., 4 cc. blood
November 26....		88‡	43.1	0.46	10:00 a.m., 4 cc. blood. 10:45 a.m., final test. Experiment discontinued

Rabbit 12, male, 1265 grams

November 22....					No food; water ad lib.
November 23....		93‡	29.3	0.54	11:45 a.m., 6 cc. blood. 1:55 p.m., renal test
November 23....	2.0				9:05 p.m., acid 3 injected as sodium salt in 20 cc. water
November 24....	2.0				10:00 a.m., acid 3 injected as sodium salt in 20 cc. water
November 24....		77‡	41.1	0.48	12:30 p.m., 6 cc. blood. 6:15 p.m., renal test
November 25....			48.0	0.50	4:00 p.m., 5 cc. blood
November 26....		92Δ	42.9	0.49	10:15 a.m., 7 cc. blood. 10:50 a.m., renal test. Experiment discontinued

\* First period, 10 cc. urine containing 74 per cent dye; second period, 5 cc. urine containing 12 per cent dye; total, 86 per cent.

† First period, 6 cc. urine containing 49 per cent dye; second period, 3 cc. urine containing 25 per cent dye; total, 74 per cent.

‡ First period, 17 cc. urine containing 81 per cent dye; second period, 13 cc. urine containing 7 per cent dye; total, 88 per cent.

§ First period, 14 cc. urine containing 87 per cent dye; second period, 9 cc. urine containing 6 per cent dye; total, 93 per cent.

¶ First period, 4 cc. urine containing 59 per cent dye; second period, 5 cc. urine containing 18 per cent dye; total, 77 per cent.

Δ First period, 3 cc. urine containing 82 per cent dye; second period, 2 cc. urine containing 10 per cent dye; total, 92 per cent.

TABLE 8  
dl-malic acid  
Rabbit 4, female, 1790 grams

DATE	ACID ADMIN- ISTERED	URINARY EXCRETION	BLOOD					NOTES, ETC.
			Non- protein nitro- gen	Org. N	Crysta- line	Hemo- g.	Hct <sup>1</sup>	
	grams	per cent	mgm.	mgm.	mgm.	per cent	per cent	
October 18.....								No food; water ad lib.
October 19.....		78 <sup>2</sup>	41.1	21.4	1.6	0.127	0.55	10:10 a.m., renal test. 3:00 p.m., 6 cc. blood removed
October 20 to 22.....								Animal fed oats and hay
October 23.....								No food
October 24.....	1.0							10:00 a.m., acid 2 injected as sodium salt in 15 cc. water
October 25.....		70 <sup>1</sup>	50.2			0.140	0.56	10:00 a.m., 2 cc. blood. 11:10 a.m., renal test
October 26.....	3.0	60 <sup>1</sup>						Acid 2 given as sodium salt in two equal doses, 9:40 a.m. and 9:00 p.m. 12:40 p.m., renal test
October 27.....		75 <sup>1</sup>	48.6	23.4	1.7	0.127	0.45	9:30 a.m., 8 cc. blood. 10:00 a.m., renal test. Experiment discontinued

Rabbit 8, male, 2100 grams

November 17.....								No food; water ad lib.
November 18.....		76 <sup>1</sup>	52.8				0.46	10:00 a.m., 4 cc. blood. 10:50 a.m., renal test.
November 18.....	3.0							Acid 2 injected as sodium salt in two equal doses at 4:40 and 9:30 p.m.
November 19.....	1.5							1:00 p.m., acid 2 as sodium salt

November 19 .....			80.5			0.46	4:00 p.m., 8 cc. blood
November 20 .....							
November 21 .....	1.5						9:40 a.m., acid 2 as sodium salt
November 21 .....		78Δ	92.8	1.7	0.47		2:50 p.m., renal test. 9:30 p.m., 8 cc. blood. Experiment discontinued

\* First period, 8 cc. urine containing 60 per cent dye; second period, 5 cc. urine containing 18 per cent dye; total, 78 per cent.

† First period, 12 cc. urine containing 50 per cent dye; second period, 7 cc. urine containing 20 per cent dye; total, 70 per cent.

‡ First period, 7 cc. urine containing 28 per cent dye; second period, 5 cc. urine containing 32 per cent dye; total, 60 per cent.

§ Total dye elimination for the two periods combined was 75 per cent.

¶ First period, 42 cc. urine containing 60 per cent dye; second period, 5 cc. urine containing 10 per cent dye; total, 70 per cent.

Δ First period, 10 cc. urine containing 45 per cent dye; second period, 9 cc. urine containing 33 per cent dye; total, 78 per cent.

effect that rabbits require a tolerance for tartaric acid. We shall refer to this aspect of the problem in a subsequent communication.

*Malic acid.* In tables 7 and 8 are shown the results of two experiments with l-malic acid and two with dl-malic acid respectively. In the case of the former, only non-protein nitrogen and chlorides were determined. The effects of the levo acid are exceedingly slight, or indeed negligible as far as the blood picture is concerned. In each case following the administration of the acid, there was apparently a slight decrease in the rate of elimination of phenolsulphonophthalein although the total output did not become subnormal. Whether this is to be interpreted as indicative of temporary renal injury is questionable. We are inclined to believe that it does *not* represent nephropathic action. Probably the apparent decreases in dye elimination may be associated with the unusually high "normals" for these animals of 86 and 93 per cent respectively. The excretion during the first seventy minutes is in each case as large as the "normals" for most of the other animals.

Contrary to our expectations, dl-malic acid appeared to exert slightly more influence than did the levo compound upon renal activity. Both rabbits 4 and 8 (table 8), especially the latter, showed appreciable increases in non-protein nitrogen, as well as decreases in the rate of phenolsulphonophthalein elimination. The less marked effects upon blood composition in rabbit 4 are probably to be explained by the fact that the first dose of the acid was only 1 gram, and tended to increase the tolerance of the animal, so that subsequent injections failed to occasion decided changes. Whenever we have administered large amounts (3 grams per day) of dl-malic acid in animals which have not previously received a smaller injection, we have never failed to obtain distinct increases in the non-protein nitrogen of the blood. In none of our malic acid experiments, however, has the kidney injury been sufficiently extensive to occasion an appreciable rise in blood creatinine. In one experiment, the details of which are omitted, a rabbit was given doses of dl-malic acid exactly equal to, and at the same relative times as those employed in

rabbit 8. Four hours after the last injection, the animal was killed, and the kidneys were examined microscopically.<sup>2</sup> The examination showed a rather marked excess of blood with small areas of hemorrhage in the cortex, a limited degeneration of the tubular epithelium, and obliteration of some of the glomerular spaces. In view of the histological and chemical findings, it appears that dl-malic acid is slightly nephropathic to rabbits. Tomita (30) found that following the injection of dl-malic acid in rabbits and dogs, the dextro form could be recovered from the urine. He concluded that l-malic acid is more easily destroyed than is its isomer. Dakin (7) has shown that when the sodium salt of dl-malic acid is subjected to the action of muscle enzymes, the specific rotation of the residual malic acid becomes progressively dextro-rotatory. This he ascribes to the preferential conversion of the levo component of inactive malic acid into fumaric acid. These observations, together with our own results, make it seem probable that, if dl-malic acid is indeed nephropathic, the dextro isomer is the active agent. Owing to the difficulty of preparing d-malic acid, we have been unable to secure sufficient amounts to test its renal-toxic action.<sup>3</sup>

*Succinic acid.* In table 9 are shown the results of two typical experiments with succinic acid. Despite the fact that unusually large doses of this substance were administered, no effects upon blood composition or dye excretion were observed. Not even the rate of elimination of phenolsulphonephthalein showed a change. Certainly succinic acid is not a nephropathic agent. Battelli and Stern (4), and Einbeck (8) have shown that muscle extracts transform succinic acid into malic acid, probably through the intermediate stage of fumaric acid. In both papers the malic acid is said to be the optically inactive form. If this were correct, and represented the normal method of oxidation of succinic acid, one would expect the administration of the latter to be fol-

<sup>2</sup> The author is indebted to Professor Henry Hartman of the School of Medicine, University of Texas, for the histological examination.

<sup>3</sup> In a recent paper, Dakin (Jour. Biol. Chem., 1924, lix, 7) has provided a simple method of resolving inactive malic acid into its active components. By means of his procedure, we hope to secure enough d-malic acid to test its nephropathic action.

TABLE 9  
*Succinic acid*  
 Rabbit 10, female, 2185 grams

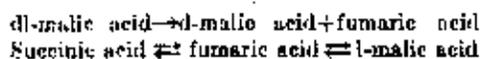
DATE	ACID ADMINISTERED	BLOOD			NOTES, ETC.	
		SUCCINATE PER CENT	Non-protein nitrogen mgm.	Creatinine mgm.		NaCl per cent
November 22					No food; water ad lib.	
November 23		85 <sup>+</sup>	31.5	0.30	11:00 a.m., 4 cc. blood. 1:45 p.m. renal test	
November 23	2.0				10:00 p.m., acid 4 injected subcutaneously as sodium salt in 20 cc. water	
November 24	2.0				10:00 a.m., acid 4 injected subcutaneously as sodium salt in 20 cc. water	
November 24		83 <sup>+</sup>	41.1	0.40	12:15 p.m., 5 cc. blood. 6:10 p.m., renal test	
November 25	3.0				1:00 p.m., acid 4 injected subcutaneously as sodium salt in 20 cc. water	
November 25			42.9	0.47	3:30 p.m., 5 cc. blood	
November 26		83 <sup>+</sup>	39.5	0.46	9:45 a.m., 6 cc. blood. 10:40 a.m. renal test. Experiment discontinued	

Rabbit 35, female, 1980 grams

April 21					No food; water ad lib.
April 22		78 <sup>+</sup>	45.5	1.3	0.49 10:05 a.m., 8 cc. blood removed. 10:20 a.m., renal test
April 23			46.5	1.3	0.41 10:05 a.m., 8 cc. blood
April 23	4.0				2:00 p.m., acid 4 injected subcutaneously as sodium salt
April 24		78 <sup>+</sup>	40.5	1.3	0.45 10:10 a.m., 8 cc. blood. 12:40 p.m., renal test
April 25			38.7	1.3	0.43 10:15 a.m., 5 cc. blood
April 26			36.1	1.3	0.39 12:30 p.m., 8 cc. blood
April 26	4.0				2:50 p.m., acid 4 injected subcutaneously as sodium salt
April 27		74 <sup>+</sup>	45.8	1.4	0.39 10:35 a.m., 8 cc. blood. 11:25 a.m., renal test
April 28			40.0	1.4	0.45 10:55 a.m., 8 cc. blood
April 29			44.4	1.4	0.48 10:50 a.m., 8 cc. blood. Experiment discontinued

For references see bottom of p. 113.

lowed by renal effects, since di-malic acid appears to be slightly nephropathic. It is of interest, therefore, to observe that Dakin (7) has presented conclusive proof of the fact that only l-malic acid results from the action of muscle enzymes on fumaric acid. Hence, d-malic acid would not arise in succinic acid oxidation. The probable relationship of succinic, malic, and fumaric acids in the animal organism may be indicated as follows (7):



Our data are thus entirely in accord with the interesting observation of Dakin.

#### DISCUSSION

The experiments outlined above emphasize the fact that tartrates are exceedingly violent nephropathic agents. Concerning their mode of action, the literature contains very little information. Umeda and Ringer (31) expressed the opinion that the effects following tartrate administration are due to precipitation in the convoluted tubules of the relatively insoluble calcium tartrate, resulting in complete destruction of the renal cells. As far as one may judge from the brief report available, the authors' conclusions were based upon the indirect evidence that oxalic acid produces a tubular nephritis accompanied by clogging of the tubules with crystals of calcium oxalate. On the other hand, Simpson (29) believes that calcium precipitation is probably not a factor in the etiology of tartrate nephritis. Salant (24)

\* First period, 8 cc. urine containing 70 per cent dye; second period, 4 cc. urine containing 48 per cent dye; total, 88 per cent.

† First period, 3 cc. urine containing 74 per cent dye; second period, 4 cc. urine containing 9 per cent dye; total, 83 per cent.

‡ First period, 10 cc. urine containing 70 per cent dye; second period, 7 cc. urine containing 7 per cent dye; total, 83 per cent.

§ The experiment upon rabbit 33 was made by A. J. Sells, formerly Assistant in Physiological Chemistry, University of Illinois.

¶ First period, 5 cc. urine containing 67 per cent dye; second period, 5 cc. urine containing 11 per cent dye; total, 78 per cent.

Δ First period, 14 cc. urine containing 69 per cent dye; second period, 5 cc. urine containing 9 per cent dye; total, 78 per cent.

⊙ First period, 9 cc. urine containing 52 per cent dye; second period, 7 cc. urine containing 22 per cent dye; total, 71 per cent.

reports that the administration of calcium chloride counteracts the effect of tartrate on the heart and blood pressure. In a later communication, Salant and Swanson (28) present the results of comparative studies of the action of tartrates, citrates, and oxalates. Having observed that tartrate administration may lead to the development of a tolerance in rabbits, while citrate and oxalate are cumulative in effect, the authors state that "the different behavior of tartrate, citrate, and oxalate as regards tolerance and cumulation is held to be unfavorable to the theory which assumes that the physiological effects of their acids and soluble salts are due to calcium precipitation, or to the transformation of ionic calcium into non-ionized calcium in the cells." Hara (13) believes that the action of tartrate, citrate, and oxalate, is due not to their calcium precipitating power *per se*, but to the disturbance which they indirectly produce in the balance of calcium and magnesium ions.

In the case of the three acids used by us in the above experiments, the nephropathic action is inversely proportioned to the solubilities of their calcium salts. This fact at first led us to believe that the conception of Umeda and Ringer (31) might be correct. On the other hand, later observations with glutaric acid, whose calcium salt is fairly soluble, have served to convince us that if calcium precipitation plays a part, it is not the only factor involved. We shall refer to this question in the following paper.

Omitting from further consideration the question of the *modus operandi* of tartrates in manifesting nephropathicity, it is interesting to observe that the progressive removal of the hydroxyl groups of tartaric acid (with the production of malic and succinic acids respectively), is accompanied by a simultaneous decrease in renal-toxic action. We are unable to state whether the presence of hydroxyl groups usually tends to increase the nephropathic action of dicarboxylic acids or not. Results which we will present in a subsequent paper indicate that sometimes this may be the case. On the other hand, it seems likely that the real factor involved is the influence which hydroxylation exerts on the ease of oxidation of the acid. Of organic compounds, the difficultly oxidizable ones appear more prone to manifest renal-toxic effects. Probably compounds like succinic acid, which are readily

burned in the organism, are disposed of rapidly by oxidation, and with the doses employed, do not reach the kidneys in appreciable quantities. But tartaric acid, which is slowly and incompletely oxidized, is perhaps disposed of chiefly by excretion, and during the course of its removal from the body leads to injury of the renal cells. If this tentative theory is correct, any alteration in chemical constitution which would result in a *more rapid* oxidation might be expected to diminish nephropathic properties. Conversely, any structural change which would render oxidation *more difficult*, might increase renal-toxic action. We are endeavoring to test this point at the present time.

#### CONCLUSIONS

1. The subcutaneous administration to rabbits of doses of 0.25 to 1 gram of d-tartaric acid as its sodium salt occasions enormous increases in non-protein nitrogen, urea, creatinine, sugar, and cholesterol of the blood, accompanied by diminished excretion of total nitrogen in the urine. The blood chlorides show distinct decreases in amounts, and the phenolsulphonaphthalein elimination may drop to zero. With recovery of renal function, the chlorides tend to return to normal.

2. L-malic acid is practically non-toxic, and dl-malic acid exerts only slight effects upon renal excretion. Following parenteral administration of doses of 2 to 3 grams of inactive malic acid, moderate increases may occur in total non-protein nitrogen of the blood, but the creatinine values remain unaffected. The rate of elimination of phenolsulphonaphthalein diminishes, and the total output for the two periods may be slightly decreased. These data, together with the histological findings, are interpreted as indicating that dl-malic acid is slightly nephropathic to rabbits.

3. Succinic acid in doses of 4 gms. is non-nephropathic to rabbits.

4. It is suggested that the renal-toxic action of certain acids is associated with their slow or incomplete oxidation in the body. Readily oxidizable substances are probably burned to carbon dioxide and water without exerting detrimental renal effects. Acids which are oxidized with difficulty may be disposed of by excretion only. In the course of the body's efforts to eliminate them, damage to the kidney cells may result.

## REFERENCES

- (1) BARR, J., AND BLUM, L.: Beitr. chem. Physiol. u. Path., 1907, x, 80.
- (2) BARR, J., AND BLUM, L.: Beitr. chem. Physiol. u. Path., 1908, xi, 191.
- (3) BARR, J., AND BLUM, L.: Arch. exp. Path. u. Pharm., 1911, lxx, 1.
- (4) BATTLELLI, F., AND STERN, L.: Biochem. Z., 1909-1910, xxx, 172.
- (5) BARRON, A.: K. physiol. Chem., 1898, xxv, 263.
- (6) CHARRIÉ, C.: Compt. rend. Acad., 1903, cxvi, 1410.
- (7) DAVIS, H. H.: Jour. Biol. Chem., 1922, lxx, 183.
- (8) ELSNER, H.: Biochem. Z., 1919, xcv, 296.
- (9) FOLIX, O.: A Laboratory Manual of Biological Chemistry, D. Appleton and Co., New York, 1922.
- (10) FOLIX, O., AND WU, H.: Jour. Biol. Chem., 1919, xxxviii, 81.
- (11) FOLIX, O., AND WU, H.: Jour. Biol. Chem., 1920, xli, 367.
- (12) GERAGHTY, J. Y., AND ROWNTREE, L. G.: Jour. Amer. Med. Assoc., 1911, lvii, 811.
- (13) HANA, S.: Acta Schol. Med., Univ. Kioto, 1919, iii, 213.
- (14) KARNWEL, H. T., AND DENTS, W.: Jour. Exper. Med., 1914, xiv, 259.
- (15) KILLIAN, J. A.: Jour. Lab. and Clin. Med., 1921, vii, 126.
- (16) MYERS, V. C., FINE, M. S., AND LOUGH, W. G.: Arch. Int. Med., 1916, xvii, 570.
- (17) NEUBERG, C., AND SANAYOSHI, S.: Biochem. Z., 1911, xxxvi, 32.
- (18) OHTA, K.: Biochem. Z., 1912, xliv, 481.
- (19) PEARCE, R. M., AND RINGER, A. L.: Jour. Med. Res., 1913, xxix, 57.
- (20) POHL, J.: Arch. exp. Path. u. Pharm., 1896, xxxvii, 413.
- (21) POST, W. E.: Jour. Amer. Med. Assoc., 1914, lxi, 592.
- (22) POTTER, A. C., AND BELL, E. T.: Amer. Jour. Med. Sci., 1915, cxliii, 236.
- (23) RINGER, A. L., FRANKEL, E. M., AND JONAS, L.: Jour. Biol. Chem., 1913, xiv, 330.
- (24) SALANT, W.: Jour. Amer. Med. Assoc., 1914, lxi, 1076.
- (25) SALANT, W., AND SMITH, C. S.: Amer. Jour. Physiol., 1914, xxxv, 230.
- (26) SALANT, W., AND SWANSON, A. M.: Jour. Pharm. and Exper. Therap., 1918, xi, 27.
- (27) SALANT, W., AND SWANSON, A. M.: Jour. Pharm. and Exper. Therap., 1918, xi, 43.
- (28) SALANT, W., AND SWANSON, A. M.: Jour. Pharm. and Exper. Therap., 1918, xi, 131.
- (29) SIMPSON, G. E.: Jour. Biol. Chem., 1920, lxi, xvi.
- (30) TOMITA, N.: Biochem. Z., 1921, cxviii, 281.
- (31) UMEDA, N., AND RINGER, A. L.: Proc. Soc. Exper. Biol. and Med., 1916, xiv, 31.
- (32) UNDERHILL, F. P.: Jour. Biol. Chem., 1912, xli, 115.
- (33) UNDERHILL, F. P., AND BLATHERWICK, N. R.: Jour. Biol. Chem., 1914, xix, 30.
- (34) UNDERHILL, F. P., AND WAKEMAN, E. T.: Jour. Biol. Chem., 1922, lxx, 701.
- (35) UNDERHILL, F. P., WELLS, H. G., AND GOLDSCHMIDT, S.: Jour. Exper. Med., 1913, xviii, 317.
- (36) UNDERHILL, F. P., WELLS, H. G., AND GOLDSCHMIDT, S.: Jour. Exper. Med., 1913, xviii, 322.
- (37) UNDERHILL, F. P., WELLS, H. G., AND GOLDSCHMIDT, S.: Jour. Exper. Med., 1913, xviii, 347.
- (38) WATFORD, J. C.: Jour. Biol. Chem., 1920-1921, lxx, 449.
- (39) WISE, I. E.: Jour. Biol. Chem., 1916-1917, xxxviii, 182.

Note on glycogen formation from succinic acid.

by Richard Stoehr

Feeding tests with Na-acetate (1), which have the question of the metabolism of acetic acid in carbohydrate as the theme, were the motive for the present short communication, and in keeping with the Wieland-Thunberg'schen dehydration theory, glycogen formation from acetic acid\* must proceed through succinic acid, fumaric acid, malic acid, oxaloacetic acid and pyruvic acid (2), which have been shown in animal experiments to be glycogen formers (3); in this connection it is of interest to us to determine to what extent feeding of succinic acid is capable of producing an increase in the glycogen content of the liver.

Succinic acid was first demonstrated in muscle by Einbeck (4); in the literature there are tests on its origin from acetic acid in vitro (5), in fungi (6), and with yeast (7); with more highly organized creatures, proof of its origin by this route is yet missing; Toenniessen and Brinkmann (8) found no formation of succinic acid with perfusion of muscle with acetic acid; according to Ringer, Frankel and Jonas (9), succinic acid led to the excretion of extra sugar by phlorhizinized animals; while our own tests were already in progress, Ponsford and Smedley-Maclean (10) reported on an increased glycogen formation in the liver of rats which had received a carbohydrate-poor diet with supplements of succinic acid\*\*.

Succinic acid can arise in organisms also by other routes; thus Toenniessen and Brinkmann (8) observed its appearance by the perfusion of muscle with pyruvic acid; further, various considerations and in vitro tests by Mueller(11), Spiro (12), Clutterbuck and Raper (13) and Smedley-Maclean and Pearce (14) do not allow the exclusion of the assumption of the formation of succinic acid by a delta-oxidation of fatty acids.

Our own tests on young, fasting, male rats resulted, after feeding of 0.15 g of succinic acid, in a strong increase in liver glycogen (0.909 g% per 100 g liver compared to 0.152 g% for the controls) and thereby confirmed the results of Ponsford and Smedley-Maclean which had been obtained under other test conditions.

#### Experimental

A. Method. The succinic acid was dissolved in 2.5 ml H<sub>2</sub>O for the feeding and was neutralized with the calculated quantity of Na<sub>2</sub>CO<sub>3</sub>; sacrifice of the animals and the determination of the liver glycogen were carried out according to our earlier reports (15); blood sugar according to Hagedorn-Jensen.

B. Animal tests. The tests were carried out on young white or speckled male rats, which had been fasted 24 hours; the test duration amounted to 4 hours; the urine excreted during the test period reacted strongly alkaline. The following table shows the tests:

**Table**  
**Liver glycogen in fasted rats 4 hours after feeding of 0.15 g of succinic acid (neutralized with Na<sub>2</sub>CO<sub>3</sub>),**  
**14 controls: 0.152 g% per 100 g liver**

① Nr.	② Protokoll Nr.	④ Vorperiode			⑥ Blut- zucker mg-%	⑦ Leber- glycogen pro 100 g Leber g-%	⑧ Anmerkung
		③ Gewicht nach 24 Stdn. Hungern g	⑤ Gewichts- verlust g				
1	251	153	10	65	0,920	sch. R. ⑩	
2	252	131	14	67	1,113	w. R. ⑩	
3	253	136	15	82	1,111	w. R.	
4	254	116	11	69	0,668	sch. R.	
5	255	150	14	76	0,623	w. R.	
6	256	130	15	85	0,977	w. R.	

⑪ Mittel: 0,920

1. Number
2. Pre-test period
3. Protocol number
4. Weight after 24 hours of fasting
5. Weight loss
6. Blood sugar
7. Liver glycogen per 100 g liver
8. Remarks
9. Speckled rats
10. White rats
11. Average

#### Summary

0.15 g succinic acid, neutralized with Na<sub>2</sub>CO<sub>3</sub> caused a marked increase in liver glycogen in young male rats 4 hours after feeding.

\* As to the possibility of a conversion of acetic acid in carbohydrate to acetoacetic acid and subsequent coupling with methylglyoxal see the previous work.

\*\* Fumaric acid and malic acid, after administration by these authors under analogous conditions, also led to an increase in liver glycogen.

#### Literature

1. R. Stöckl, *Diets Z.* 217, 111 (1933).
2. Vgl. H. Jentz, *Recher. Biophys. d. Journ. n. path. Physiol.* 5, 664 (1928).
3. R. Stöckl, *Diets Z.* 204, 15 (1932).
4. H. Einbeck, *Diets Z.* 57, 13 (1927); 59, 291 (1929).
5. C. Meritt u. H. Wolff, *Zeitsch. Ber. chem. Ges.* 32, 2531 (1900); E. Knapp u. M. Gebike, *Diets Z.* 156, 63 (1925); K. Bernhäuser u. W. Stein, *Biochem. Z.* 219, 210 (1928).
6. Zügel u. K. Bernhäuser u. W. Stein, *Biochem. Z.* 213, 219 (1928).
7. H. Wieland u. R. Sondorhoff, *Liedig. Ann.* 339, 219 (1902).
8. E. Fomaleiro u. E. Brindley, *Diets Z.* 187, 137 (1930).
9. A. J. Ringler, E. M. Frankel u. L. Jenko, *J. offic. Chem.* 15, 509 (1913).
10. A. Ph. Penzford u. L. St. Gley-Maclean, *Biochem. J.* 26, 1219 (1932).
11. H. Müller, *Verh. d. ch. Ges.* 5, 100 (1923).
12. K. Spira, *Ch. News Z.* 137, 299 (1923).
13. P. W. Clutterbuck u. R. St. Gley-Maclean, *J. Biol. Chem.* 19, 283 (1925).
14. L. St. Gley-Maclean u. M. S. O. Pedree, *Biochem. J.* 25, 1207 (1931).
15. R. Stöckl u. M. Heuse, *Diets Z.* 305, 1 (1932).

Evaluation of Available Energy of Aliphatic Chemicals by Rats;  
An Application of Bioassay of Energy to Mono-gastric Animal

By Minoru YOSHIDA, Haruhisa IKUMO and Osamu SUZUKI\*

National Institute of Animal Industry, Chiba-shi

\*Fukui Prefectural Animal Husbandry Experiment Station

Received January 8, 1971

Bioassay procedure to evaluate biologically available energy of chemicals applicable to rats was established, and available energy of 36 chemicals was determined and compared with that estimated by chicks previously. Rats can utilize energy of propionic and butyric acids and *n*-hexyl propionate and butyrate well, while chicks cannot. Succinic acid, lauryl alcohol and dilauryl succinate at 5% dietary level were available by rats, though at 10% level lauryl alcohol was toxic. Ethyl lactate, octyl and decyl acetates and 1,2-propanediol dilaurate were available by both rats and chicks. Availability of other 6 esters including ethyl succinate and citrate was low. Availability and digestibility of aldehydes by rats were also low.

Growth response of chicks on aliphatic chemicals reported previously<sup>1-4</sup> revealed some difference between chicks and rats in certain chemicals, though most of the chemicals available by chicks are also available by rats. Chicks looked like lacking the ability to utilize propionic and butyric acids.<sup>1</sup> The findings were confirmed repeatedly by using esters of these acids with glycols<sup>2</sup> and alcohols.<sup>3</sup> Rats can utilize these acids or their esters well,<sup>4</sup> although Ozaki<sup>5</sup> reported the death of rats on acetic, butyric, caprylic and capric acids at the dietary level of 10%. Chicks can utilize lauryl and myristyl alco-

hols<sup>6</sup> which are toxic to rats.<sup>6</sup> These findings indicated the necessity of the direct comparison of availabilities of chemicals by chicks and rats.

In the experiments with rats cited above, growth response of rats to a sample was compared with that to control diet. Therefore, it is hard to compare the data obtained by such a comparative feeding experiment with those obtained by bioassay technique with chicks. For example, Ozaki<sup>7</sup> prepared a test diet containing 5% of lauric acid in place of starch in the control diet, and fed the same amount of each of the diets to the test and control groups of rats. Average body weight gain of 3 rats on both of the test and control diets for 50 days was the same, being 54 g. Ozaki described that the nutritional value of lauric acid was comparable with that of the control diet. Nevertheless, the data suggested rather low availability of lauric acid, only 40% ( $= 3.52/8.75 \times 100$ ). Since caloric value of lauric acid and starch was

1) M. Yoshida, H. Hoshii and H. Morimoto, *Japan. Poultry Sci.*, 6, 73 (1969).

2) M. Yoshida, H. Morimoto and N. Oda, *Agr. Biol. Chem.*, 34, 1301 (1970).

3) M. Yoshida, H. Morimoto, M. Matsui and R. Oda, *ibid.*, 34, 1308 (1970).

4) M. Yoshida, H. Morimoto, M. Matsui and R. Oda, *ibid.*, 34, 1313 (1970).

5) M. Yoshida, H. Morimoto, and R. Oda, *ibid.*, 34, 1608 (1970).

6) K. Ashida, private communication (1968).

7) J. Ozaki, *Nippon Nogeikagaku Kaishi*, 8, 1206 (1932).

8) M. Miyazaki, *Nippon Nogeikagaku Kaishi*, 29, 301 (1955).

4.75<sup>2</sup> and 3.52<sup>2</sup> kcal/g, respectively, Ozaki's test diet contained higher caloric value than the control diet, although both of the diets contained the same amount of nutrients. The difference in dietary caloric value should reflect on the difference in growth response of rats under such an experimental condition. Therefore, body weight gain of rats on the test diet should be heavier than that on the control diet, if more than 40% of dietary lauric acid be utilized by rats. Ozaki's finding of the same body weight gain indicated only low availability of lauric acid by rats.

Even though the nutritional values of the test and control diets are exactly the same, the same body weight gain on both of the diets can be obtained only by chance, considering relatively large individual variation in biological experiment. When growth response of rats on the test diet is higher or lower than that on the control, the availability of energy in the sample can not be estimated by such a comparative feeding experiment with only one control diet. For such an estimation of availability, at least two control diets with higher and lower energy levels than that of the test diet are necessary.

For the quantitative estimation, the experimental condition should be such that the response of rats is in direct proportion to the dietary energy level.

The optimum experimental condition suitable to the bioassay procedure to estimate available energy of chemicals by rats was examined and the procedure of mini-test for chicks described previously<sup>9</sup> was applied to rats. In this paper, the available energy of 26 samples was determined by this bioassay procedure with rats and compared directly with that estimated by chicks.<sup>10,11</sup>

## EXPERIMENTAL

**Bioassay procedure.** Preliminary tests revealed that

9) M. Yoshida and H. Murimoto, *Agr. Biol. Chem.*, **34**, 681 (1970).

body weight gain of male weanling rats responded linearly with the change in dietary energy level of the standard diets prepared for starting chicks in mini-test.<sup>9</sup> The response of male rats to the change in dietary energy level was much more sharp than that of female rats, and the response of body weight gain to the dietary energy level was already clear at 6th day on the diets. Based on these preliminary findings, the following bioassay procedure was adopted as the standard procedure in this paper.

Male weanling albino rats of Wistar strain of about 50 to 60 g body weight were purchased and divided into lots of 4 rats each with uniform average body weight as far as possible. Each lot was reared in a cage with wire-net floor with free access to water. Daily supply of feed was so adjusted according to appetite of rats that most of the lots took the same amount of feed for 12 days, except those given experimental diet of low palatability. Daily feed supply of these diets of low palatability was so adjusted that the rats took most of the diet within 24 hr. Amount of feed consumed by the rats who left part of feed was measured every day by subtracting the amount of feed left in the feed cup from the amount of feed supplied. Individual body weight was measured at the beginning and every 6 days.

**Test material.** All of the sample tested were the same as used previously in mini-tests by chicks.<sup>9-11</sup>

**Composition of diet.** The composition of standard and test diets was exactly the same as mentioned previously.<sup>9</sup> The low energy standard diet, designated as SO-0 diet, consisted of the following: corn starch, 25.8%; soybean oil, 2.0%; protein mixture, 43.0%; cellulose, 22.0%; mineral mixture, 4.1%; and vitamin supplement, 1.1%. Four or 8% of cellulose in SO-0 diet was replaced by soybean oil to prepare medium and high energy standard diets, designated SO-4 and SO-8, respectively. Five percent of cellulose in SO-0 diet was replaced by test material to prepare test diet in the standard bioassay procedure. For evaluation of nutritional value of lauryl and myristyl alcohols, 10% of cellulose was also replaced to prepare the test diets. All of the standard and test diets were prepared just before the beginning of the assay, except the test diets containing volatile samples such as acetic acid. For these volatile samples, a basal diet was prepared which had the same composition as SO-0 diet, except 5% of cellulose omitted in the basal diet. Five parts of a volatile sample was mixed with 95

parts of the basal diet every day just before feeding to the rats. The procedure lead to minimize the loss of the sample by evaporation. The amount of sample lost by evaporation during 24 hr was determined as described later. All of the diets were fed *ad libitum* form.

**Calculation of available energy, availability and palatability index.** Although feed intake of the lots of rats fed the standard diets and mean of the test diets was exactly the same in the procedure mentioned above, body weight gain per 100 g feed taken was calculated and used statistical regression analysis. The procedure partially compensates the difference in growth response on a sample due to low palatability. The calculation of available energy in the standard procedure of bioassay was essentially the same as described previously with chicks.<sup>10</sup> Following Equation 1 was induced from the data on the standard diets;

$$y = a + bX \quad (1)$$

where  $X$  is dietary level (%) of soybean oil used in place of cellulose,  $y$  is body weight gain per 100 g feed, and  $a$  and  $b$  are the constants. Amount of soybean oil corresponding to a sample in the test diet can be estimated by replacing  $y$  in Equation 1 with growth response of this on the test diet. Since digestible energy of soybean oil was determined to be 9.25 kcal/g and dietary level of the test material in the standard procedure of this bioassay was constant at 5%, available energy of a sample,  $x$  kcal/g, can be estimated by the following Equation 2,

$$x = 9.25X/5 = 1.85X \quad (2)$$

In the standard procedure of the bioassay, it is convenient to use Equation 3 instead of Equation 1.

$$y = a + bx/1.85 = c + d'x \quad (3)$$

where  $c$ ,  $d$  and  $b$  are as in Equation 1,  $x$  is available energy of the sample, kcal/g, now  $d'$  is a new constant. In this Equation 3,  $x$ -values of the 3 standard diets, SO-0, SO-4 and SO-8, containing 0, 4 and 8% of soybean oil, respectively, are estimated to be 0, 7.4 (=9.25 × 4/5) and 14.8 (=9.25 × 8/5) kcal/g sample, respectively.

Confidence interval of available energy at (100 -  $\alpha$ )% level can be calculated by solving Equation 4 for  $x$ .<sup>10</sup>

<sup>10</sup> In the previous paper,<sup>10</sup> degrees of freedom were shown erroneously as  $n-1$ .

$$y - a + b'x = \pm t_{(n-2), \alpha/2} s_{y,x} \sqrt{1/n + (x - \bar{x})^2 / S_{xx}} \quad (4)$$

where  $x$  is caloric value of the sample,  $y$  is growth response of rats, and  $a$  and  $b'$  are the constants;  $t_{(n-2), \alpha/2}$  is  $t$ -value at  $\alpha\%$  level with  $n-2$  degrees of freedom<sup>10</sup>;  $s_{y,x}$  is deviation from regression;  $n$  and  $p$  are number of groups on the standard and test diets, respectively; and  $\bar{x}$  and  $S_{xx}$  are mean and sum of squares of  $x$  on the standard diets, respectively.

Availability was calculated by dividing available energy of the sample by its gross energy and multiplying by 100.

Palatability index of the test diet was calculated by dividing average intake of the test diet by average intake of SO-4 diet and multiplying by 100. Since supply of feed was adjusted as mentioned above, palatability index of most of the samples tested was 100. In the preliminary test before the standard procedure mentioned above had been established, all of the rats were fed *ad libitum*. Therefore, palatability index of 6 samples tested by this procedure was slightly different from 100. These samples are shown in footnote of Table II.

**Determination of digestibility.** When the simultaneous determination of digestibility was intended in a bioassay, 0.05% of chromic oxide was mixed in all of the standard and test diets, and feces being not contaminated by urine for optional 2 days in the latter half of the experimental period was collected on wire net placed under the cage. Contents of crude fat and chromic oxide in the diet and feces were determined and true digestibility was calculated as mentioned previously.<sup>11</sup> The procedure was only applicable to the non-volatile sample soluble in ethyl ether.

**Estimation of loss by evaporation in the diet.** Twenty grams of SO-0 and test diets were prepared and placed in animal room kept at 20°C for 24 hr. Weight of the samples before and after the storage was weighed exactly using a chemical balance. To compensate the weight change of the diets by evaporation or absorption of moisture, loss of the sample in the test diet was calculated by subtracting the weight change in SO-0 diet from the change in the test diet. The loss of the sample by evaporation was shown as percentage in the initial amount of the sample, i.e. 1 g.

## RESULTS

*Linearity of standard curve*

In Fig. 1, standard curve obtained in one of 7 trials is shown for an example. In this trial, triplicated lots of 4 rats each, whose average initial body weight was from 57.5 to 58.0g, were fed one of 8 experimental diets, 3 standard and 5 test diets. The rats on the standard diets took 105.9g of feed per head during 12 days of experimental period. Linear relationship between body weight gain per 100

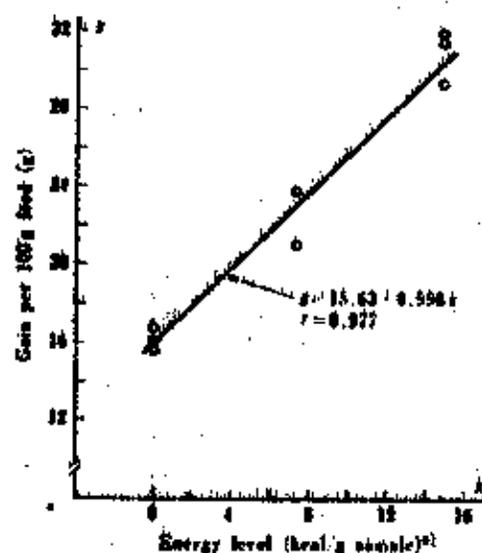


FIG. 1. Growth Response of Rats for 12 Days on Standard Diets.

\* See text.

g feed and dietary energy level was clear as shown in Fig. 1 with highly significant ( $P < 0.01$ ) correlation coefficient,  $r = 0.977$ . Highly significant correlation coefficients from 0.927 to 0.999 were obtained in other 6 trials with rats reported in this paper. In Fig. 1, x-axis was kcal of available energy per g of sample as in Equation 3, so that the available energy of a sample could be read directly from  $y$  on the sample.

In the trial shown in Fig. 1, 5 fatty acids were tested for the availability. Response of rats on the acids and means and confidence intervals of available energy and availability are shown in Table I. As expected from Equation 4, the confidence interval was a function of available energy. The closer was the available energy to  $\bar{x}$ , i.e. 7.4 kcal/g, the narrower was the confidence interval. The confidence interval of available energy in Table I was narrower than that of the corresponding interval estimated previously with chicks.<sup>11</sup> However, it should be pointed out that triplicated lots of rats were used in this trial, while duplicated lots of chicks were used in the previous one.<sup>11</sup> Larger  $n$  and  $p$  in Equation 4 resulted in the narrower confidence interval. Actually no difference was observed in the accuracy of estimation of available energy between rats and chicks with the same  $n$  and  $p$ . Since availability was the ratio of available energy to gross energy, confidence interval of availability was a func-

TABLE I. GROWTH RESPONSE OF RATS TO FATTY ACIDS, AVAILABLE ENERGY, AVAILABILITY AND THEIR 95% CONFIDENCE INTERVAL

Fatty acids	Gibbs energy kcal/g	Body wt. gain		Av. energy		Availability	
		100g feed	Mean	Mean	95% confidence interval	Mean	95% confidence interval
Acetic	3.15	16.3	0.7	3.1	2.3	22	98-73
Propionic	4.79	21.1	3.5	7.8	5.0	115	103-63
n-Butyric	6.00	21.4	5.8	8.1	3.3	100	140-57
Caprylic	7.99	25.0	10.3	12.9	0.0	129	161-100
Lauric	9.75	16.3	0.7	3.1	2.3	8	35-26

TABLE II. GROSS ENERGY, AVAILABILITY, PALATABILITY, DIGESTIBILITY AND LOSS BY EVAPORATION OF CHEMICALS TESTED BY RATS

Chemicals	Carbon skeleton	No. of rats	Gross energy kcal/g	Available energy kcal/g	Availability AEE/EK %	Palatability index	Digestibility coeff. %	Loss by evaporation %
<b>Acids:</b>								
Acetic	C <sub>2</sub>	12	3.15	0.7	22(-) <sup>1)</sup>	100		25
Propionic	C <sub>3</sub>	12	4.79	3.5	115(-)	98		21
n-Butyric	C <sub>4</sub>	12	5.80	5.8	100(-)	100		18
Caprylic	C <sub>8</sub>	12	7.99	10.8	129(39)	100	114	
Capric	C <sub>10</sub>	8	8.46	9.9	117(107)	100	92	
Lauric	C <sub>12</sub>	12	8.75	0.7	8(108)	78	102	
Myristic <sup>2)</sup>	C <sub>14</sub>	14	2.96	3.4	112(173)	96 <sup>3)</sup>		
<b>Alcohol:</b>								
n-Hexyl	C <sub>6</sub>	8	9.19	5.7	62(-)	100		
n-Octyl	C <sub>8</sub>	4 <sup>4)</sup>	9.74	—	—(-)	—		
n-Decyl	C <sub>10</sub>	8	9.86	3.3	34(32)	84	96	
Lauryl	C <sub>12</sub>	8	9.99	9.9	99(112)	84	91	
Myristyl	C <sub>14</sub>	3,8	10.09	—	—(94)	—		
Octyl	C <sub>8</sub>	8,8	10.23	—	—(23)	—		
Stearyl	C <sub>18</sub>	8	10.56	2.2	21(47)	100		
Lauryl <sup>5)</sup>	C <sub>12</sub>	5,8	—	—	—	—		
Myristyl <sup>5)</sup>	C <sub>14</sub>	2,8	—	—	—	—		
<b>Carbonyl compound:</b>								
Acetaldehyde	C <sub>2</sub>	8,8	5.36	—	—(-)	—		
Panacetalddehyde <sup>6)</sup>	C <sub>7</sub>	6	5.97	1.2	20(45)	103 <sup>4)</sup>		
Acetal	C <sub>2</sub>	8	7.46	2.2	29(64)	100		
Diacetone alcohol <sup>6)</sup>	C <sub>6</sub>	2	7.36	-13.4	—(-)	89 <sup>4)</sup>		
n-Octyl aldehyde	C <sub>8</sub>	8	9.49	8.8	78(-)	98	54	
Pantpropionaldehyde <sup>6)</sup>	C <sub>7</sub>	2	7.29	-19.3	—(-)	75 <sup>4)</sup>		
n-Decyl aldehyde	C <sub>10</sub>	8	9.73	4.9	50(42)	100	32	
Laurylaldehyde	C <sub>12</sub>	8	9.92	3.4	34(64)	100	47	
<b>Ester:</b>								
Ethyl lactate	C <sub>5</sub>	1,8	5.39	9.3	172(90)	102		
" succinate	C <sub>6</sub>	1,8	5.80	—	—(22)	68		
" citrate	C <sub>6</sub>	1,8	5.26	—	—(32)	83		
n-Butyl acetate	C <sub>8</sub>	8	7.29	-1.4	—	180		
n-Amyl acetate	C <sub>9</sub>	8	7.86	1.1	14(6)	100		
n-Hexyl formate	C <sub>8</sub>	8	7.79	1.9	24(30)	100		7
" acetate	C <sub>8</sub>	8	8.06	4.2	52(37)	100		14
" propionate	C <sub>8</sub>	8	8.22	9.6	115(66)	100		9
" n-butyrate	C <sub>8</sub>	8	8.55	9.7	113(81)	100		
" valerate	C <sub>8</sub>	8	8.67	5.7	66(107)	100		
n-Octyl acetate	C <sub>10</sub>	8	8.73	10.0	113(91)	100		
n-Decyl acetate	C <sub>12</sub>	8	8.86	11.6	131(111)	100		
Dilauryl succinate <sup>7)</sup>	C <sub>22</sub>	14	9.08	9.0	99(132)	98 <sup>4)</sup>		
1,2-Propandiol dilaurate <sup>7)</sup>	C <sub>22</sub>	14	8.91	8.5	95(119)	97 <sup>4)</sup>		

<sup>1)</sup> Figures in parentheses denote availability by chicks.

<sup>2)</sup> The data were not available.

<sup>3)</sup> Number of rats (total/number of tested).

<sup>4)</sup> Dietary level was 10%.

<sup>5)</sup> Body weight decreased during the initial 6 days.

tion of gross energy as shown clearly in Table I.

#### Data of biological assay

Gross energy, available energy, availability, palatability index, digestibility coefficient and evaporation loss of 30 samples in 7 trials are summarized in Table II.

Among the fatty acids tested, propionic, *n*-butyric, caprylic and capric acids were utilized well by rats. Availability of acetic and lauric acids was unexpectedly low. Though acetic acid is volatile, loss of the acid by evaporation in the diet during 24 hr was 25%. Amount of experimental diet supplied daily was so adjusted that rats took all of the diet within 24 hr. This means that actual loss of acetic acid was far less than 25%. Therefore, the loss of acetic acid by evaporation might not be the main reason of its apparent low availability. Low palatability of lauric acid might decrease the body weight gain of rats per 100 g feed, since energy supplied through diet was first utilized for maintenance of physiological activity of rats, so that the part of dietary energy used for growth should be much lower than that expected from palatability. This might be one of the reasons of unexpectedly low availability of lauric acid.

Succinic acid was well utilized by rats. *n*-Hexyl and lauryl alcohols were utilized well at dietary level of 5%, and *n*-deyl and stearyl alcohols partially. *n*-Octyl, myristyl and cetyl alcohols were toxic at dietary level of 5%. All of the rats on cetyl alcohol died within 12 days. At dietary level of 10%, both of lauric and myristyl alcohols were toxic. All of the rats on the former died within 12 days and those on the latter within 7 days.

Acetaldol was toxic and diacetone alcohol depressed body weight gain remarkably. Having free carbonyl function, these compounds certainly reacted with other nutrients in the diet as discussed previously on the data with chicks.<sup>11</sup> Aldehydes with carbon chain equal to or longer than 8 could be partially

utilized by rats. Paraformaldehyde and acetal, with masked carbonyl function, were partially available, although the availability was estimated to be less than 30%. Parapropanaldehyde depressed growth rate significantly.

In marked contrast, availability of ethyl lactate was extremely high, while availability of diethyl succinate and triethyl citrate was negative. The rats on the latter two esters lost body weight during initial 6 days, and the gain in latter half of experimental period was only equal to or even less than that on standard SO-0 diet.

Among 5 acetates tested, those of alcohol moiety having long carbon chain of 8 and 10 were available by rats. Availability of the acetates of alcohol moiety having shorter carbon chain than 8 decreased in parallel with the decrease of carbon chain length.

*n*-Hexyl esters of fatty acid moiety having longer carbon chain than 2 were available, though slightly low availability of isovalerate may suggest the lower availability of iso-type fatty acid than that of *n*-type. *n*-Hexyl formate and acetate were both available only partially.

Dilauryl succinate and 1,2-propanediol dilaurate were both available by rats.

#### DISCUSSION

As discussed previously,<sup>11</sup> one of the purpose of the experiments reported in this paper was to re-examine the previous reports<sup>1,2</sup> on the nutritional value of volatile fatty acids by rats, and to compare the ability of rats to utilize them with that of chicks. For convenience of comparison, availability of energy in the chemicals by chicks<sup>11</sup> is shown in parentheses in Table II, so that the availabilities of a compound by chicks and by rats can be directly compared. It is clear that rats can utilize energy in propionic and *n*-butyric acids well, while chicks can not. Availability of *n*-hexyl propionate and *n*-butyrate by rats is also higher than that by chicks.

Unexpectedly low availability of free acetic acid is hard to understand, though the finding suggests the physiological effect of free acetic acid other than caloric value. The suggestion may be supported by the previous findings<sup>11</sup> that aqueous solution (10%) of acetic acid infused into the crops of chicks killed a chick out of 4 and depressed palatability of the diet.

Combining the data shown in Table II and those obtained by Ashida,<sup>6</sup> it is expected that rats have ability to utilize dietary free fatty acids of carbon chain length from 3 to 10, including those of odd numbers.

Low availability of free lauric acid, though its digestibility by rats was excellent, may be due to its acidity as discussed already by Ozaki,<sup>7</sup> or may be due to some irritating effect on the gastro-intestinal tract as pointed out by Renner and Hill.<sup>11</sup> Derivatives of lauric acid, such as sodium salt, methyl and ethyl esters and glyceride stimulated growth of rats over that on the control diet.<sup>11</sup> Laurate of 1,2-propanediol was highly available by both chicks and rats as shown in Table II. These findings suggest that derivatives of lauric and other fatty acids may be one of the hopeful groups for feedstuffs of energy source, regardless of apparent low availability of free lauric acid itself.

Succinic acid, as expected from its role in tricarboxylic acid cycle of carbohydrate metabolism, was available by rats at 5% dietary level. However, its diethyl ester was rather injurious to rats. Triethyl citrate was also injurious, while availability of ethyl lactate was excellent. Availability of diethyl succinate and triethyl citrate by chicks was unexpectedly low, while that of ethyl lactate was satisfactory.<sup>6</sup> As pointed out previously,<sup>6</sup> it is hard to understand the low availability of ethyl esters of succinic and citric acids, since both acid and alcohol moieties of these esters are expected available by both chicks and

rats. There must be some physiological functions in these esters other than their caloric value as pointed out above in case of free acetic and lauric acids.

Significantly higher availability ( $p < 0.05$ ) of ethyl lactate than 100% indicates the possibility of unidentified growth promoting effect over its caloric value, as discussed previously.<sup>6</sup> The likelihood of such an effect should be confirmed by the long-term feeding experiment with larger number of animals than those used in this test, since the procedure of mini-test sacrificed the accuracy of estimate partially as described repeatedly.<sup>2-5,9</sup> From the author's experience on long-term feeding experiment with chicks to confirm growth promoting effect of succinic acid suspected by mini-test,<sup>12</sup> it is rather likely that the apparent high availability of ethyl lactate will be due mainly to biological variation of the growth response of the rats in the test.

Partial availability of hexyl and stearyl alcohols by rats and highly toxic effect of octyl, myristyl and cetyl alcohols were as expected from the paper of Miyazaki.<sup>8</sup> However, high availability of lauryl alcohol at 5% dietary level was unexpected. Accordingly, lauryl and myristyl alcohols were fed at 10% level as Miyazaki had tested.<sup>8</sup> It was confirmed that both of them were toxic at this level. Since dilauryl succinate was available by rats, lauryl alcohol and its derivatives might be one of the hopeful chemical groups as feedstuffs for domestic mono-gastric animals, but the findings mentioned above suggest the necessity of further studies for their practical use.

It has been pointed out already by Ozaki<sup>13</sup> that acetaldehyde is not only non-toxic but also has considerable nutritional value to rats. However, availability of acetaldehyde estimated by bioassay procedure was as low as 20%. The findings presented in Table II, as well

11) R. Renner and F.W. Hill, *J. Nutr.*, **74**, 259 (1961).

12) M. Yoshida and H. Hoshii, *Agr. Biol. Chem.*, **35**, 201 (1971).

13) J. Ozaki, *Nippon Nogeikagaku Kaishi*, **3**, 977 (1927).

as those observed with chicks<sup>37</sup> suggest rather low possibility of aldehydes to be utilized as feedstuffs of energy source.

Combined effect of carbon chain length of alcohol and fatty acid moieties of esters on their availability was observed with rats again to be similar to that pointed out previously<sup>31</sup> with chicks. Available *n*-hexyl esters by rats were those of fatty acid moiety of carbon chain length of 3 and over. Availabilities of acetates decreased with decreasing carbon chain length of alcohol moiety. These findings with rats are quite agreeable with those observed with chicks,<sup>31</sup> except the availability of *n*-hexyl propionate by chicks which was somewhat low. Loss of the esters by evaporation from the experimental diet during feeding period was discussed in the previous paper<sup>31</sup> as one of the reasons of apparently low availability of these esters. However, amount of 3 esters lost by evaporation during 24 hr was less than 14% as shown in Table II. Furthermore, actual loss of these esters was suspected to be much less than those shown in Table II, as mentioned above in case of free acetic acid. Therefore, the loss of the esters by evaporation could not be the main reason of their low availability by both chicks and rats. Some unknown physiological function other than caloric value of these esters should be considered, which may or may not be related to those of diethyl succinate and

triethyl citrate.

Linear relationship between dietary energy level and growth response was confirmed with rats in this paper, as well as with chicks<sup>31</sup> and calves.<sup>141</sup> These findings suggest that the relationship is common. However, it should be pointed out that the relationship is curvilinear essentially as discussed previously on the data by chicks.<sup>150</sup> Such an curvilinear relationship was also observed in one of the preliminary experiments with rats for the establishment of bioassay procedure. Therefore, it is most important for successful application of bioassay to keep the range of dietary energy level so narrow that the linearity of the standard curve is secured. Suitable dietary level of nutrients other than energy source is also essential to secure the linearity. Rats were used in this paper to estimate the possibility to utilize the chemicals as feedstuffs for swine. If enough amounts of samples be supplied, availability of the chemicals could be estimated directly by bioassay with pigs after the preliminary trials are conducted to establish the experimental conditions. Such a direct bioassay with pigs is most important before the practical use of the hopeful chemicals for swine feed.

14) M. Yoshida, K. Osada, S. Fujishiro and R. Oda, *Agri. Biol. Chem.*, **35**, 393 (1971).

15) M. Yoshida, H. Hoshii, K. Kosaka and H. Morimoto, *Japan. Poultry Sci.*, **2**, 180 (1965).