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CITRIC ACID #119 VOL III
COPIES OF ARTICLES CITED BUT NOT USED
IN MONOGRAPH SUMMARY

VOLUME 3

# GRAS MONOGRAPH SERIES

# CITRIC ACID

(COPIES OF ARTICLES CITED BUT NOT USED IN MONOGRAPH SUMMARY)

prepared for

THE FOOD AND DRUG ADMINISTRATION
DEPARTMENT OF HEALTH, EDUCATION
AND WELFARE

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# Title 9—ANIMALS AND ANIMAL PRODUCTS

## Chapter III—Consumer and Marketing Service, Meat Inspection, Department of Agriculture

### SUBCHAPTER A—MEAT INSPECTION REGULATIONS

### PART 316—MARKING, BRANDING, AND IDENTIFYING PRODUCTS

### PART 317—LABELING

### PART 318—REINSPECTION AND PREPARATION OF PRODUCTS

#### Miscellaneous Amendments

On January 27, 1965, there was published in the FEDERAL REGISTER (30 F.R. 844) a notice of proposed amendments to Parts 316, 317, and 318 of the Federal Meat Inspection Regulations (9 CFR Parts 316, 317, and 318) to allow use of isolated soy protein in sausage and certain other meat food products. Isolated soy protein—a product of advancing food technology—is the major proteinaceous fraction of soybeans prepared from high quality, sound, clean, dehulled soybeans by removing a preponderance of the non-protein components and contains not less than 90 percent protein (N x 6.25) on a moisture-free basis.

The purposes of these amendments are to provide consumers with a broader selection of sausage and certain other meat products by including isolated soy protein in the lists of food materials that are authorized for limited use in sausage and certain other meat food products under the Meat Inspection Act, and to require that isolated soy protein used in these products processed in official establishments contain a specified amount of titanium incorporated as food grade titanium dioxide for analytical control purposes.

After due consideration of all relevant matters in connection with such notice and under authority of the Meat Inspection Act, as amended and extended (21 U.S.C. 71-91) and section 306 of the Tariff Act of 1930, as amended (19 U.S.C. 1306), §§ 316.13, 317.8, and 318.4 of said regulations are amended as follows:

1. Section 316.13(c)(1) is amended to read:

**§ 316.13 Marking of meat food products in casings.**

* * * * *

(c)(1) When cereal, vegetable starch, starchy vegetable flour, soy flour, soy protein concentrate, isolated soy protein, dried milk, nonfat dry milk, or calcium reduced dried skim milk is added to sausage within the limits prescribed under Part 317 of this subchapter, the product shall be marked with the name of each of such added ingredients, as for example, "cereal added," "potato flour added," "cereal and potato flour added," "soy flour added," "soy protein concentrate added," "isolated soy protein added," "nonfat dry milk added," "calcium reduced dried skim milk added" or "cereal and nonfat dry milk added," as the case may be. On sausage of the smaller varieties, the marking prescribed in this paragraph may be limited to links bearing the inspection legend.

* * * * *

2. Section 317.8(c) (16), (27), and (32), and the eighth sentence of § 317.8 (c)(40), and § 317.8(c)(48), are amended to read as follows:

**§ 317.8 False or deceptive labeling and practices.**

* * * * *

(16) When cereal, vegetable starch, starchy vegetable flour, soy flour, soy protein concentrate, isolated soy protein, dried milk, nonfat dry milk, or calcium reduced dried skim milk is added to sausage within the limits prescribed under this part, there shall appear on the label in a prominent manner, contiguous to the name of the product, the name of each such added ingredient, as, for example, "cereal added," "with cereal," "potato flour added," "cereal and potato flour added," "soy flour added," "soy protein concentrate added," "isolated soy protein added," "nonfat dry milk added," "calcium reduced dried skim milk added," or "cereal and nonfat dry milk added," as the case may be.

* * * * *

(27) Product labeled "Chili Con Carne" shall contain not less than 40 percent of meat computed on the weight of the fresh meat. Head meat, cheek meat, and heart meat exclusive of the heart cap may be used to the extent of 25 percent of the meat ingredient under specific declaration on the label. The mixture may contain not more than 8 percent, individually or collectively, of cereal, vegetable starch, starchy vegetable flour, soy flour, soy protein concentrate, isolated soy protein, dried milk, nonfat dry milk, or calcium reduced dried skim milk.

* * * * *

(32) Spaghetti with meat balls and sauce, spaghetti with meat and sauce, and similar products, shall contain not less than 12 percent of meat computed on the weight of the fresh meat. The presence of the sauce or gravy constituent shall be declared prominently on the label as part of the name of the product. Meat balls may be prepared with not more than 12 percent, singly or collectively, of farinaceous material, soy flour, soy protein concentrate, isolated soy protein, nonfat dry milk, calcium reduced dried skim milk, and similar substances.

* * * * *

(40) * * * Sausage may contain not more than 3½ percent, individually or collectively, of cereal, vegetable starch, starchy vegetable flour, soy protein concentrate, isolated soy protein, nonfat dry milk, calcium reduced dried skim milk, or dried milk"; and by adding immediately after such sentence a new sentence reading as follows: "In determining the maximum amount of the ingredients specified in this subparagraph which may be used, individually or collectively, in a product, 2 percent of isolated soy protein shall be considered the equivalent of 3.5 percent of any other ingredient specified in this subparagraph. * * *

* * * * *

(48) Products labeled "Pork With Barbecue Sauce" and "Beef With Barbecue Sauce" shall contain not less than 50 percent meat computed on the weight of the cooked and trimmed meat. The weight of the cooked meat used in this calculation shall not exceed 70 percent of the uncooked weight of the meat. If uncooked meat is used in formulating the products, they shall contain at least 72 percent meat computed on the weight of the fresh uncooked meat. When cereal, vegetable flour, soy flour, soy protein concentrate, isolated soy protein, nonfat dry milk, calcium reduced dried skim milk or similar substances are used in preparing the products, such fact shall be prominently stated contiguous to the name of the product.

3. Section 317.8(c)(66) is amended by changing the first sentence to read: "Cheesefurters and similar products made in simulation of sausage in casings but containing sufficient cheese to give definite characteristics to the finished article may contain cereal, vegetable starch, starchy vegetable flour, soy flour, soy protein concentrate, isolated soy protein, nonfat dry milk, calcium reduced dried skim milk, or dried milk."; and by adding immediately after the second sentence a new sentence reading as follows: "In determining the maximum amount of the ingredients specified in this subparagraph which may be used, individually or collectively, in a product, 2 percent of isolated soy protein shall be considered the equivalent of 3.5 percent of any other ingredient specified in this subparagraph."

4. Section 318.4 is amended by adding thereto a new paragraph (d) to read:

**§ 318.4 Products and chemical preparations entering official establishments; identification; disposition; shipping in commerce.**

* * * * *

(d) All isolated soy protein used in products processed in official establishments must contain not more and not less than 0.1 percent titanium incorporated as food grade titanium dioxide, and the presence of such substance must be shown on the label of the container of the isolated soy protein.

The foregoing amendments differ in some respects from the proposals set forth in the notice of proposed rule making. These differences merely reflect minor editorial changes and do not affect the substance of the amendments. Furthermore, since these amendments authorize the use of isolated soy protein in certain meat food products in which its use was previously not permitted, they are in the nature of amendments relieving restrictions and should be made effective as soon as possible. Therefore, pursuant to section 4 of the Administrative Procedure Act (5 U.S.C. 1003), it is found upon good cause that further public rule-making procedure on these amendments is unnecessary and that they may be made effective in less than 30 days.

These amendments shall become effective upon publication in the FEDERAL REGISTER.

Done at Washington, D.C., this 6th day of July 1965.

R. K. SOMERS,
*Acting Deputy Administrator, Consumer Protection, Consumer and Marketing Service.*

[F.R. Doc. 65-7232; Filed, July 8, 1965; 8:49 a.m.]

## PART 316—MARKING, BRANDING, AND IDENTIFYING PRODUCTS

## PART 318—REINSPECTION AND PREPARATION OF PRODUCTS

### Miscellaneous Amendments

On January 30, 1965, there was published in the FEDERAL REGISTER (30 F.R. 998) notice of a proposal to amend Parts 316 and 318 of the Federal Meat Inspection Regulations (9 CFR Parts 316 and 318) to permit the use of certain antioxidants in the preparation of frozen fresh pork sausage and freeze-dried meats under the Meat Inspection Act.

Interested persons were afforded 60 days to furnish views and comments. It was suggested by interested persons that Nordihydroguaiaretic acid (NDGA) be included as an antioxidant permitted to be used in these products, that citric acid be included as a synergist, and that in the case of freeze-dried meats, the level of antioxidant accepted not be based solely on the fat content since the phospholipids in the lean are also protected. Since these suggestions are incorporated in the amendments and a minor editorial change was made from the proposed amendments for the sake of clarity, the amendments differ in some respects from the original proposals. However, adoption of the above suggestions in the amendments does not represent a change in principle from the original proposal, and it appears that further public rule making procedure on the amendments would not make additional information available to this Department. Therefore, under section 4 of the Administrative Procedure Act, it

is found that further notice and public rule making procedure on the amendments are unnecessary.

After due consideration of all relevant matters in connection with the notice of proposed rule making and under the authority of the Meat Inspection Act, as amended and extended (21 U.S.C. 71 et seq.), and subsections 306(b) and (c) of the Tariff Act of 1930, as amended (19 U.S.C. 1306 (b) and (c)), Parts 316 and 318 of the Meat Inspection Regulations are hereby amended as follows:

**§ 316.13 [Amended]**

1. Paragraph (c) of § 316.13 is amended by adding after the words "unsmoked dried sausage" the words "or frozen fresh pork sausage."

**§ 318.7 [Amended]**

2. The table in subparagraph (4) of paragraph (b) of § 318.7 is hereby amended to permit the use of BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), propyl gallate, or NDGA (nordihydroguaiaretic acid) or a combination of these antioxidants, with or without citric acid, in frozen fresh pork sausage and freeze-dried meats in the amounts specified in the table, as follows:

(1) In the portion of the chart dealing with "Antioxidants" (Class of Substance), the following information on BHA, BHT, propyl gallate, and NDGA is inserted in the appropriate columns immediately following "tocopherols" and information relating thereto.

| Substance | Purpose | Products | Amount | |
|---|---|---|---|---|
| BHA (butylated hydroxyanisole). | To retard rancidity | Frozen fresh pork sausage. | 0.01 percent based on fat content. | 0.02 percent in combination based on fat content. |
| BHT (butylated hydroxytoluene). | do | do | 0.01 percent based on fat content. | |
| Propyl gallate | do | do | 0.01 percent based on fat content. | |
| Nordihydroguaiaretic acid (NDGA). | do | do | 0.01 percent based on fat content. | |
| BHA (butylated hydroxyanisole). | do | Freeze dried meats | 0.01 percent based on total weight. | 0.01 percent in combination. |
| BHT (butylated hydroxytoluene). | do | do | 0.01 percent based on total weight. | |
| Propyl gallate | do | do | 0.01 percent based on total weight. | |
| Nordihydroguaiaretic acid (NDGA). | do | do | 0.01 percent based on total weight. | |

(2) In the portion of the chart dealing with Synergists (class of substance), there is inserted in the appropriate columns further information relating to citric acid.

| Substance | Purpose | Products | Amount |
|---|---|---|---|
| Citric acid | To increase effectiveness of antioxidants. | Frozen fresh pork sausage. | 0.01 percent on basis of fat content, in combination with antioxidants. |
| | | Freeze dried meats | 0.01 percent in combination with antioxidants. |

(Sec. 306, 46 Stat. 689, as amended; 34 Stat. 1264, 41 Stat. 241; 19 U.S.C. 1306; 21 U.S.C. 89, 96; 29 F.R. 16210, as amended)

These amendments will become effective 30 days after publication in the FEDERAL REGISTER.

Done at Washington, D.C., this 6th day of July 1965.

E. K. SCHERER,
*Acting Deputy Administrator, Consumer Protection, Consumer and Marketing Service.*

[F.R. Doc. 65-7209; Filed, July 8, 1965; 8:49 a.m.]

## PART 316—MARKING, BRANDING, AND IDENTIFYING PRODUCTS

## PART 340—SPECIAL SERVICES RELATING TO MEAT AND OTHER PRODUCT

### Amendment of Regulations

Pursuant to the authority contained in the Meat Inspection Act, as amended and extended (21 U.S.C. 71 et seq.), section 306 of the Tariff Act of 1930, as amended (19 U.S.C. 1306), and the Agricultural Marketing Act of 1946, as amended (7 U.S.C. 1621 et seq.), the U.S. Department of Agriculture hereby amends Parts 316 and 340, Title 9, Code of Federal Regulations, as follows:

1. Section 316.16 of Part 316 is amended to read as follows:

**§ 316.16 Tank cars and tank trucks used in transporting edible product.**

(a) Each tank car and each tank truck carrying inspected and passed product from an official establishment shall bear a label containing the true name of the product, the inspection legend, the establishment number, and the words "date of loading," followed by a suitable space for the insertion of the date. The label shall be located conspicuously and shall be printed on material of such character and so affixed as to preclude detachment or effacement upon exposure to the weather. Before the car or truck is removed from the place where it is unloaded, the carrier shall remove or obliterate such label.

(b) Tank cars and tank trucks carrying inspected and passed product from an official establishment to another official establishment or to a location operating under the Identification Service furnished under Part 340 of this subchapter shall be equipped for sealing and securely sealed by a Division employee with an official seal of the Department bearing the inspection legend.

2. Paragraph (a) of § 340.3 of Part 340 is amended by adding thereto a new subparagraph (4) to read as follows:

**§ 340.3 Types and availability of service.**

* * * * *

(a) *Identification service.* * * *

(4) The service will be available for products moved in tank cars and tank trucks from an official establishment or from a location operating under this service only if such tank cars or tank trucks are equipped for sealing and are securely sealed by an employee of the Meat Inspection Division of the Consumer and Marketing Service with an official seal of the Department bearing the inspection legend before leaving such official establishment or such other location.

* * * * *

(Sec. 306, 46 Stat. 689, as amended; 34 Stat. 1264; 41 Stat. 241; secs. 203, 205, 60 Stat. 1087, 1090, as amended; 19 U.S.C. 1306(b) and (c); 21 U.S.C. 89 and 96; 7 U.S.C. 1622, 1624; 29 F.R. 16210, as amended)

*Effective date.* The foregoing amendments shall become effective 10 days after publication in the FEDERAL REGISTER.

The Meat Inspection Division has reason to believe that the present regulation (7 CFR 316.16) requiring labeling only, for each tank car and tank truck transporting inspected and passed product from an official establishment, does not adequately insure that such product will not be adulterated or that other product will not be substituted therefor en route to destination. Accordingly, the amendment of § 316.16 imposes an additional requirement that such tank cars and tank trucks must be sealed with an official seal of the Department if the product is transported from one official establishment to another such establishment or to a location where identification service is maintained under the supervision of the Division. Further, the regulations under which identification service is furnished are amended by making such service available with respect to product moved in tank cars or tank trucks from an official establishment or from a location operating under such service only if such cars or trucks are sealed before leaving such establishment or location. Inasmuch as these amendments are necessary to afford additional safeguards to maintain the integrity of products which are federally inspected or identified, it is found upon good cause under section 4 of the Administrative Procedure Act (5 U.S.C. 1003) that notice and other public procedure with respect to the amendments are impracticable and contrary to the public interest, and good cause is found for making the amendments effective less than 30 days after publication in the FEDERAL REGISTER.

# Title 21—FOOD AND DRUGS

## Chapter I—Food and Drug Administration, Department of Health, Education, and Welfare

### SUBCHAPTER B—FOOD AND FOOD PRODUCTS

### PART 19—CHEESES, PROCESSED CHEESES, CHEESE FOODS, CHEESE SPREADS, AND RELATED FOODS

### Creamed Cottage Cheese; Order Amending Identity Standard to List Diacetyl and Other Flavors; Cottage Cheese Whey, and Sodium Citrate as Optional Ingredients

In the matter of amending the standard of identity for creamed cottage cheese (21 CFR 19.530) by listing diacetyl, starter distillate, and other safe and suitable flavoring substances that enhance the characteristic flavor and aroma of the food as optional ingredients of the creaming mixture; and by listing cottage cheese whey and sodium citrate to provide another citrated medium in which to culture flavor- and aroma-producing bacteria for addition to the creaming mixture:

A notice of proposed rulemaking in the above-identified matter was published in the FEDERAL REGISTER of February 8, 1967 (32 F.R. 2346), based on a petition submitted by the Milk Industry Foundation, 1012 14th Street NW., Washington, D.C. 20005.

The information furnished by the petitioner, the comments filed in response to the proposal, and other available information have been considered, and it is concluded that it will promote honesty and fair dealing in the interest of consumers to adopt the amendments as set forth below.

Therefore, pursuant to the authority vested in the Secretary of Health, Education, and Welfare by the Federal Food, Drug, and Cosmetic Act (secs. 401, 701, 52 Stat. 1046, 1055, as amended 70 Stat. 919, 72 Stat. 948; 21 U.S.C. 341, 371) and delegated by him to the Commissioner of Food and Drugs (21 CFR 2.120): *It is ordered*, That § 19.530 be amended by revising paragraph (b)(4), by adding a new subparagraph (7) to paragraph (b), by redesignating paragraph (d)(2) as (d)(3), with changes, and by adding to paragraph (d) a new subparagraph (2). The affected portions read as follows:

**§ 19.530 Creamed cottage cheese; identity; label statement of optional ingredients.**

* * * * *

(b) * * *

(4) A preparation of pasteurized skim milk or cottage cheese whey with added citric acid or sodium citrate, which preparation has been cultured with harmless flavor- and aroma-producing bacteria.

* * * * *

(7) Singly or in combination: Diacetyl, starter distillate, or other safe and suitable flavoring substances which contribute to the characteristic flavor and aroma associated with the food.

* * * * *

(d) * * *

(2) When any ingredient named under paragraph (b)(7) is used, the label shall bear the statement "artificially flavored" or "artificial flavor added" or "with added artificial flavoring".

(3) Wherever the name "creamed cottage cheese" appears on the label so conspicuously as to be easily seen under customary conditions of purchase, the label declarations prescribed in subparagraphs (1) and (2) of this paragraph, showing the optional ingredients present, shall immediately and conspicuously precede or follow such name without intervening written, printed, or graphic matter.

Any person who will be adversely affected by the foregoing order may at any time within 30 days following the date of its publication in the FEDERAL REGISTER file with the Hearing Clerk, Department of Health, Education, and Welfare, Room 5440, 330 Independence Avenue SW., Washington, D.C. 20201, written objections thereto. Objections shall show wherein the person filing will be adversely affected by the order and specify with particularity the provisions of the order deemed objectionable and the grounds for the objections. If a hearing is requested, the objections must state the issues for the hearing, and such objections must be supported by grounds legally sufficient to justify the relief sought. Objections may be accompanied by a memorandum or brief in support thereof. All documents shall be filed in six copies.

*Effective date.* This order shall become effective 60 days from the date of its publication in the FEDERAL REGISTER, except as to any provisions that may be stayed by the filing of proper objections. Notice of the filing of objections or lack thereof will be announced by publication in the FEDERAL REGISTER.

(Secs. 401, 701, 52 Stat. 1046, 1055, as amended 70 Stat. 919, 72 Stat. 948; 21 U.S.C. 341, 371)

Dated: May 9, 1967.

JAMES L. GODDARD,
*Commissioner of Food and Drugs.*

[F.R. Doc. 67-5443; Filed, May 15, 1967; 8:50 a.m.]

Fed. Regist. 21 JAN. 1970 35(14): 806-807



(Secs. 401, 701, 52 Stat. 1046, 1055, as amended 70 Stat. 919, 72 Stat. 948; 21 U.S.C. 341, 371)

Dated: January 12, 1970.

SAM D. FINE,
*Acting Associate Commissioner for Compliance.*

[F.R. Doc. 70-736; Filed, Jan. 20, 1970; 8:45 a.m.]

## PART 37—FISH

### Canned Tuna, Identity Standard; Provision for Use of Lemon Flavoring

In the matter of amending the standard of identity for canned tuna (§ 37.1) to provide for the use of lemon flavoring:

A notice of proposed rule making in the above-identified matter was published in the FEDERAL REGISTER of June 28, 1969 (34 F.R. 9996), in response to a petition submitted by Star-Kist Foods, Inc., Terminal Island, Calif. 90731. For reasons given, the notice included a proposal by the Commissioner of Food and Drugs that the listing of lemon oil be made optional rather than mandatory and that the words "lemon flavored," or words of similar import, be made part of the name of the food. In the only comment received regarding the proposal, the petitioner asserted that the firm would not object to the Commissioner's modifications.

Based on information submitted by the petitioner, the comment received, and other relevant information, the Commissioner concludes that it will promote honesty and fair dealing in the interest of consumers to amend the standard as set forth below. Therefore, pursuant to provisions of the Federal Food, Drug, and Cosmetic Act (secs. 401, 701, 52 Stat. 1046, 1055, as amended 70 Stat. 919, 72 Stat. 948; 21 U.S.C. 341, 371) and under authority delegated to the Commissioner (21 CFR 2.120): *It is ordered*, That § 37.1 be amended by adding to paragraph (f) a new subparagraph (8), by revising paragraph (h) (6) and (8), and by adding to paragraph (h) a new subparagraph (9), as follows:

**§ 37.1 Canned tuna; definition and standard of identity; label statement of optional ingredients.**

* * * * * * *

(f) * * *

(8) Lemon flavoring to be prepared from lemon oil and citric acid together with safe and suitable carriers for the lemon oil which are present at nonfunctional and insignificant levels in the finished canned food. When lemon flavoring is added, a safe and suitable solubilizing and dispersing ingredient may be added in a quantity not exceeding 0.005 percent by weight of the finished food. A substance used in accordance with this paragraph is deemed to be suitable if it is used in an amount no greater than necessary to achieve the intended flavor effect, and is deemed to be safe if it is not a food additive as defined in section 201(s) of the act or, if it is a food additive as so defined, it is used in conformity with regulations established pursuant to section 409 of the act.

* * * * * * *

(h) * * *

(6) Where the canned tuna contains one or more of the ingredients provided for in paragraph (f) of this section, the label shall bear the statement "Seasoned with _____," the blank being filled in with the name or names of the ingredient or ingredients used, except that if the ingredient designated in paragraph (f) (6) of this section is used, the blanks shall be filled in with the term "vegetable broth"; and if the ingredient designated in paragraph (f) (5) of this section is used alone, the label may alternatively bear either the statement "spiced" or the statement "with added spice"; and if salt is the only seasoning ingredient used, the label may alternatively bear any of the statements "salted," "with added salt," or "salt added." If the flavoring ingredients designated in paragraph (f) (8) of this section are used, the words "lemon flavored" or "with lemon flavoring" shall appear as a part of the name on the label; for example, "lemon flavored chunk light tuna". Citric acid and any optional solubilizing and dispersing agent used as specified in paragraph (f) (8) of this section in connection with lemon flavoring ingredients shall be designated on the label by its common or usual name.

* * * * * * *

(8) Wherever the name of the food appears on the label so conspicuously as to be easily seen under customary conditions of purchase, the names of the optional ingredients used, as specified in subparagraphs (3), (6), and (7) of this paragraph (except if lemon flavoring is added, this subparagraph applies only to the terms "lemon flavored" or "with lemon flavoring," not to the constituent ingredients of that flavoring or to any optional solubilizing or dispersing ingredient used in connection with lemon flavoring ingredients), shall immediately and conspicuously precede or follow such name without intervening, written, printed, or graphic matter, except that the common name of the species of tuna fish may so intervene; but the species name "albacore" may be employed only for canned tuna of that species which meets the color designation "white" as prescribed by paragraph (d) (1) of this section.

(9) Statements of optional ingredients present required by subparagraph (6) of this paragraph, but not subject to the provisions of subparagraph (8) of this paragraph shall be set forth on the label with such prominence and conspicuousness as to render them likely to be read and understood by the ordinary individual under customary conditions of purchase.

Any person who will be adversely affected by the foregoing order may at any time within 30 days from the date of its publication in the FEDERAL REGISTER file with the Hearing Clerk, Department of Health, Education, and Welfare, Room 5440, 330 Independence Avenue SW., Washington, D.C. 20201, written objections thereto. Objections shall show wherein the person filing will be adversely affected by the order and specify with particularity the provisions of the order deemed objectionable and the grounds for the objections. If a hearing is requested, the objections must state the issues for the hearing, and such objections must be supported by grounds legally sufficient to justify the relief sought. Objections may be accompanied by a memorandum or brief in support thereof. All documents shall be filed in six copies.

*Effective date.* This order shall become effective 60 days from the date of its publication in the FEDERAL REGISTER, except as to any provisions that may be stayed by the filing of proper objections. Notice of the filing of objections or lack thereof will be announced by publication in the FEDERAL REGISTER.

(Secs. 401, 701, 52 Stat. 1046, 1055, as amended 70 Stat. 919, 72 Stat. 948; 21 U.S.C. 341, 371)

Dated: January 12, 1970.

SAM D. FINE,
*Acting Associate Commissioner for Compliance.*

[F.R. Doc. 70-746; Filed, Jan. 20, 1970; 8:46 a.m.]

# 9

# Animals and Animal Products

Revised as of January 1, 1974

CONTAINING
A CODIFICATION OF DOCUMENTS
OF GENERAL APPLICABILITY
AND FUTURE EFFECT

AS OF JANUARY 1, 1974

*With Ancillaries*

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as a Special Edition of
the Federal Register

Code of Federal Regulations

spected and passed, may be used for meat food products. The defibrination of blood intended for food purposes shall not be performed with the hands.

(8) Intestines shall not be used as ingredients in any meat food product for which a standard is prescribed in Part 319 of this subchapter and shall not be used in other products unless the products are labeled in accordance with § 317.8(b)(3) of this subchapter.

(9) Poultry products and egg products (other than shell eggs) which are intended for use as ingredients of meat food products shall be considered acceptable for such use only when identified as having been inspected and passed for wholesomeness by the Department under the regulations in 7 CFR Part 59 or 70 or 9 CFR Part 381 and when found to be sound and otherwise acceptable when presented for use. Poultry products and egg products (other than shell eggs) which have not been so inspected and passed for wholesomeness shall not be used in the preparation of such meat food products.

(10) Dry milk products which are intended for use as ingredients of meat food products shall be considered acceptable for such use only when produced in a plant approved by the Department under the regulations in 7 CFR Part 58, and when found to be sound and otherwise acceptable when presented for use. Dry milk products prepared in a plant not so approved shall not be used in the preparation of such meat food products.

(11) All isolated soy protein used in products prepared in any official establishment shall contain not more and not less than 0.1 percent titanium incorporated as food grade titanium dioxide. and the presence of such substance must be shown on the label of the container of the isolated soy protein at all times that the article is in the official establishment.

(12) Ingredients for use in any product may not bear or contain any pesticide chemical or other residues in excess of level permitted in § 318.16.

[35 FR 15586, Oct. 3, 1970, as amended at 38 FR 14368, June 1, 1973; 38 FR 29214, Oct. 23, 1973]

### § 318.7 Approval of substances for use in the preparation of products.

(a)(1) No chemical substance may be used in the preparation of any product unless it is approved in this part or Part 319 of this subchapter or by the Administrator in specific cases.

(2) No product shall bear or contain any substance which would render it adulterated or which is not approved in this part or Part 319 of this subchapter or by the Administrator in specific cases.

(b) [Reserved]

(c) Under appropriate declaration as required in Parts 316 and 317 of this subchapter, the following substances may be added to products:

(1) Common salt, approved sugars (sucrose, cane or beet sugar), maple sugar, dextrose, invert sugar, honey, corn syrup solids, corn syrup and glucose syrup), wood smoke, vinegar, flavorings, spices, sodium nitrate, sodium nitrite, potassium nitrate, potassium nitrite, and other substances specified in the chart in subparagraph (4) of this paragraph may be added to products under conditions, if any, specified in this part or in Part 317 of this subchapter.

(2) Other harmless artificial flavorings may be added to products with the approval of the Administrator in specific cases.

(3) Coloring matter and dyes other than those specified in the chart in subparagraph (4) of this paragraph, may be applied to products, mixed with rendered fat, applied to natural and artificial casings, and applied to such casings enclosing products, if approved by the Administrator in specific cases. When any coloring matter or dye is applied to casings, there shall be no penetration of coloring into the product. When any coloring matter or dye is added to meat fat shortening containing artificial flavoring, the product shall be packed in conventional, round shortening containers having a capacity no greater than 3 pounds.

(4) The substances specified in the following chart are acceptable for use in the preparation of products, provided they are used for the purposes indicated, within the limits of the amounts stated and under other conditions specified in this part and Part 317 of this subchapter. In addition to the substances listed in the following chart, Part 319 of this subchapter specifies other substances that are acceptable in preparing specified products.

| Class of substance | Substance | Purpose | Products | Amount | |
|---|---|---|---|---|---|
| Anticoagulants | Citric acid<br>Sodium citrate. | To prevent clotting | Fresh beef blood | 0.2 percent—with or without water. When water is used to make a solution of citric acid or sodium citrate added to beef blood not more than 2 parts of water to 1 part of citric acid or sodium citrate shall be used. | |
| Antifoaming agent | Methyl polysilicone | To retard foaming | Soups | 10 parts per million. | |
| | | | Rendered fats | Do. | |
| | | | Curing pickle | 50 parts per million. | |
| Antioxidants and oxygen interceptors. | BHA (butylated hydroxyanisole) | To retard rancidity | Dry sausage | 0.003 percent based on total weight. | 0.006 percent in combination. |
| | BHT (butylated hydroxytoluene). | do | do | do | |
| | Propyl gallate | do | do | do | |
| | BHA (butylated hydroxyanisole). | do | Rendered animal fat or a combination of such fat and vegetable fat. | 0.01 percent | 0.02 percent in combination. |
| | BHT (butylated hydroxytoluene). | do | do | do | |
| | Glycine | do | do | do | |
| | Propyl gallate | do | do | do | |
| | Resin guaiac | do | do | do | |
| | Tocopherols | do | do | 0.03 percent. A 30 percent concentration of tocopherols in vegetable oils shall be used when added as an antioxidant to products designated as "lard" or "rendered pork fat." | |
| | BHA (butylated hydroxyanisole). | do | Fresh pork sausage, brown and serve sausage, pre-grilled beef patties, and fresh sausage made from beef or beef and pork. | 0.01 percent based on fat content. | 0.02 percent combination based on fat content. |
| | BHT (butylated hydroxytoluene). | do | do | do | |
| | Propyl gallate | do | do | do | |
| | BHA (butylated hydroxyanisole). | do | Dried meats | 0.01 percent based on total weight. | 0.01 percent in combination. |
| | BHT (butylated hydroxytoluene). | do | do | do | |
| | Propyl gallate | do | do | do | |
| Binders | Algin | To extend and stabilize product. | Breading mix; sauces. | Sufficient for purpose. | |
| | Carrageenan | do | do | Do. | |
| | Carboxymethyl cellulose (cellulose gum). | do | Baked pies | Do. | |
| | Gums, vegetable | do | Egg roll | Do. | |
| | Methyl cellulose | To extend and to stabilize product (also carrier). | Meat and vegetable patties. | 0.15 percent. | |
| Binders | Isolated soy protein | To bind and extend product. | Sausage, as provided for in Part 319 of this subchapter. | 2 percent. | |
| | do | do | Imitation sausage; nonspecific loaves; soups; stews. | Sufficient for purpose. | |
| | Sodium caseinate | do | do | Do. | |
| | Whey (dried) | do | do | Do. | |
| Bleaching agent | Hydrogen peroxide | To remove color | Tripe (substance must be removed from product by rinsing with clear water). | Do. | |

| Class of substance | Substance | Purpose | Products | Amount |
|---|---|---|---|---|
| Catalysts (substances must be eliminated during process). | Nickel | To accelerate chemical reaction. | Rendered animal fats or a combination of such fats and vegetable fats. | Sufficient for purpose. |
| | Sodium amide | Rearrangement of fatty acid radicals. | do | Do. |
| | Sodium methoxide | do | do | Do. |
| Coloring agents (natural). | Alkanet, annatto, carotene, cochineal, green chlorophyl, saffron and tumeric. | To color casings or rendered fats; marking and branding product. | Sausage casings, oleomargarine, shortening, marking or branding ink on product. | Sufficient for purpose. (may be mixed with approved artificial dyes or harmless inert material such as common salt and sugar). |
| Coloring agents (artificial). | Coal tar dyes approved under the Federal Food, Drug, and Cosmetic Act (operator must furnish evidence to inspector in charge that dye has been certified for use in connection with foods by the Food and Drug Administration). | do | do | Sufficient for purpose. (may be mixed with approved natural coloring matters or harmless inert materia such as common salt o sugar). |
| | Titanium dioxide | do | Canned ham salad spread and creamed type canned products. | 0.5 percent. |
| Cooling and retort water treatment agents. | Calcium chloride | To prevent staining on exterior of canned goods. | Any | Sufficient for purpose. |
| | Citric acid | do | do | Do. |
| | Dioctyl sodium sulfosuccinate. | do | do | 0.05 percent. |
| | Disodium-calcium ethylenediamine-tetraacetate. | do | do | Sufficient for purpose. |
| | Disodium ethylenediamine-tetraacetate. | do | do | Do. |
| | Disodium phosphate | do | do | Do. |
| | Ethylenediamine-tetraacetic acid. | do | do | Do. |
| | Isopropanol | do | do | 0.002 percent. |
| | Potassium pyrophosphate. | do | do | Sufficient for purpose. |
| | Propylene glycol | do | do | Do. |
| | Sodium bicarbonate | do | do | Do. |
| | Sodium bisulfate | To inhibit corrosion on exterior of canned goods. | do | 0.001 percent. |
| | Sodium carbonate | To prevent staining on exterior of canned goods. | do | Sufficient for purpose. |
| | Sodium dodecylbenzene sulfonate. | do | do | 0.05 perenct. |
| | Sodium gluconate | do | do | Sufficient for purpose. |
| | Sodium hexametaphosphate. | do | do | Do. |
| | Sodium laurylsulfate | do | do | 0.0[illegible] percent. |
| | Sodium metasilicate | do | do | Sufficient for purpose. |
| | Sodium n-alkylbenzene sulfonate (alkyl group predominantly $C_{12}$ and $C_{13}$ and not less than 95 percent $C_{10}$ to $C_{18}$). | do | do | 0.05 percent. |
| | Sodium nitrite. (The dry nitrite must be decharacterized with 0.05 percent powdered charcoal or 0.03 percent nigrosine. Bulk decharacterized sodium nitrite when in cook room shall be held in a locked container conspicuously labeled "Decharacterized Sodium Nitrite—to be used by authorized personnel only.") | To inhibit corrosion on exterior of canned goods. | do | 600 parts per million. |

| Class of substance | Substance | Purpose | Products | Amount |
|---|---|---|---|---|
| | Sodium pyrophosphate. | To prevent staining on canned goods. | do | 0.05 percent. |
| | Sodium tripolyphosphate. | do | do | Do. |
| | Zinc oxide | do | do | 0.01 percent. |
| | Zinc sulfate | do | do | Do. |
| Curing accelerators; must be used only in combination with curing agents. | Sodium Acid Pyrophosphate. | To accelerate color fixing. | Frankfurters, weiners, vienna, bologna, garlic bologna, knockwurst, and similar products. | Not to exceed, alone or in combination with other curing accelerators, the following: 8 ozs. in 100 lbs. of the meat, or meat and meat byproducts, content of the formula; nor 0.5 percent in the finished product. |
| Curing agents | Ascorbic acid | To accelerate color fixing or preserve color during storage. | Cured pork and beef cuts, cured comminuted meat food product. | 75 ozs. to 100 gals. pickle at 10 percent pump level ¾ oz. to 100 lbs. meat or meat byproduct; 10 percent solution to surfaces of cured cuts prior to packaging (the use of such solution shall not result in the addition of a significant amount of moisture to the product). |
| | Erythorbic acid | do | do | Do. |
| | Glucono delta lactone | To accelerate color fixing. | Cured, comminuted meat or meat food product. | 8 ozs. to each 100 lbs. of meat or meat byproduct. |
| | | | Genoa salami | 16 ozs. to 100 lbs. of meat (1.0 percent). |
| | Sodium ascorbate | To accelerate color fixing or preserve color during storage. | Cured pork and beef cuts, cured comminuted meat food product. | 87.5 ozs. to 100 gals. pickle at 10 percent pump level; ⅞ ozs. to 100 lbs. meat or meat byproduct; 10 percent solution to surfaces of cured cuts prior to packaging (the use of such solution shall not result in the addition of a significant amount of moisture to the product). |
| | Sodium erythorbate | do | do | Do. |
| | Citric acid or sodium citrate. | do | do | May be used in cured products or in 10 per cent solution used to spray surfaces of cured cuts prior to packaging to replace up to 50 percent of the ascorbic acid, erythorbic acid, sodium ascorbate, or sodium erythorbate that is used. |
| | Sodium or potassium nitrate. | Source of nitrite | Cured products | 7 lbs. to 100 gals. pickle; 3½ ozs. to 100 lbs. meat (dry cure); 2¾ ozs. to 100 lbs. chopped meat. |
| | Sodium or potassium nitrite (supplies of sodium nitrite and potassium nitrite and mixtures containing them must be kept securely under the care of a responsible employee of the establishment. The specific nitrite content of such supplies must be known and clearly marked accordingly). | To fix color | do | 2 lbs. to 100 gals. pickle at 10 percent pump level; 1 oz. to 100 lbs. meat (dry cure); ¼ oz. to 100 lbs. chopped meat and/or meat byproduct. The use of nitrites, nitrates, or combination shall not result in more than 200 parts per million calculated as sodium nitrite, in finished product. |
| Denuding agents; may be used in combination. Must be removed from tripe by rinsing with potable water. | Lime (calcium oxide, calcium hydroxide). | To denude mucous membrane. | Tripe | Sufficient for purpose. |

| Class of substance | Substance | Purpose | Products | Amount |
|---|---|---|---|---|
| | Sodium carbonate | do | do | Sufficient for purpose. |
| | Sodium gluconate | do | do | Do. |
| | Sodium hydroxide | do | do | Do. |
| | Sodium metasilicate | do | do | Do. |
| | Sodium persulfate | do | do | Do. |
| | Trisodium phosphate | do | do | Do. |
| Emulsifying agents. | Acetylated monoglycerides. | To emulsify product. | Shortening | Do. |
| | Diacetyl tartaric acid esters of mono and diglycerides. | do | Rendered animal fat or a combination of such fat with vegetable fat. | Do. |
| | Glycerol-lacto stearate, oleate, or palmitate. | do | do | Do. |
| | Lecithin | To emulsify product (also as antioxidant). | Oleomargarine, shortening. | Do. |
| | Mono and diglycerides (glycerol palmitate, etc.). | To emulsify product. | Rendered animal fat or a combination of such fat with vegetable fat. | Sufficient for purpose in lard and shortening; 0.5 percent in oleomargarine. |
| | Polyglycerol esters of fatty acids (polyglycerol esters of fatty acids are restricted to those up to and including the decaglycerol esters and otherwise meeting the requirements of § 121.1120 (a) of the Food Additive Regulations). | do | Rendered animal fat or a combination of such fat with vegetable fat when use is not precluded by standards of identity or composition. | Sufficient for purpose. |
| | olysorbate 80 (polyoxyethylene (20) sorbitan monooleate). | do | hortening[f] or use in nonstandardized baked goods, baking mixes, icings, fillings, and toppings and in the frying of foods. | 1 percent when used alone. If usedwith polysorbate. 60 the combined total shall not exceed 1 percent. |
| | Propylene glycol mono and diesters of fats and fatty acids. | do | Rendered animal fat or a combination of such fat with vegetable fat. | Sufficient for purpose. |
| | Polysorbate 60 (polyoxeyethylene (20) sorbitan monostearate). | do | Shortening for use in nonstandardized baked goods, baking mixes, icings, fillings, and toppings and in the frying of foods. | 1 percent when used alone. If used with polysorbate 80 the combined total shall not exceed 1 percent. |
| | Stearyl-2-lactylic acid | do | Shortening to be used for cake icings and fillings. | 3.0 percent. |
| | Stearyl monoglyceridyl citrate. | do | Shortening | Sufficient for purpose. |
| Flavoring agents; protectors and developers. | Program approved artificial smoke flavoring.[1] | To flavor product | Various[2] | Do. |
| | Program approved smoke flavoring.[1] | do | do | Do. |
| | Autolyzed yeast extract. | do | do | Do. |
| | Harmless bacteria starters of the acidophilus type, lactic acid starter or culture of Pediococcus cerevisiae. | To develop flavor | Dry sausage, pork roll, thuringer, lebanon bologna, cervelat, and salami. | 0.5 percent. |
| | Benzoic acid, sodium benzoate. | To retard flavor reversion. | Oleomargarine | 0.1 percent. |
| | Citric acid | To protect flavor | do | Sufficient for purpose. |
| | do | Flavoring | Chili con carne | Sufficient for purpose. |
| | Corn syrup solids, corn syrup, glucose syrup. | To flavor | Sausage, hamburger, meat loaf, luncheon meat, chopped or pressed ham. | 2.0 percent individually or collectively, calculated on a dry basis. |

See footnotes at end of table.

| Class of substance | Substance | Purpose | Products | Amount |
|---|---|---|---|---|
| | Dextrose | To flavor product | Sausage, ham and cured products. | Sufficient for purpose. |
| | Diacetyl | do | Oleomargarine | Do. |
| | Disodium guanylate | do | Various[3] | Do. |
| | Disodium inosinate | do | Various[3] | Do. |
| | Hydrolyzed plant protein. | do | Various[3] | Do. |
| | Isopropyl citrate | To protect flavor | Oleomargarine | 0.02 percent. |
| | Malt syrup | To flavor product | Cured products | 2.5 percent. |
| | Milk protein hydrolysate. | do | Various[3] | Sufficient for purpose. |
| | Monosodium glutamate. | do | do | Do. |
| | Sodium sulfoacetate derivative of mono and diglycerides. | do | do | 0.5 percent. |
| | Sodium tripolyphosphate. | To help protect flavor. | "Fresh Beef," "Beef for Further Cooking," "Cooked Beef," and similar products which are frozen after processing. | Do. |
| | Mixtures of sodium tripolyphosphate and sodium hexametaphosphate. | do | do | Do. |
| | Sorbitol | To flavor, to facilitate the removal of casings from product and to reduce caramelization and charring. | Cooked sausage labeled frankfurter, frank, furter, wiener, knockwurst. | Not more than 2 percent of the weight of the formula, excluding the formula weight of water or ice; not permitted in combination with corn syrup, and/or corn syrup solids. |
| | Starter distillate | To help protect flavor. | Oleomargarine | Sufficient for purpose. |
| | Stearyl citrate | To protect flavor | do | 0.15 percent. |
| | Sugars (sucrose and dextrose). | To flavor product | Various[3] | Sufficient for purpose. |
| Gases | Carbon dioxide solid (dry ice). | To cool product | Chopping of meat, packaging of product. | Do. |
| | Nitrogen | To exclude oxygen | Sealed container | Do. |
| Hog scald agents; must be removed by subsequent cleaning operations. | Caustic soda | To remove hair | Hog carcasses | Do. |
| | Dioctyl sodium sulfosuccinate. | do | do | Do. |
| | Lime | do | do | Do. |
| | Methyl polysilicone | do | do | Do. |
| | Sodium carbonate | do | do | Do. |
| | Sodium dodecylbenzene sulfonate. | do | do | Do. |
| | Sodium hexametaphosphate. | do | do | Do. |
| | Sodium lauryl sulfate. | do | do | Do. |
| | Sodium metasilicate | do | do | Do. |
| | Sodium n-alkylbenzene sulfonate (alkyl group predominantly $C_{12}$ and $C_{13}$ and not less than 95 percent $C_{10}$ to $C_{16}$). | do | do | Do. |
| | Sodium sulfate | do | do | Do. |
| | Sodium tripolyphosphate. | do | do | Do. |
| | Sucrose | do | do | Do. |
| | Trisodium phosphate. | do | do | Do. |
| Miscellaneous | Potassium sorbate | To retard mold growth. | Dry sausage | 2.5 percent in water solution may be applied to casings after stuffing or casings may be dipped in solution prior to stuffing. |
| | | To preserve product and to retard mold growth. | Oleomargarine or margarine | 0.1 percent by weight of the finished oleomargarine or margarine. |

| Class of substance | Substance | Purpose | Products | Amount |
|---|---|---|---|---|
| | Calcium disodium, EDTA (calcium disodium ethylenediaminetetraacetate). | To preserve product and to protect flavor. | do | 75 parts per million by weight of the finished oleomargarine or margarine. |
| | Propyl paraben (propyl p-hydroxybenzoate). | To retard mold growth. | Dry sausage | 3.5 percent in water solution may be applied to casings after stuffing, or casings may be dipped in solution prior to stuffing. |
| | Sodium bicarbonate | To neutralize excess acidity, cleaning vegetables. | Rendered fats, soups, curing pickle. | Sufficient for purpose. |
| | Calcium propionate | To retard mold growth. | Pizza crust | 0.32 percent alone or in combination based on weight of the flour used. |
| | Sodium propionate | do | do | |
| | Sodium hydroxide | To decrease amount of cooked out juices. | Cured hams, pork shoulder picnics and loins, canned hams and pork shoulder picnics, and products covered by § 319.104(d); chopped ham and bacon. | May be used only in combination with phosphates in ratio of four parts phosphate to one part sodium hydroxide; the combination shall not exceed 5 percent in pickle at 10 percent pump level; 0.5 percent in product. |
| Phosphates | Disodium phosphate | do | do | 5 percent of phosphate in pickle at 10 percent pump level; 0.5 percent of phosphate in product (only clear solution may be injected into product). |
| | Monosodium phosphate. | do | do | Do. |
| | Sodium hexametaphosphate. | do | do | Do. |
| | Sodium tripolyphosphate. | do | do | Do. |
| | Sodium pyrophosphate. | do | do | Do. |
| | Sodium acid pyrophosphate. | do | do | Do. |
| Proteolytic enzymes. | Aspergillus oryzae | To soften tissues | Beef cuts | Solutions consisting of water, salt, monosodium glutamate, and approved proteolytic enzymes applied or injected into cuts of beef shall not result in a gain of more than 3 percent above the weight of the untreated product. |
| | Aspergillus flavus-oryzae group. | do | do | Do. |
| | Bromelin | do | do | Do. |
| | Ficin | do | do | Do. |
| | Papain | do | do | Do. |
| Refining agents (must be eliminated during process of manufacturing). | Acetic acid | To separate fatty acids and glycerol. | Rendered fats | Sufficient for purpose. |
| | Bicarbonate of soda | do | do | Do. |
| | Carbon (purified charcoal). | To aid in refining of animal fats. | do | Do. |
| | Caustic soda (sodium hydroxide). | To refine fats | do | Do. |
| | Diatomaceous earth; Fuller's earth. | do | do | Do. |
| | Sodium carbonate | do | do | Do. |
| | Tannic acid | do | do | Do. |
| Rendering agents | Tricalcium phosphate | To aid rendering | Animal fats | Do. |
| | Trisodium phosphate | do | do | Do. |
| Artificial sweeteners. | Saccharin | To sweeten product | Bacon | 0.01 percent. |
| Synergists (used in combination with antioxidants). | Citric acid | To increase effectiveness of antioxidants. | Lard and shortening | 0.01 percent alone or in combination with antioxidants in lard or shortening. |
| | | | Dry sausage | 0.003 percent in dry sausage in combination with antioxidants. |

## Subpart P—Fats, Oils, Shortenings

### § 319.700 Oleomargarine or margarine.

(a) Oleomargarine or margarine is the plastic food which is prepared in accordance with the provisions of subparagraphs (1), (2), (3), and (4) of this paragraph. In this section the term "oleomargarine" is used to refer to such product whether it is sold as "oleomargarine" or "margarine."

(1) It is prepared with one or more of the fat ingredients named in any one of the subdivisions (i), (ii), (iii), and (iv) of this subparagraph.

(i) The rendered fat or oil, or stearin derived therefrom (any or all of which may be hydrogenated), of cattle, sheep, swine, or goats, or any combination of two or more of such articles;

(ii) Any vegetable food fat or oil, or oil or stearin derived therefrom (any or all of which may be hydrogenated), or any combination of two or more of such articles;

(iii) Any combination of ingredients named under subdivision (i) and (ii) of this subparagraph in such proportion that the weight of the ingredients named under subdivision (i) of this subparagraph either equals the weight of the ingredients named under subdivision (ii) of this subparagraph or exceeds such weight by a ratio not greater than 9 to 1;

(iv) Any combination of ingredients named under subdivisions (i) and (ii) of this subparagraph in such proportion that the weight of the ingredients named under subdivision (ii) of this subparagraph exceeds the weight of the ingredients named under subdivision (i) of this subparagraph by a ratio not greater than 9 to 1.

(2) One of the articles (or combinations) named under subdivisions (i), (ii), (iii), (iv), (v), (vi), (vii), (viii), or (ix) of this subparagraph is intimately mixed with the fat ingredient or ingredients. The ingredients named under subdivisions (i), (ii), (iii), (iv), (v), (vi), and (vii) of this subparagraph are pasteurized and then may be subjected to the action of harmless bacterial starters. The term "milk" as used in this subparagraph means "cow's milk."

(i) Cream;

(ii) Milk;

(iii) Skim milk;

(iv) Liquid sweet cream buttermilk;

(v) Any combination of dry or condensed sweet cream buttermilk and water with a total solids content of not less than 8.5 percent.

(vi) Any combination of nonfat dry milk and water in which the weight of the nonfat dry milk is not less than 10 percent of the weight of the water;

(vii) Any combination of two or more of the articles (or combination) named under subdivisions (i), (ii), (iii), (iv), (v), and (vi) of this subparagraph;

(viii) In case only of the fat ingredient named in subparagraph (1)(ii) of this paragraph, any combination of finely ground soybeans and water, in which the weight of the finely ground soybeans is not less than 10 percent of the weight of the water. The finely ground soybeans are subjected to a heat treatment before or after mixing with the water. The soybeans may or may not be dehulled;

(ix) Water in lieu of any of the articles (or combinations) designated in subdivisions (i), (ii), (iii), (iv), (v), (vi), (vii), or (viii) of this subparagraph.

Congealing is effected, either with or without contact with water, and the congealed mixture may be worked.

(3) It may contain one or more of the following optional ingredients in addition to the ingredients and articles named in subparagraphs (1) and (2) of this paragraph.

(i) Artificial coloring. For the purpose of this subdivision, pro-Vitamin A shall be deemed to be artificial coloring;

(ii) Sodium benzoate, or benzoic acid, or a combination of these, in a quantity not to exceed 0.1 percent of the weight of the finished product;

(iii) Vitamin A (with or without any accompanying Vitamin D and with or without Vitamin D concentrate), in such quantity that the finished oleomargarine contains not less than 15,000 United States Pharmacopeia units of Vitamin A per pound, as determined by the method prescribed in the Pharmacopeia of the United States for total biological Vitamin A activity. The Vitamin A potency prescribed may be furnished by fish liver oil; by concentrates of Vitamin A or its fatty

acid esters from animal sources; by synthetic Vitamin A or its fatty acid esters; by mixtures of synthetic Vitamin A or its fatty acid esters with harmless substances formed during the synthesis of the Vitamin A, if the Vitamin A or its fatty acid ester constitutes not less than 50 percent of the mixture; by pro-Vitamin A; or by any combination of two or more of these. For the purposes of this subdivision, the term "fatty acid" may include acetic acid;

(iv) Any safe and suitable artificial flavoring substance that imparts to the food a flavor in semblance of butter. Such artificial flavoring substances are deemed to be safe for use in oleomargarine which contains any fat ingredient named in subparagraph (1)(i) of this paragraph if they are not food additives as defined in section 201(s) of the Federal Food, Drug, and Cosmetic Act, or if they are used in conformity with regulations established pursuant to section 409 of that Act, and in either case if they have been approved for such use by the Administrator; and they are deemed to be safe for use in other oleomargarine if they are used in conformity with regulations established pursuant to section 409 of the Federal Food, Drug, and Cosmetic Act or their use is otherwise lawful under that Act;

(v) (*a*) Lecithin, in an amount not exceeding 0.5 percent of the weight of the finished oleomargarine; or

(*b*) Monoglycerides or diglycerides of fat-forming fatty acids, or a combination of these, in an amount not exceeding 0.5 percent of the weight of the finished oleomargarine; or

(*c*) Such monoglycerides and diglycerides in combination with the sodium sulfoacetate derivatives thereof in a total amount not exceeding 0.5 percent of the weight of the finished oleomargarine; or

(*d*) A combination of the substances specified in (*a*) and (*b*) of this subdivision in which the amount of neither exceeds that stated in (*a*) and (*b*); or

(*e*) A combination of the substances specified in (*a*) and (*c*) of this subdivision in a total amount not exceeding 0.5 percent of the weight of the finished oleomargarine. The weight of the diglycerides in each of the ingredients specified in (*b*), (*c*), (*d*), and (*e*) of this subdivision is calculated at one-half actual weight;

(vi) Butter;

(vii) Salt;

(viii) Citric acid incorporated in the fat or oil ingredient used;

(ix) Isopropyl citrates incorporated in the fat or oil ingredient used, in an amount not to exceed 0.02 percent by weight of the finished oleomargarine;

(x) Stearyl citrate incorporated in the fat or oil ingredient in an amount not to exceed 0.15 percent by weight of the finished oleomargarine;

(xi) Potassium sorbate, in an amount not to exceed 0.1 percent by weight of the finished oleomargarine;

(xii) Calcium disodium EDTA (calcium disodium ethylenediaminetetraacetate) in an amount not to exceed 75 parts per million by weight of the finished oleomargarine.

(xiii) BHA (butylated hydroxyanisole) or BHT (butylated hydroxytoluene), or a combination of these, incorporated in any animal fat ingredient permitted by subparagraph (1)(i) of this paragraph, in an amount not to exceed 0.02 percent by weight of such animal fat content.

(4) The finished oleomargarine contains not less than 80 percent fat, as determined by the method prescribed in the current "Official Methods of Analysis of the Association of Official Analytical Chemists."[8]

(b) The name of the food for which a definition and standard of identity are prescribed by this section is "oleomargarine" or "margarine." The presence of ingredients, provided for in paragraph (a) of this section, in the finished product shall be declared as follows:

(1) Fat ingredients shall be declared first in the ingredient statement by the name of the specific fat or oil or stearin used. Where combinations of fat ingredients are used, the names shall be arranged in order of decreasing predominance. If any fat ingredient is hydrogenated, the ingredient statement shall include the word "hydrogenated" or "hardened" at such place or places in the

[8] Copies of this publication are available from the Association of Official Analytical Chemists, Post Office Box 540, Benjamin Franklin Station, Washington, D.C. 20044.

shall designate the type of plan and the program employee shall select the specific plan to be used in accordance with instructions issued by the Administrator.[1]

**§ 381.146 Sampling at official establishments.**

Inspectors may take, without cost to the Department, such samples as are necessary of any poultry product, or other article for use as an ingredient of any poultry product, at any official establishment to determine whether it complies with the requirements of the regulations.

**§ 381.147 Restrictions on the use of substances in poultry products.**

(a) All ingredients and other substances used in the processing or handling of poultry products at official establishments shall be such as will not result in adulteration or misbranding of the poultry products.

(b) Poultry products and poultry broth used in the processing of poultry products shall have been processed in the United States only in an official establishment, or imported from a foreign country listed in § 381.196(b), and inspected and passed, in accordance with the regulations. Detached ova and offal shall not be used in the processing of any poultry products, except that poultry feet may be processed for use as human food when handled in a manner approved by the Administrator in specific cases, and detached ova may be used in the processing of poultry products if the processor demonstrates that such ova comply with the requirements under the Federal Food, Drug, and Cosmetic Act.

(c) Liquid, frozen, and dried egg products used in the processing of any poultry product shall have been prepared under inspection and be so marked in accordance with the Egg Products Inspection Act.

(d) Carcasses, parts thereof, meat and meat food products of cattle, sheep, swine, goats, or equines may be used in the processing of poultry products only if they were prepared in the United States only in an official meat packing establishment, or imported, and were inspected and passed, in accordance with the Federal Meat Inspection Act, and the regulations under such Act (Subchapter A of this chapter) and are so marked.

(e) All isolated soy protein used in poultry products processed in any official establishment shall contain not more and not less than 0.1 percent titanium, incorporated as food grade titanium dioxide, and the presence of such substance must be shown on the label of the container of the isolated soy protein at all times that the article is in the official establishment.

(f) (1) No substance may be used as an ingredient or otherwise in the processing of any raw or cooked poultry product unless its use is approved as shown in Table 1 in subparagraph (3) of this paragraph or elsewhere in this part, or by the Administrator in specific cases.

(2) Additives, to be used in the processing of poultry products, will be approved only if they comply with the following criteria:

(i) No food additive or other substance subject to section 408, 409, or 706 of the Federal Food, Drug, and Cosmetic Act may be used if it is deemed "unsafe" under that Act. No other additive may be used if, in the judgment of the Administrator, it is an added poisonous or deleterious substance which may render the poultry products injurious to health or otherwise unfit for human food.

(ii) The additive shall not promote deception or cause the product to be otherwise adulterated or misbranded. Scientific data acceptable to the Administrator showing that the additive meets the criteria specified in this section shall be submitted by the person interested in having the additive approved.

(3) The substances specified in the following table are acceptable for use in the processing of poultry products provided they are used within the limits of the amounts stated and under other conditions as stated in the regulations.

[1] Further information concerning sampling plans which have been adopted for specific products may be obtained from the Circuit Supervisor. These sampling plans are developed for individual products by the Washington staff and will be distributed for field use as they are developed. The type of plan applicable depends on factors such as whether the product is in containers, stage of preparation, and procedures followed by the establishment operator. The specific plan applicable depends on the kind of product involved.

TABLE 1

| Class of substance | Substance | Purpose | Products | Amount |
|---|---|---|---|---|
| Antifoaming agent | Methyl polysilicone | To retard foaming | Soups | 10 ppm. |
| | | | Rendered fats | 10 ppm. |
| | | | Curing pickle | 50 ppm. |
| Antioxidants and oxygen interceptors. | BHA (Butylated hydroxyanisole) | To retard rancidity | Various | 0.01 percent based on fat content. (0.02 percent in combination with any other antioxidant listed in this table based on fat content.) |
| | BHT (Butylated hydroxytoluene) | do | do | Do. |
| | Propyl gallate | do | do | Do. |
| | Tocopherols | do | do | 0.03 percent based on fat content. (0.02 percent in combination with any other antioxidant listed in this Table based on fat content.) |
| Binders and extenders | Algin | To extend and stabilize product. | do | Sufficient for purpose. |
| | Carrageenan | do | do | Do. |
| | Carboxymethyl cellulose (cellulose gum). | do | do | Do. |
| | Gums, vegetable | do | do | Do. |
| | Methyl cellulose | To extend and to stabilize product (also carrier). | do | 0.15 percent. |
| | Isolated soy protein | To bind and extend product. | do | Sufficient for purpose. |
| | Sodium caseinate | do | do | Do. |
| | Whey (dried) | do | do | Do. |
| Chilling media | Salt (NaCl) | To aid in chilling | Raw poultry products | 700 lbs. to 10,000 gals. of water.[1] |
| Coloring agents (natural) | Annatto, Carotene | To color products | Various | Sufficient for purpose. |
| Coloring agents (artificial) | Coal tar dyes (FD&C certified), Titanium dioxide. | To color products to whiten products. | do | Do. |
| Cooling and retort water treatment agents. | Calcium chloride | To prevent staining on exterior of canned goods. | Salads and spreads | 0.5 percent. |
| | | | Various | Sufficient for purpose. |
| | Citric acid | do | do | Do. |
| | Dioctyl sodium sulfosuccinate | do | do | 0.05 percent. |
| | Disodium-calcium ethylenediamine-tetraacetate. | do | do | Sufficient for purpose. |
| | Disodium ethylenediamine-tetraacetate. | do | do | Do. |
| | Disodium phosphate | do | do | Do. |
| | Ethylenediamine-tetraacetic acid | do | do | Do. |
| | Isopropanol | do | do | 0.002 percent. |

See footnotes at end of table.

TABLE I—Continued

| Class of substance | Substance | Purpose | Products | Amount |
|---|---|---|---|---|
| | Potassium pyrophosphate | do | do | Sufficient for purpose. |
| | Propylene glycol | do | do | Do. |
| | Sodium bicarbonate | do | do | Do. |
| | Sodium carbonate | do | do | Do. |
| | Sodium dodecylbenzene sulfonate | do | do | 0.05 percent. |
| | Sodium gluconate | do | do | Sufficient for purpose. |
| | Sodium hexametaphosphate | do | do | Do. |
| | Sodium laurylsulfate | do | do | 0.05 percent. |
| | Sodium metasilicate | do | do | Sufficient for purpose. |
| | Sodium n-alkylbenzene sulfonate (alkyl group predominantly $C_{12}$ and $C_{13}$ and not less than 95 percent $C_{10}$ to $C_{15}$). | do | do | 0.05 percent. |
| | Sodium nitrite (The sodium nitrite must be decharacterized with 0.05 percent powdered charcoal. Bulk decharacterized sodium nitrite when in cook room shall be held in locked metal bin or container conspicuously labeled "Decharacterized sodium nitrite—To be used by authorized personnel only."). | To inhibit corrosion on exterior of canned goods. | do | 600 parts per million. |
| | Sodium pyrophosphate | To prevent staining on canned goods. | do | 0.05 percent. |
| | Sodium tripolyphosphate | do | do | Do. |
| | Zinc oxide | do | do | 0.01 percent. |
| | Zinc sulfate | do | do | Do. |
| Curing agents | Ascorbic acid | To accelerate color fixing and to preserve color in storage. | Various | 75 ozs. to 100 gals. pickle at 10% pump level; ¾ oz. to 100 lbs. of poultry product; 10% solution to surfaces of the product prior to packaging (the use of such solution shall not result in the addition of a significant amount of moisture to the product). |
| | Erythorbic acid | do | do | Do. |
| | Sodium ascorbate | do | do | 87.5 ozs. to 100 gals. pickle at 10% pump level; ⅞ oz. to 100 lbs. of poultry product; 10% solution to surfaces of product prior to packaging (the use of such solution shall not result in the addition of a significant amount of moisture to the product). |
| | Sodium erythorbate | do | do | Do. |
| | Citric acid or sodium citrate | Accelerate color fixing | Cured products | May be used in cured products to replace up to 50% of the ascorbic acid or sodium ascorbate that is used. |
| | Sodium or potassium nitrate | Source of nitrite | do | 7 lbs. to 100 gals. pickle; 3½ ozs. to 100 lbs. of poultry product (dry cure); 2¾ ozs. to 100 lbs. of chopped poultry meat. |

| | | | | |
|---|---|---|---|---|
| Curing agents—Continued | Sodium or potassium nitrite (supplies of sodium nitrite and potassium nitrite and mixtures containing them must be kept securely under the care of a responsible employee of the establishment. The specific nitrite content of such supplies must be known and clearly marked accordingly). | To fix color | do | 2 lbs. to 100 gals. pickle at 10% pump level; 1 oz. to 100 lbs. of poultry product (dry cure); ¼ oz. to 100 lbs. chopped poultry meat. The use of nitrites, nitrates, or combination shall not result in more than 200 p.p.m. sodium nitrite in finished product. |
| Emulsifying agents | Acetylated monoglycerides | To emulsify product | Various | Sufficient for purpose. |
| | Diacetyl tartaric acid esters of mono- and diglycerides. | do | Rendered poultry fat or a combination of such fat with vegetable fat. | Do. |
| | Glycerol-lacto stearate, oleate or palmitate. | do | do | Do. |
| | Lecithin | To emulsify product (also as antioxidant). | Various | Do. |
| | Mono- and diglycerides (glycerol palmitate, etc.). | To emulsify product | do | Do. |
| | Polysorbate 80 (polyoxyethylene (20) sorbitan monooleate). | do | do | 1 percent when used alone. If used with polysorbate 60, the combined total shall not exceed 1 percent. |
| | Propylene glycol mono- and diesters of fats and fatty acids. | do | Rendered poultry fat or a combination of such fat with vegetable fat. | Sufficient for purpose. |
| | Polysorbate 60 (polyoxyethylene (20) sorbitan monostearate). | do | do | 1 percent when used alone. If used with polysorbate 80, the combined total shall not exceed 1 percent. |
| Flavoring agents, protectors and developers. | Approved artificial smoke flavorings.[2] | To flavor product | Various | Sufficient for purpose. |
| | Approved smoke flavoring [3] | do | do | Do. |
| | Autolyzed yeast extract | do | do | Do. |
| | Citric acid | To protect flavor | do | Do. |
| | Corn syrup solids | To flavor product | do | Do. |
| | Disodium inosinate | do | do | Do. |
| | Disodium guanylate | do | do | Do. |
| | Hydrolyzed plant protein | do | do | Do. |
| | Malt syrup | do | do | Do. |
| | Milk protein hydrolysate | do | do | Do. |
| | Monosodium glutamate | do | do | Do. |
| | Sodium sulfoacetate derivative of mono and diglycerides. | do | do | 0.5 percent. |
| | Sugars approved (sucrose and dextrose). | do | do | Sufficient for purpose. |

See footnotes at end of table.

TABLE I—Continued

| Class of substance | Substance | Purpose | Products | Amount |
|---|---|---|---|---|
| Gases | Carbon dioxide solid (dry ice) | To cool product or facilitate chopping or packaging. | Various | Do. |
| | Carbon dioxide liquid | Contact freezing | do | Do. |
| | Nitrogen | To exclude oxygen from sealed containers. | do | Do. |
| | Nitrogen liquid | Contact freezing | do | Do. |
| Miscellaneous | Sodium bicarbonate | To neutralize excess acidity; cleaning vegetables. | Rendered fat, soups, curing pickle. | Do. |
| | Calcium propionate | To retard mold growth | Fresh pie dough | 0.3 percent of calcium propionate or sodium propionate alone, or in combination, based on weight of the flour used. |
| | Sodium propionate | do | do | Do. |
| Phosphates | Disodium phosphate | To decrease the amount of cooked out juices and protect flavor. | Various | 0.5 percent in product. |
| | Monosodium phosphate | do | do | Do. |
| | Sodium hexametaphosphate | do | do | Do. |
| | Sodium tripolyphosphate | do | do | Do. |
| | Sodium pyrophosphate | do | do | Do. |
| | Sodium acid pyrophosphate | do | do | Do. |
| Synergists (used in combination with antioxidants). | Citric acid | To increase effectiveness of antioxidants. | Poultry fats | 0.01 percent alone or in combination with antioxidants in poultry fats. |
| | Malic acid | do | do | Do. |
| | Monoisopropyl citrate | do | do | 0.01 percent poultry fats. |
| | Phosphoric acid | do | do | 0.0 percent. |
| | Monoglyceride citrate | do | do | 0.02 percent. |

[1] Special labeling requirements are prescribed in § 381.120 for raw poultry products chilled in a medium with more than 70 lbs. of salt to 10,000 gals. of water.
[2] These are proprietary products, and a list thereof can be obtained from the Laboratory Services Division, Animal and Plant Health Inspection Service, U.S. Department of Agriculture, Washington, D.C. 20250.

**§ 381.148 Processing and handling requirements for frozen poultry products.**

Procedures with respect to processing of frozen ready-to-heat-and-eat poultry products or stuffed ready-to-roast poultry shall be in accordance with sound operating practices and carried out in a manner which will assure freedom from adulteration of the products. Products to be frozen shall be moved into the freezer promptly under such supervision by an inspector as is necessary to assure preservation of the products by prompt and efficient freezing. Adequate freezing facilities shall be provided within the official establishment where products to be frozen are prepared, except that, upon written request, and under such conditions as may be prescribed by the Administrator in specific cases, such products may be moved from the official establishment prior to freezing: *Provided*, That the official establishment and freezer are so located and the necessary arrangements are made so that the Inspection Service will have access to the freezing room and adequate opportunity to determine that the products are being properly handled and frozen.

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# STUDIES ON THE EXPERIMENTAL PRODUCTION OF ARTERIOSCLEROSIS BY THE DISTURBANCE OF THE TCA CYCLE (KREBS)

## REPORT 3.

## The Production of arteriosclerosis by prolonged administration of citric acid in rabbits

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(Received on March 11, 1958)

It is already a known fact that the so-called TCA cycle (KREBS) plays an important role in metabolism. It was previously reported by ARAI and YAO[1] that the prolonged administration of fumaric acid, which belongs to this cycle, to rabbits produced relatively marked arteriosclerosis. ARAI and TAKENAKA[2] also reported similar results, administering pyruvic acid, which has an important relation with this cycle.

The authors administered citric acid, which also belongs to the TCA cycle and has a close relation with pyruvic acid in carbohydrate metabolism, to rabbits for a long period and obtained similar results, and moreover, specific findings. The results of the experiment are as follows:

TABLE I

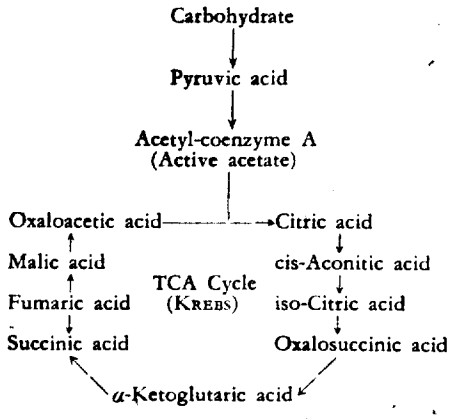
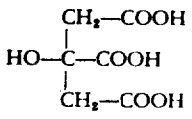



TABLE II




Citric acid

* Prof. of Pathology.

## MATERIAL AND METHOD

The rabbits were fed with bean-curd residuum, treated as in Table III, and sacrificed by air embolism. Gross findings were observed and H.E. staining, elastica stainig (WEIGERT), collagen fiber staining (VAN GIESON), polysaccharide staining (PAS), mucopolysaccharide (HIGHMAN's method, pH 3.7 and OHNO's method, pH 2.5, 4.1) were carried out on formalin fixed, paraffin embedded materials. Sudan III was used for fat staining.

TABLE III

| Animal No. | Sex | Body weight (g) | Treatment | Period (wks.) | Degree of Arteriosclerosis |
|---|---|---|---|---|---|
| CA 5 | m. | 1900 | | 5 | − |
| CA 3 | m. | 1800 | | 10 | − |
| CA 8 | f. | 2300 | 3.0% | 10 | + |
| CA 7 | m. | 1350 | sodium citrate solution | 13 | ++ |
| CA 6 | f. | 2100 | 2.0cc | 15 | + |
| CA 9 | m. | 1500 | thrice a week | 19 | +++ |
| CA 2 | f. | 2000 | intraabdominal inj. | 20 | ± |
| CA 4 | f. | 1350 | | 22 | ++++ |
| CA 1 | m. | 2400 | | 25 | ++ |

## RESULTS

*I. Gross Findings.*

Arteriosclerotic lesions of the aorta were observed in prolonged cases. Namely, in the beginning portion, ascending portion or from the arch to the descending portion of the aorta, intimal thickening, roughness of intimal surface, slightly edematous lesions and miliary calcifications were observed.

*II. Microscopical Findings.*

In the short cases, edematous degeneration was observed under the intima to the media, but the amount of fibers in the media was normal (H.E. staining). According to the period of administration, the intima showed slight thickening, and severe diffuse continuous edematous degeneration was found under the intima extending to the media. In the media, degeneration was conspicuous, and some portions demonstrated a necrotic tendency, relatively marked calcification and slight infiltration of histiocytic cells (Fig. 1).

In the prolonged cases, special staining revealed increased collagen fibers in the degenerated lesions of the media and increased reticulin fibers in the same area, but only sparse elastic fibers. The latter became

irregular and were swollen, disrupted or faded in the degenerative portions of the media (Fig. 2~4). PAS positive substances markedly increased in the degenerated portions. Mucopolysaccharide was examined with toluidin blue metachromasia. The adjacent portion to the intima of media, especially the degenerated portion showed positive reaction at pH 3.7 (mucin) and pH 4.1 (hyaluronic acid), but indicated slight positive metachromasia or almost negative reaction at pH 2.5 (chondroitin sulfate). No lipid was found in any of the cases. There was no remarkable findings in the adventitia.

The aforementioned findings are considered to be arteriosclerotic lesions with relatively marked calcification in the media.

## Discussion

As aforementioned, the prolonged continuous administration of citric acid produces arteriosclerotic lesions with relatively marked calcification in the aorta of rabbits.

In other words, the intima of the aorta were thickened and the media were diffusely swollen or degenerated and showed relatively marked calcification. In some portions, collagen fibers and argentaffine fibers increased, but the elastic fibers were fragmentated, became irregular and swollen and showed a tendency of vanishing. PAS positive substances also markedly increased. Mucin and hyaluronic acid were found in a certain amount, but chondroitin sulfate was indefinite and lipid was not demonstrated at all.

Recently, as for the etiology of arteriosclerosis, acid mucopolysaccharides or metachromatic substances, which are the interfibrous ground substance of the arterial wall, are considered to be an important initial change of arteriosclerosis (Rinehart and Greenberg[3], Taylor[4], Bunting and Bunting[5], Nishiyama and Kusayanagi[6]). However, as for the etiology in the case administered with citric acid, the metabolic disturbance of polysaccharide in the arterial wall, is considered to be the fundamental factor assumed from the relation of citric acid to the TCA cycle (Arai et al[7], Ishigami[8]).

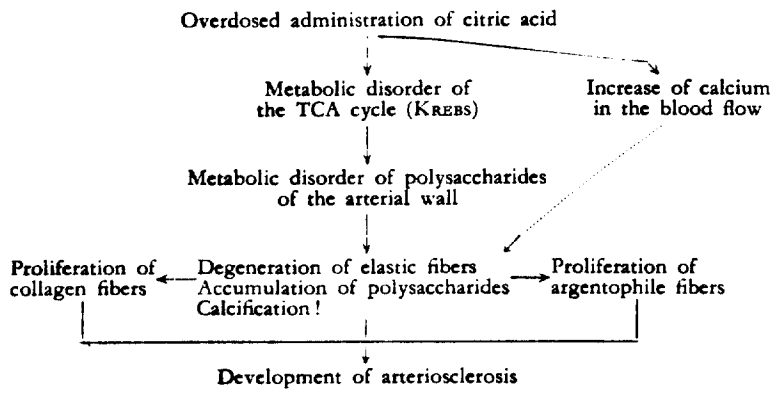
In other words, the metabolic disturbance of polysaccharide in the arterial wall is considered to be induced as a partial phenomenon of the overdosed administration of citric acid. Severe calcification is regarded to be due to the deposition of calcium, in the blood flow, which is increased by the bounding action of calcium of citric acid.

Nishiyama and Kusayanagi[6] considered that the familiarity between lipid deposition and calcification in arteriosclerosis was due to mucoid degeneration which preceded them. Deposition and bounding of mucoid

substance to elastic fibers are considered to be the preceded factor for calcification.

For the treatment of overdosed citric acid, abnormal activation or increase of aconitase is necessary and therefore a relative vitamin C deficiency is caused by the marked consumption of vitamin C, which activates aconitase (SUDA and TANAKA[9]) and a vicious circle is established. This experiment is considered to be fairly affected by this. Especially at a time when the metabolic relationship between citric acid, vitamin D and calcium is being disputed, the fact that arteriosclerosis can be induced by the administration of citric acid is of special interest in the relation with vitamin D type arteriosclerosis and for the clarification of arteriosclerosis with severe calcification in human subjects.

TABLE IV




## CONCLUSION

Prolonged continued administration of citric acid which belongs to the TCA cycle caused arteriosclerosis of the aorta with severe calcification.

As to the etiology of arteriosclerosis, metabolic disturbance of polysaccharides in the aortic wall follows the metabolic disturbance of the TCA cycle, and disintegration, swelling and vanishing of elastic fibers and deposition of polysaccharides occur and arteriosclerotic changes are established. At the same time, the calcium, which is increased in the blood by the action of citric acid, is deposited around the arteriosclerotic lesions.

The authors are of the opinion that these facts provide many suggestions in the clarification of the cause of arteriosclerosis in human beings.

An outline of this subject was reported at the 45th General Japanese Pathological Meeting in July, 1956.

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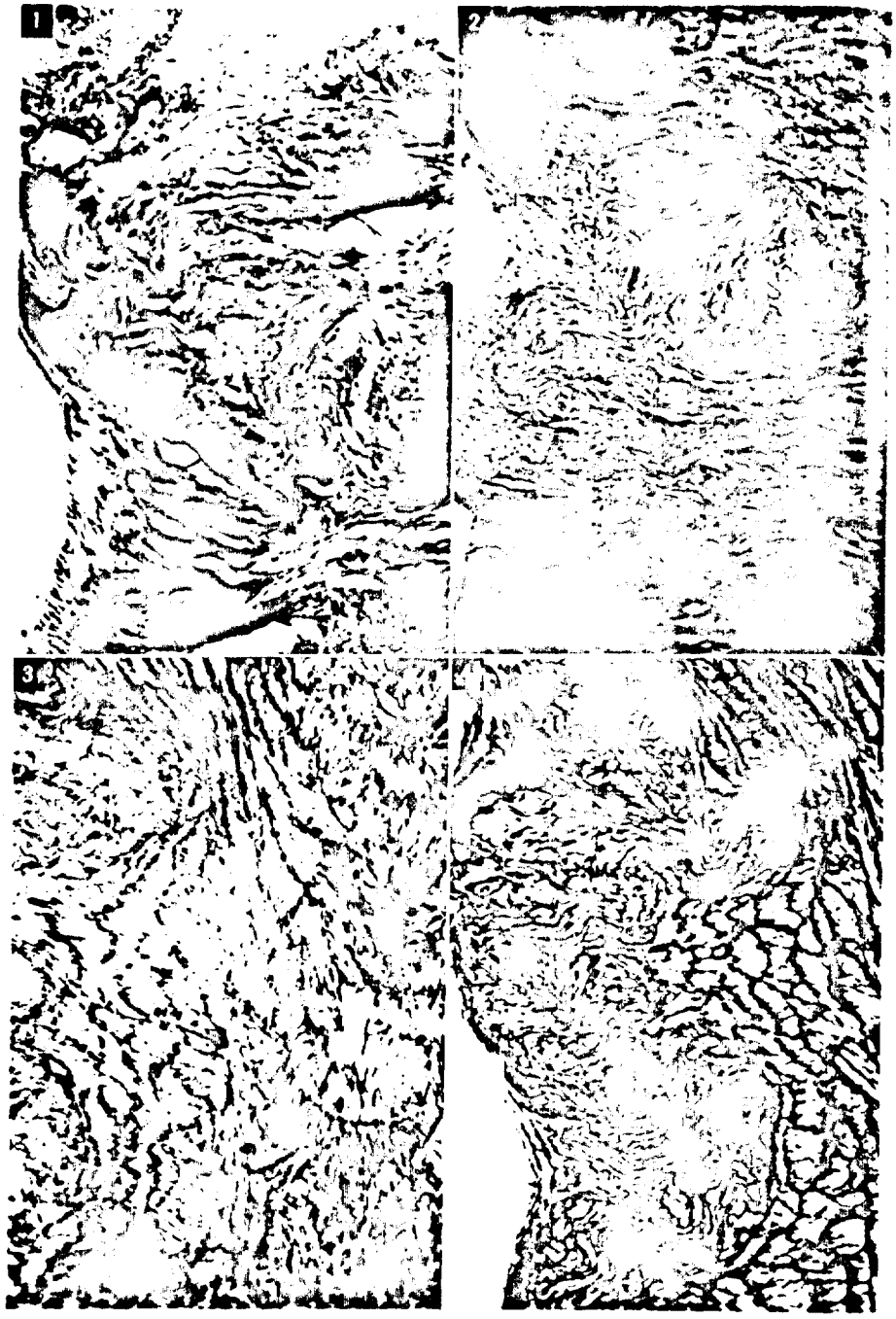
## EXPLANATION OF PLATES

Fig. 1. Aorta, CA 4, H.E., low magnification. The intima is thickened, the media is degenerated and swollen accompanied by marked calcification.

Fig. 2. Aorta, CA 4, VAN GIESON, low magnification. Collagen fibers are increased in the degenerated region.

Fig. 3. Aorta, CA 4, WEIGERT staining, low magnification. Elastic fibers are irregular, fragmented or show a tendency of vanishing.

Fig. 4. Aorta, CA 4, BIELSCHOWSKY staining, low magnification. Reticulin fibers are increased in the degenerated region.


1
2
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Thromb. Diath Haemorrh. 4: 462-72.

# The Effects of Sodium Citrate and an Excess of Plasminogen on Fibrinolytic Agents*

*(From the Biological Institute, Carlsberg Foundation, Copenhagen, Denmark)*

Tage Astrup and Ida Sterndorff

This paper reports experiments on the enhancing effects of citric acid and of added plasminogen on the fibrinolytic activity as estimated by the fibrin plate method.

When neutral salts of citric acid or other polycarboxylic acids are present in the fibrin substrate, an enhancement of the fibrinolytic activity produced by streptokinase activated human globulin or by spontaneously active human globulin is observed (9, 11). The effect of trypsin is only slightly and variably increased. Other proteases (bovine plasmin, chymotrypsin, an aspergillus protease, a B. subtilis protease) are insignificantly influenced. It was therefore suggested that the enhancing effect is caused by an acceleration of the activation of the plasminogen present in the fibrin substrate, while the direct effect of proteases on fibrin is independent of the presence of citrate (11).

Since these observations were made, a number of other activators of plasminogen have been made available in concentrated form. The effect of citric acid on these activators has therefore been studied in order to see whether an accelerating effect is a phenomenon common to all plasminogen activators.

The fibrinolytic effect produced on fibrin plates by plasminogen activators depends upon the concentration of plasminogen in the fibrin substrate. When plasminogen has been destroyed by heating of the fibrin (8), an effect of plasminogen activators can again be produced when plasminogen is added to the activator solution applied to the heated fibrin. Using this principle it could be demonstrated that the fibrinolytic activity of most tissue extracts is caused by an activator of plasminogen and not by proteases (2, 3); that trypsin (3) and the fibrinolytic agent in urine are plasminogen activators (4); and that an activator of plasminogen is produced by addition of streptokinase to human globulin (13).

When a test solution containing plasminogen was applied to normal fibrin plates an enhancement of activator effect could also be observed, but the increase

* This investigation was aided by grants from the Josiah Macy, Jr. Foundation, New York, and from the National Danish Association against Rheumatic Diseases.

was only slight because of the large amounts of plasminogen already present in the substrate (10). The present paper describes experiments which demonstrate that the effect of activators of plasminogen can be greatly enhanced, when an excess of plasminogen is present in the fibrin substrate.

## Materials and Methods

Fibrinolytic activity was estimated on normal fibrin plates containing 0.1 per cent bovine fibrinogen (1). The bovine thrombin used for clotting was kindly supplied by the *Leo Pharmaceutical Co.*, Copenhagen. Protease activity was estimated on fibrin plates heated for 45 minutes at 85° C in order to destroy plasminogen (8). Estimations were performed in triplicates and recorded in sq.mm as products of two perpendicular diameters of the lysed zones after 18 hours at 37°.

The stock solution of fibrinogen (usually containing 0.6 to 0.9 per cent fibrinogen) was diluted to the final concentration by a saline-barbital buffer (0.05 M sodium barbital; pH 7.8; 0.1 M NaCl; total ionic strength: 0.15).

Fibrinogen for the citrate containing fibrin plates was diluted with a mixture of equal volumes of the saline-barbital buffer and a 0.03 M solution of trisodium citrate. The citrate concentration in the final solution then became approximately 0.015 M.

In a few experiments protease activity was estimated on casein using precipitation with perchloric acid and estimating the optimal density of the supernatant at 275 mμ with a Hilger Uvispek spectrophotometer (12). In these experiments a phosphate buffer of pH 7.35 and ionic strength 0.15 was used.

Bovine plasminogen was prepared from bovine fibrinogen dissolved in 0.9 per cent NaCl (instead of the barbital buffer). After heating at 54° C for 10 min. and dialysis of the supernatant against saturated ammonium sulphate the precipitate was dissolved in phosphate buffer, dialysed against the buffer and lyophilized. In the experiments this preparation was dissolved in varying concentrations in the final fibrinogen solution.

Streptokinase (Varidase®) was kindly supplied by the *Lederle Laboratories*. The stock solution contained 1 mg per ml in phosphate buffer and was stored in the ice-box.

Proactivator was prepared from human milk as described before (5), except that phosphate buffer was used.

Bovine plasmin was prepared from bovine plasminogen by activation with trypsin followed by neutralization of excess of trypsin with soy bean inhibitor (Astrup and Sterndorff, to be published). Small amounts of trypsin inhibitor do not significantly affect fibrinolytic agents (6).

The plasminogen activators used will be described below.

## Results

### *1. Trypsin*

The variable but usually slight effect of citrate on the activity of trypsin previously observed (9, 11) could be confirmed. A pronounced increase in trypsin activity was always obtained when excess of plasminogen was present in the fibrin (Fig. 1). The increase depended upon the purity of the plasminogen preparation and the presence of inhibitors.

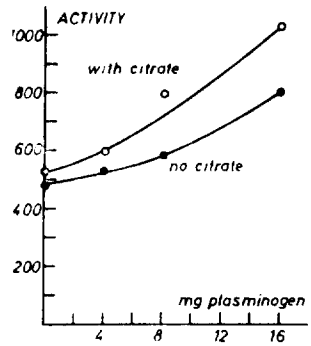


Fig. 1: Effect of trypsin (Armour; 0.05 per cent in 0.025 M HCl; diluted 20 times with buffer before use) on normal fibrin plates in the presence of citrate and increasing amounts of bovine plasminogen. Abscissa: Amount of plasminogen preparation added to the fibrinogen before clotting in mg per 9 ml of fibrinogen solution, which is the amount used for each fibrin plate. Ordinate: Fibrinolytic activity in sq.mm of diameter products of lysed zones (average of triplicates)

### 2. *Other proteases*

None of the other proteases investigated were significantly influenced by citrate or by excess of plasminogen. This applies to chymotrypsin, an aspergillus protease and a B. subtilis protease (same preparations as used before (9, 11); fresh solutions were prepared for each experiment). Bovine plasmin was also very slightly influenced, though occasionally preparations were encountered which gave some enhancement of activity, indicating contamination with an activator of plasminogen.

### 3. *Streptokinase activated milk activator*

A solution containing 20 mg proactivator preparation per ml barbital buffer was activated with an equal volume of streptokinase solution (the stock solution was diluted 50 times) for 30 min. at room temperature. Serial dilutions were then prepared and to each dilution an equal volume of a solution of bovine plasminogen (containing 15 mg per ml barbital buffer) was added in order to study the effect of plasmin on heated fibrin. The plasminogen solution, which was present in constant concentrations in all the activator dilutions, had a slight spontaneous activity. The activities were estimated on normal and heated fibrin, with as well as without citrate (Fig. 2).

It is seen that the effect on normal fibrin is significantly enhanced by the presence of citrate. When interpolated upon the normal curve it is apparent

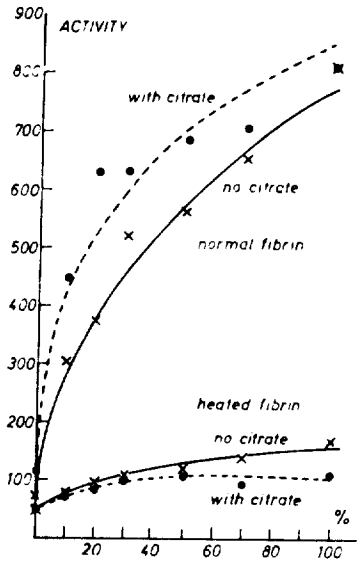


Fig. 2: Effect of citrate on activity of streptokinase activated human milk pro-activator in the presence of a small amount of bovine plasminogen in the solutions. Tested on normal and heated fibrin (with and without added citrate). Abscissa: Concentrations of activator solution in percentages of stock solution. Ordinate: Activity

that equal activities are obtained with about the half concentration of activating solution, when citrate is present. In this experiment some increase in activity was also obtained when citrate was present in the heated fibrin. This was probably due to the presence of added unconverted plasminogen, since other experiments did not show any enhancing effect of citrate on the protease activity estimated on heated fibrin as also shown in the following experiments.

### 4. *Spontaneously active human milk activator*

This activator was prepared from spontaneously active human milk by adsorption on tricalcium phosphate and resolution in phosphate buffer followed by lyophilization (Astrup and Sterndorff, to be published). The stock solution contained 40 mg of this activator preparation per ml. Serial dilutions were prepared with barbital buffer and mixed with an equal volume of a solution of a bovine plasminogen preparation (15 mg per ml). The activities were estimated on normal and heated fibrin with and without citrate (Fig. 3). A considerable increase (3 to 4 times) in activity in the presence of citrate is seen on the normal fibrin, but there is no difference on the heated fibrin.

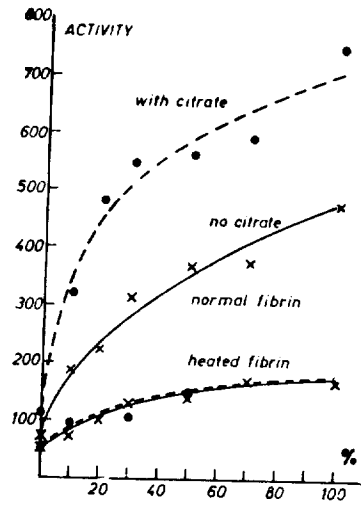


Fig. 3: Effect of citrate on the activity of spontaneously active human milk activator. Abscissa and Ordinate as in Fig. 2. For details see text and Fig. 2. No difference on heated fibrin with and without citrate

### 5. *Urine activator*

The plasminogen activator from human urine was prepared as described before (4). The stock solution contained 0.1 mg per ml. Dilutions were prepared and bovine plasminogen added as described above. The activities were estimated as before (Fig. 4). There is a significant increase in activity in citrate containing

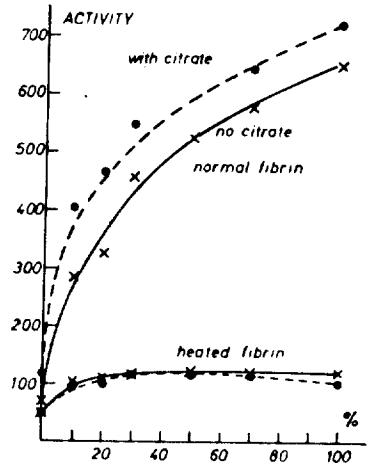


Fig. 4: Effect of citrate on the activity of the plasminogen activator from human urine. For details see text and Fig. 2

fibrin though much less than with the previous activators (about 50 per cent). No difference is seen on the heated fibrin.

### 6. *Soluble tissue activator*

This activator was prepared from pig hearts by extraction with potassium thiocyanate followed by precipitation with acetone as described before (2). The stock solution contained 30 mg per ml in saline barbital buffer. Dilutions were prepared and mixed with a solution of bovine plasminogen as described above (Fig. 5). The increase in activity on normal citrate containing fibrin is considerable (about 3 times). There is no significant difference on heated fibrin.

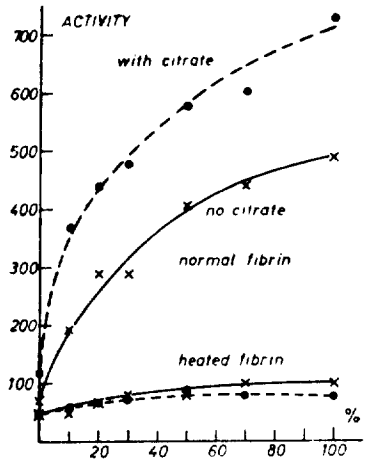


Fig. 5: Effect of citrate on the activity of a soluble plasminogen activator from pig heart. For details see text and Fig. 2

### 7. *Saline lung extract*

Human lung tissue (75 g) was cut into small pieces and washed repeatedly with 0.9 per cent NaCl. After treatment for 2 min. in a Waring blendor with 200 ml 0.9 per cent NaCl, the mixture was passed through a cotton plug to remove coarse particles and centrifuged. The supernatant was dialysed against tap water followed by distilled water, and 90 ml of the dialysate was precipitated with 90 ml saturated ammonium sulphate solution. After centrifugation the precipitate was dissolved in 25 ml 0.9 per cent NaCl, dialysed against the same solution and centrifuged. Serial dilutions of the supernatant were prepared and the activities estimated on normal fibrin with and without citrate (Fig. 6). It is seen that the activity is increased about 4 times by the citrate.

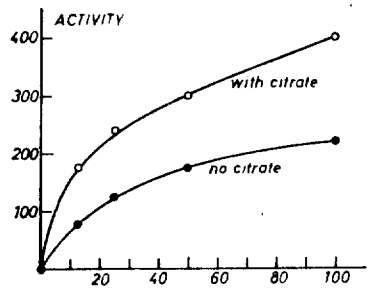


Fig. 6: Effect of citrate on the activity of a saline extract of human lung. Serial dilutions tested on normal fibrin with or without addition of citrate. Abscissa: Concentration of diluted activator solution in percentages of stock solution. Ordinate: Activity

### 8. *Saline brain extract*

Pig brain (125 g) was rinsed and the membranes removed. The brain substance was then ground with sand under slow addition of 70 ml 0.9 per cent NaCl. After further dilution with 60 ml 0.9 per cent NaCl the mixture was centrifuged for 30 min. and the turbid supernatant separated and dialysed against 0.9 per cent NaCl. Serial dilutions of the extract were prepared and the activity estimated on normal fibrin. Similar results as described above for the human lung extract were obtained.

### 9. *Addition of plasminogen*

The addition of excess of plasminogen to the fibrin substrate has already been mentioned in section 1 and 2. When excess of plasminogen was present the effect of mixtures containing an activator of plasminogen was always greatly increased. This is seen for trypsin in Fig. 1. A few examples of the effect on some of the other activators of plasminogen will be described below.

Fig. 7 shows the effect of increasing concentrations of plasminogen on the activity of serial dilutions of human urine on normal fibrin plates. The following amounts of the bovine plasminogen preparation were added to the fibrinogen solution per plate: 0; 7.5; 15; and 30 mg. Similar results were obtained with other activators (tissue activator from pig heart [T]; spontaneously active human milk [H]; activator prepared from human urine (U); streptokinase activated human milk [A]). In this experiment (Fig. 8) the activators were used in constant concentration and only the concentration of plasminogen in the plates was varied.

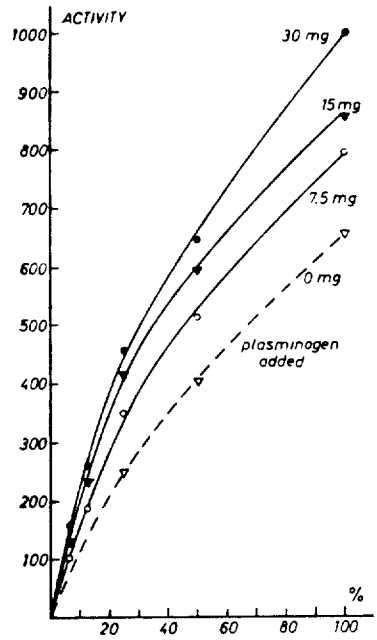


Fig. 7: Effect of addition of plasminogen to the fibrin plate on the activity of human urine. The amount of bovine plasminogen preparation in mg added to each fibrin plate is indicated. Abscissa: Dilutions of normal human urine in percentages of undiluted urine. Ordinate: Activity

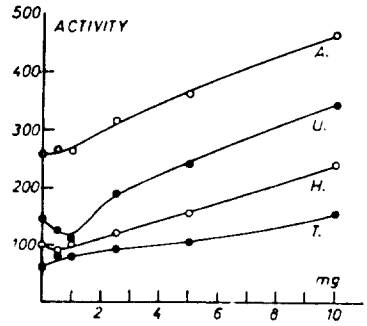


Fig. 8: Effect of addition of plasminogen to the normal fibrin plate on the activity of some plasminogen activators. Abscissa: mg bovine plasminogen preparation per fibrin plate. Ordinate: Activity. T = Tissue activator prepared from pig heart. H = Activator prepared from spontaneously active human milk. U = Activator prepared from human urine. A = Activator prepared by activation of human milk proactivator by streptokinase

No enhancing effect was obtained when the activation of plasminogen was performed in the presence of citrate, and the mixtures then applied to the heated plates. When applied to normal plates a slight effect was produced by the small amounts of citrate added in the test solutions applied to the plates, and acting on the plasminogen in the fibrin substrate. Citrate did not influence the proteolytic splitting of casein by bovine plasmin (activated by urine activator), which again substantiates the lacking effect of citrate on simple proteolysis. Citric acid could be exchanged with maleic acid or fumaric acid and the enhancing effects remained approximately the same when 0.05 M solutions were used instead of the 0.03 M citrate solution. The fibrin substrate was however slightly less solid than with citrate.

## Discussion

So far the results have demonstrated that the enhancing effect of citrate is common to all the activators of plasminogen investigated, though there are quantitative differences. The urine activator, and also the streptokinase activated human milk activator, are apparently less affected than most other activators. This observation could suggest qualitative differences between various types of activators of plasminogen.

It is apparent that the protease effect on heated fibrin is generally not enhanced by the presence of citrate. The result shown in Fig. 2 is probably caused by technical errors. These results confirm the previous observations (9, 11), and they demonstrate that the reaction influenced by citrate must be the activation of plasminogen present in the fibrin substrate.

When increasing concentrations of plasminogen were present in the fibrin substrate, none of the proteases tested produced increased lysis, except trypsin (as described above) and samples of bovine plasmin, when these were contaminated with activator. These results confirm those previously obtained on heated fibrin plates (3), and they suggest that trypsin is the only true protease, which also acts as an activator of plasminogen.

In our previous investigations we have, as mentioned above, used the heated fibrin as a means of discriminating between fibrinolytic activities caused by true proteases and by activators of plasminogen, since only the proteases show any activity on heated fibrin, while addition of plasminogen to the test solution is required in order to obtain any effect of activators of plasminogen.

The results presented above demonstrate that two other principles can be used in order to assist in the discrimination between activator and protease. These two principles are based on the increased activities recorded on normal

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(plasminogen containing) fibrin plates, when either citrate or excess of plasminogen is present in the fibrin substrate. While destruction of plasminogen by heating of the fibrin abolishes the activity of plasminogen activators, the two new methods are based on a specific enhancement of activator activity. These principles are therefore mutually supplementary.

## Summary

1. The presence of citrate in the normal fibrin enhanced the fibrinolytic activity of plasminogen activators, including trypsin. The effect of proteases (on normal or on heated fibrin, containing citrate) was not significantly influenced.

2. The effect of plasminogen activators was also increased when excess of plasminogen was present in the normal fibrin plates.

3. Fumaric acid and maleic acid belong to the polycarboxylic acids producing an enhancing effect.

## Résumé

1. La présence de citrate dans la fibrine normale augmente l'activité fibrinolytique des activateurs du plasminogène, trypsine inclue. L'effet des protéases sur la fibrine normale ou chauffée et contenant du citrate n'est pas influencé de manière significative.

2. L'action des activateurs du plasminogène est également augmentée quand la fibrine contient un excès de plasminogène.

3. Les acides fumariques et maléiques appartenant au groupe des acides polycarboxyliques, produisent le même effet.

## Zusammenfassung

1. Die Gegenwart von Zitrat in normalen Fibrinplatten fördert die fibrinolytische Aktivität von Plasminogenaktivatoren, einschließlich Trypsin. Die Wirkung der Proteasen wird nicht signifikant beeinflußt (auf normalen oder erhitzten Fibrinplatten, die Zitrat enthalten).

2. Die Wirkung von Plasminogenaktivatoren wurde ebenfalls gesteigert, wenn ein Überschuß an Plasminogen in normalen Fibrinplatten enthalten war.

3. Fumarsäure und Maleinsäure gehören ebenfalls zu den Polykarbonsäuren, die eine fördernde Wirkung ausüben.

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# CITRATE AND THE CITRATE CYCLE IN THE REGULATION OF ENERGY METABOLISM

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The metabolic capabilities of an organism, like its other phenotypic characteristics, must have been shaped by evolutionary processes. It does not seem unreasonable to believe that every aspect of an organism's metabolic pattern, even every kinetically or structurally significant group on every enzyme, is or has been adaptive. Phenotypic characters, across the whole range from a highly developed nervous system to a mere sulphydryl group at the catalytic site of an enzyme, are purposive and functional. As biochemists we must be thorough-going and unashamed teleologists, despite the frequent misunderstandings of our colleagues in other branches of chemistry and physics. The first strong statement of the complete dependence of metabolic chemistry on evolutionary teleology of which I am aware was made by Sir Hans Krebs. He pointed out also that this kind of teleology contains no trace of vitalism. I first read his Herter lectures (Krebs, 1954a,b,c) at about the time that I began thinking of metabolism in an integrated way. For me, and surely for many others, the frankly philosophical third lecture was a major factor in the development of a personal scientific viewpoint. Application of this functionalistic credo had earlier contributed very importantly to the recognition of integrated reaction sequences as metabolic units with specific functional, and hence selective, value. The citrate cycle not only elucidated the oxidation of acetate, but also provided one of the earliest recognized examples of an integrated process in which all intermediates are merely catalysts in the overall process. The individual reactions, and indeed the individual compounds, have significance only in terms of the roles of the cycle, and only the cycle as a functioning unit confers selective advantage on the organism. Similarly, the ability of certain mammalian tissues to synthesize arginine at rates many times in excess of their biosynthetic needs for this amino acid might to a non-teleologically oriented biochemist appear merely as an interesting curiosity, but on the recognition of the urea cycle (Krebs & Henseleit, 1932) this ability was seen to be strongly adaptive. Again the individual reactions make no sense alone; their significance can be seen only in terms of the integrated sequence.

## METABOLIC REGULATION: GENERAL CONSIDERATIONS

Regulation is, of course, an essential condition of life. Metabolic regulation is not a late development somehow grafted on to pre-existing metabolic

sequences; the sequences and their regulation necessarily evolved together, with mutation and selection acting in most cases on the same molecules in the development of catalytic efficiency and of regulatory response.

### *Teleology*

Hardly any reaction or pathway can be imagined to be adaptive in the absence of regulation. To take our previous examples, both the oxidation of acetate and the production of urea would be disadvantageous to the organism under many conditions if they proceeded at a constant rapid rate. Thus, we expect that the evolution of every metabolic sequence has been accompanied by evolution of control characteristics that cause the sequence to respond appropriately to the needs of the organism under a wide range of internal and external conditions. Just as the individual reaction has meaning only in terms of the sequence in which it participates, the sequence is useful only by virtue of its integration into the overall metabolism of the cell or organism. The function of regulatory enzymes is to effect this integration.

### *Complexity through multiplicity*

Biochemistry is necessarily based on faith in the postulate, which we attribute to Sir Frederick Gowland Hopkins, that metabolic processes, however complex, are composed of understandable reactions involving simple compounds (or at least simple reactive groups). A biochemical approach to problems of metabolic regulation can be fruitful only if regulatory responses, however complex, similarly result from the simultaneous occurrence of numerous regulatory interactions, each relatively simple in principle. Present evidence suggests that many of the elementary effects are of the simplest type that one could suggest—direct interaction between a strategically-placed enzyme and a metabolite of low molecular weight. It is by no means possible to exclude more complex effects; protein-protein interactions, in particular, seem likely to be important in the intact cell. These may also be simple in principle, involving conformational changes, for example, that alter the kinetic properties of catalytic sites on one or both proteins, or the accessibility of one active site to a metabolite produced at the other. But even if simple in principle, such effects cannot be observed within the intact cell by presently available techniques. Our views on metabolic regulation are therefore based predominantly on interactions between enzymes and metabolites that have been observed in cell-free preparations. My discussion will deal with only this type of effect.

### *Metabolic branchpoints*

Concepts derived from work on biosynthetic control have been useful in the study of the regulation of energy metabolism. The most important of these generalizations is that regulation occurs at metabolic branchpoints (which is obviously just where an engineer would wish to control an analogous technical process) and that the usual element of metabolic control is a branchpoint metabolite that is competed for by two or more enzymes, at least one

of which is kinetically modulated by one or more metabolites (modifiers or effectors). Product feedback inhibition is a special case of this general situation.

The most important factor in determining the steady-state concentration of a metabolite is probably the effective Michaelis constant of the enzyme or enzymes that catalyse its further reactions. This generalization was first enunciated, to my knowledge, by Trevelyan (1958). It turns out to be very important in metabolic regulation because most modifiers so far discovered modulate the substrate affinities of their target enzymes much more strongly than their maximal velocities. Thus in a generalized case (see Fig. 1) enzymes I and II compete for branchpoint metabolite $M$. The outcome of the competition depends on the relative affinities of the enzymes for $M$, and these

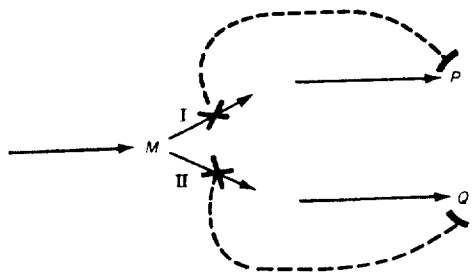


Fig. 1. Generalized representation of regulation at a metabolic branchpoint.

affinities are regulated by the concentrations of the products $P$ and $Q$. The pool level of $P$ is determined largely by the affinity for $P$ of the regulatory site on enzyme I; thus a mutation that increased this affinity would tend to reduce the steady-state concentration of $P$. Similarly the concentration of $Q$ depends upon its affinity for the regulatory site of enzyme II. The concentration of $M$ will be a joint function of the substrate affinities of enzymes I and II, and thus will depend indirectly on the cell's momentary need for $P$ and $Q$. Since the level of $M$ will tend to drop when the requirement for biosynthesis of $P$ and $Q$ rises, one appropriate type of further control would be for $M$ similarly to regulate its own synthesis at an earlier point, and such cases are known.

## THE ADENYLATE CONTROL HYPOTHESIS

Cori, Colowick & Cori reported in 1938 that AMP strongly stimulated the catalytic activity of polysaccharide phosphorylase. Kornberg & Pricer (1951) discovered NAD-specific isocitrate dehydrogenase, in yeast, and reported AMP to be essential to its activity. We (Hathaway & Atkinson, 1963) later found that AMP is not required by this enzyme if the isocitrate level is high; the effect of the nucleotide is to cause an increase in the affinity of the enzyme for isocitrate. Mansour (Mansour & Mansour, 1962; Mansour, 1963) and Passonneau & Lowry (1962) found that AMP similarly increases the affinity of phosphofructokinase for its substrate, fructose-6-phosphate.

These effects posed an intriguing problem. Phosphorylase catalyses the phosphorylytic cleavage of an acetal bond; phosphofructokinase catalyses transfer of a phosphate group; and the reaction catalysed by isocitrate dehydrogenase is an NAD-linked electron transfer. The reactions have nothing in common chemically, yet they are all affected similarly by AMP. Clearly the explanation must be sought in terms of functional metabolic relationships, rather than in chemical similarity. All three reactions participate in the oxidation of polysaccharide, which furnishes electrons for electron-transport phosphorylation. Thus they are involved in the regeneration of ATP. By analogy with the product feedback systems already known to regulate biosynthesis, it would seem reasonable for glycolysis and the citrate cycle to be inhibited by ATP. Since the concentrations of AMP and ATP vary oppositely in the intact cell, stimulation by AMP is metabolically equivalent to inhibition by ATP. The three reactions are strategically well located for control purposes. Phosphorylase catalyses the first step in the mobilization of polysaccharide; phosphofructokinase catalyses an essentially irreversible step and one that had previously been implicated on the basis of various types of evidence as a major control point in glycolysis; and isocitrate dehydrogenase catalyses the first oxidative step in the citrate cycle. These considerations, together with the possibility that the AMP level may affect the production of storage fat and glycogen indirectly through effects on the concentrations of citrate and glucose-6-phosphate, led us (Hathaway & Atkinson, 1963; Ramaiah, Hathaway & Atkinson, 1964) to propose that the level of AMP may play a critical role in partitioning metabolic intermediates between oxidation, with consequent immediate production of ATP, and the production of storage compounds, which may be expended for ATP regeneration later as needed.

At the same time, ATP had been shown to stimulate, and AMP to inhibit, the action of fructose diphosphate phosphatase (Taketa & Pogell, 1963; Newsholme, 1963; Mendicino & Vasarhely, 1963; Salas, Viñuela, Salas & Sols, 1964). On the basis of its oppositely-directed effects on phosphofructokinase and fructose diphosphate phosphatase, Krebs (1964) suggested that AMP plays a decisive role in determining the direction of metabolism between hexose phosphate and phosphoenylpyruvate, glycolysis being favoured when the ATP level is low and glyconeogenesis when it is high.

Krebs & Lowenstein (1960) had previously pointed out that synthesis of citrate seems to be the rate-limiting step in the citrate cycle. We (Hathaway & Atkinson, 1965) were led by the adenylate control hypothesis, and Shepherd & Garland (1966) by different but related considerations, to look for adenylate control of citrate synthase. This enzyme was found to be strongly inhibited by ATP, which increases the apparent Michaelis constant of the enzyme for acetyl coenzyme A.

Each of these effects is in the right direction to contribute to metabolic stability and to regulation of the rate of ATP regeneration in response to the cell's momentary need for ATP. At this level, then, the adenylate control hypothesis seems reasonably satisfactory. We will return to it after

considering some enzyme-modifier interactions that have been implicated in citrate metabolism.

## REGULATION OF THE PRODUCTION AND UTILIZATION OF CITRATE

Although we may tend to think of it as merely an intermediate in the oxidation of acetate, citrate actually occupies a metabolic branch-point of considerable importance. If, as seems likely, the action of aconitase keeps the citrate/isocitrate concentration ratio nearly constant, these two isomers can be considered to be essentially a single metabolite. Figure 2 illustrates some of the metabolic possibilities open to these acids. First, of course, they participate in the Krebs citrate cycle by virtue of the oxidation catalysed by NAD-linked isocitrate dehydrogenase, and in this way contribute to the regeneration of ATP. When a source of oxaloacetate is available, this reaction can lead also to net production of α-oxoglutarate and succinyl coenzyme A as biosynthetic intermediates. Isocitrate may also contribute to NADPH regeneration through action of the NADP-linked isocitrate

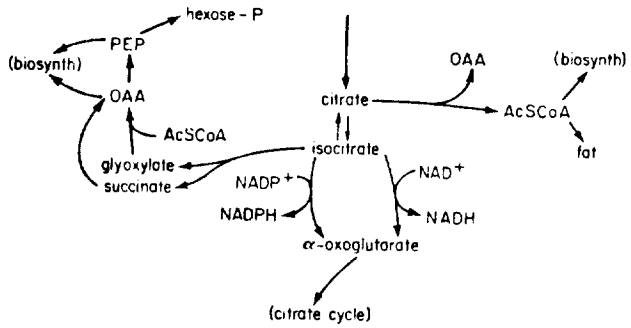


**Fig. 2. Schematic representation of major pathways of citrate metabolism.**

dehydrogenase. In some micro-organisms, and in some higher plants under certain conditions, isocitrate participates in gluconeogenesis and in the production of oxaloacetate, α-oxoglutarate, phosphoenolpyruvate and succinyl coenzyme A as biosynthetic intermediates by way of the glyoxylate cycle proposed by Kornberg & Krebs (1957) for the utilization of two-carbon compounds. Finally, ATP citrate lyase catalyses the cleavage of citrate to oxaloacetate and acetyl coenzyme A at the expense of ATP. When this reaction was discovered by Srere & Lipmann (1953) its function was obscure. Why should a cell use ATP to break citrate down into the very compounds from which it had just been synthesized? Srere & Bhaduri (1962), Bhaduri & Srere (1963) and Spencer & Lowenstein (1962) suggested some years later that this reaction is the primary source of the extra-mitochondrial acetyl coenzyme A that is needed in various biosyntheses and in the production of storage fat. Apparently citrate traverses the mitochondrial membrane much more readily than do coenzyme A esters. There is now convincing evidence of several kinds in favour of this role for the

cleavage enzyme. If this enzyme catalyses the first step in the production of storage fat, we expect it to be modulated by the adenylate control system, and indeed it is strongly inhibited by ADP (Atkinson & Walton, 1967). Thus fat production should be favoured when the ATP level is high, as is obviously appropriate. We will consider the details of this control later.

At least seven enzyme-modifier interactions are known that seem to be directly involved in regulation of the rate of production or the rate of utilization of citrate. Six of these are illustrated schematically in Fig. 3. Three of the interactions in the figure affect citrate production. The citrate-synthesizing step itself is inhibited by ATP (a). This may be the primary

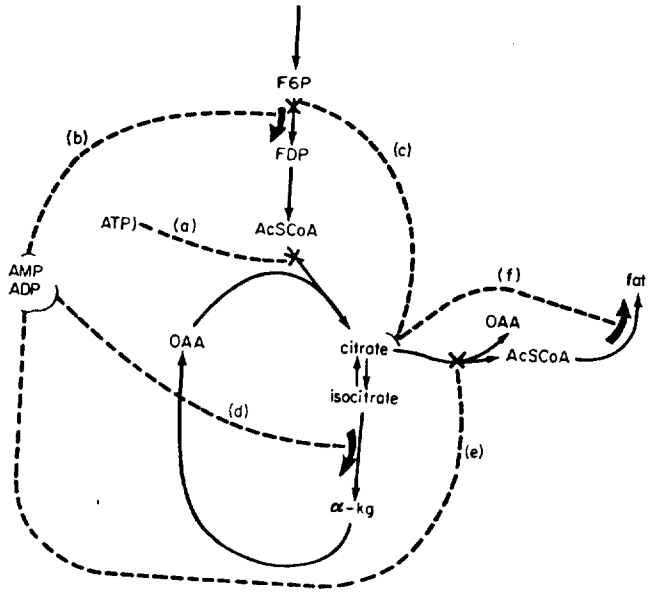


Fig. 3. Some regulatory interactions affecting the production and utilization of citrate. Broken lines link modifiers to the reactions that they control. Negative modifier action is indicated by a cross, and positive modifier action by an arrow.

means by which the entry of acetate into the cycle is kept in step with the energy needs of the cell. In addition, the rate of production of pyruvate and acetyl coenzyme A from hexose precursors is regulated at the phosphofructokinase step, where AMP is a positive modifier (b). Citrate itself is a negative modifier for the same step (c); presumably this effect insures that glycolysis will tend to be accelerated if the concentration of citrate falls below its usual level.

The other three interactions shown here affect processes that utilize citrate. AMP is a positive modifier for NAD-specific isocitrate dehydrogenase (d). As we noted earlier, this effect is in the right direction to increase the rate of regeneration of ATP if its concentration tends to fall. ADP is a negative modifier for ATP citrate lyase (e); thus citrate and ATP will be

consumed in the cleavage reaction only when the ATP level is high. Taken together, these last two effects appear to be responsible for partitioning citrate between oxidation (with resultant regeneration of ATP) and use in biosynthesis or in the production of storage compounds (which consume ATP). In addition, citrate itself is a positive modifier for acetyl coenzyme A carboxylase (f), which catalyses the first step in the production of fatty acids. Presumably this interaction helps to determine the effectiveness with which this storage process competes with biosynthetic uses of acetyl coenzyme A. Because of the action of AMP on isocitrate dehydrogenase, the citrate level should fall as the cell's energy supply is depleted, and it is appropriate that storage processes should be among the first to be curtailed under these conditions.

As I mentioned earlier, the Kornberg-Krebs glyoxylate cycle provides for the synthesis of oxaloacetate, α-oxoglutarate, phosphoenol pyruvate, and carbohydrate from two-carbon compounds. This pathway has been studied extensively by Kornberg and his colleagues, and the results have recently been reviewed by Kornberg (1966). Isocitrate lyase, which catalyses the first step in the sequence, is inhibited by phosphoenol pyruvate. This effect appears appropriate in view of the roles of phosphoenol pyruvate as a biosynthetic intermediate and as a key compound in glyconeogenesis.

Although on first thought it may seem surprising that seven regulatory interactions affecting citrate metabolism are already known, this is not an excessive number in view of the central position of citrate in metabolism. I have no doubt that a number of other regulatory interactions remain to be discovered. Indeed, a few other effects not shown in Fig. 3 have been observed. Weitzman (1966) reported that citrate synthase of *Escherichia coli* is inhibited also by NADH, and preliminary experiments in our laboratory indicate that NADPH inhibits the yeast enzyme (G. M. Walton & D. E. Atkinson, unpublished). We have found that the response of the *E. coli* enzyme to ATP is very strongly affected by pH (Jangaard, 1966), and P. A. Srere (personal communication) observed a similar effect of potassium ion.

These regulatory interactions affecting citrate metabolism were discussed in a recent review (D. E. Atkinson, 1967) and more thorough citations to the original literature may be found there. Instead of repeating that documentation here, it seems preferable to present some recent results from our laboratory that are compatible with a rather more explicit way of considering the interactions of the adenylate control system with glycolysis and the citrate cycle.

## THE ENERGY CHARGE

The usual experimental approach in the study of enzyme modulation by AMP, ADP or ATP has been to add graded levels of each compound individually and compare the behaviour of the enzyme to that of the 'control' treatment with no added adenylate. Such an approach is suitable in the study of biosynthetic product inhibition, but in the case of adenylate control nothing could be less physiological. Thus, while the results that

have been obtained give us a rather solid basis for the belief that the adenylate system is indeed of primary importance in the regulation of energy metabolism, they can shed little light on interactions among the regulated enzymes.

In the cell the total adenylate pool (AMP + ADP + ATP) can be considered to be essentially constant, at least over short time spans, and the energy level of the cell is reflected in the relative concentrations of the three components. The amount of energy stored in the adenylate system is obviously proportional to the average number of anhydride-bound phosphate groups per adenosine moiety. This number varies between zero and two. Because we prefer an index that ranges from zero to one, we have defined the *energy charge* of the adenylate system as half of the number of anhydride-bound phosphate groups per adenosine (Atkinson & Walton, 1967). This parameter is conceptually analogous to the charge of an electrochemical storage or accumulator cell; this analogy may sometimes be helpful in visualizing metabolic relationships operationally. In terms of concentrations of individual compounds, the energy charge is defined as (ATP + $\frac{1}{2}$ADP)/(ATP + ADP + AMP). On the assumption that adenylate kinase is present and in excess, so that the concentrations of the three adenylates are near equilibrium at all times, their relative concentrations will vary with the energy charge of the system as shown in Fig. 4. Starting at any given point

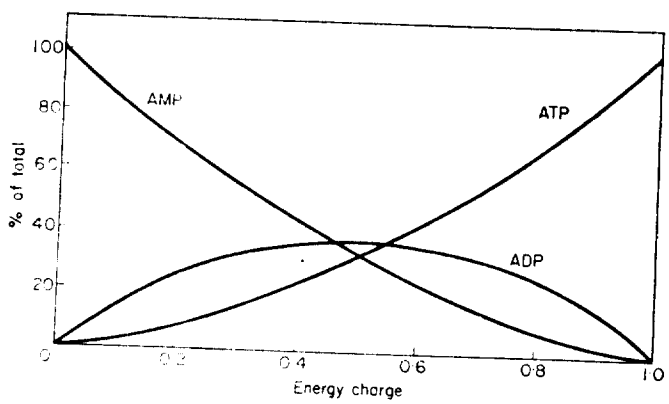


Fig. 4. Relative concentrations of ATP, ADP and AMP as functions of the energy charge (half of the number of anhydride-bound phosphate groups per adenosine moiety), assuming equilibration of the adenylate kinase reaction. The equilibrium constant for this reaction was taken as 0·8 (Markland and Wadkins, 1966). From Atkinson and Walton (1967).

on the charge scale, a process that utilizes ATP will move the system to the left, and the concentrations of the individual adenylates will increase or decrease as indicated by their curves. A process that regenerates ATP will shift the system to the right. The function of the overall complex of regulatory interactions in energy metabolism is to keep these two types of processes—those that utilize ATP and those that regenerate it—in balance. It

therefore seemed of interest to test the responses to variations in energy charge of several enzymes that have been previously implicated in regulation of energy metabolism.

### *ATP citrate lyase*

As previously discussed, ATP citrate lyase appears to be responsible for making acetyl coenzyme A available for extramitochondrial biosyntheses and for the production of fatty acids. It requires ATP and is inhibited by ADP, which is a product of the reaction. As indicated by Fig. 5, the reaction exhibits normal Michaelis kinetics with regard to ATP concentration. There is no trace of the sigmoidicity that characterizes the behaviour of many

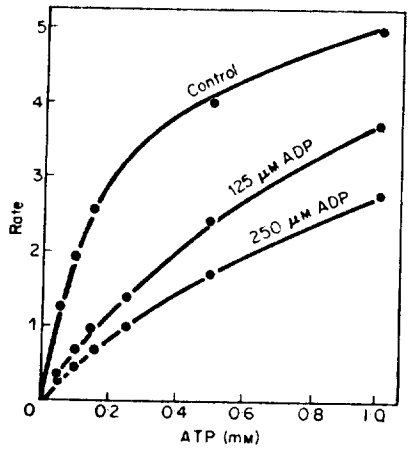


Fig. 5. Rate of the reaction catalysed by rat liver ATP citrate lyase as a function of ATP concentration; effect of ADP. Assay mixtures contained 200 mM Tris Cl, pH 7·5, 5 mM $MgCl_2$, 40 μM coenzyme A, 100 μM NADH, 5 mM sodium citrate, excess commercial malate dehydrogenase, enzyme preparation, and ATP and ADP as indicated. The enzyme preparation was obtained from rat liver homogenate by precipitation with sodium citrate (20–50% saturation) and passage through a Sephadex G-200 column. Adenylate kinase and ATPase assays were negative. From Atkinson and Walton (1967).

regulatory enzymes. Inhibition by ADP is equally classical: $V_{max}$ is unchanged, the apparent $K_m$ for ATP is increased, and the double reciprocal plot has the pattern of competitive inhibition. Thus the response is entirely compatible with simple competition at the catalytic site between ATP and the product ADP. Because of preconceptions based on the types of interactions that have been observed in biosynthetic regulation, we have not previously considered seriously the possibility that simple competitive inhibition by the immediate product of the reaction might be involved in metabolic regulation. However, there are several situations in energy metabolism in which such direct inhibition seems on evolutionary grounds to be the most likely type of control. ATP citrase lyase is a case in point. This enzyme is involved in a storage sequence; it would be appropriate, and

presumably would confer selective advantage, if it were constructed so as to function only when the ATP level is high. Of the several ways by which this objective might be attained, inhibition by ADP is by far the simplest. As ADP is a product, the catalytic site must necessarily have some affinity for it. The degree of inhibition exerted by ADP will depend, of course, on the relative affinities of the catalytic site for ATP and for ADP. As with any other phenotypic character of the cell, this affinity ratio is subject to variation as a consequence of random mutation. Selection will then suffice to produce the affinity ratio, and hence the degree of competitive inhibition, that is most advantageous to the organism. Thus a regulatory interaction can be evolved directly. The evolution of such features as separate regulatory sites and intersite interactions must be, in contrast, a much more complex process.

Sensitive control requires steep response curves. In the cases of most regulatory enzymes yet studied, steep response is achieved by means of intersite interactions, and it might therefore be thought that a reaction controlled by simple product inhibition would necessarily respond less sensitively to its control parameter. This, however, is not true when the energy charge of the system, rather than the concentration of any individual compound, is the relevant variable.

In order to study enzyme behaviour as a function of the energy charge of the adenylate system, it is necessary first to establish the ATP/ADP/AMP equilibrium corresponding to the desired charge, and then to observe the effect of this condition on the enzyme in question. This might be done by supplying the three adenylates at the relative concentrations indicated by Fig. 4 for each desired charge. However, when only ATP and AMP are present the energy charge equals (ATP)/(ATP + AMP), and thus a desired charge is most simply established by supplying ATP and AMP in the appropriate ratio, adding adenylate kinase in excess, and allowing equilibrium between ATP, ADP and AMP to be attained before the enzyme under investigation is added.

The results of two experiments of the type just described are shown in Fig. 6. To obtain the curve marked 4, the total concentration of the initial AMP + ATP mixture was fixed at 4 mM, containing a varying amount of ATP as indicated on the abscissa. The topmost horizontal scale gives corresponding energy charge values. The rate of the cleavage reaction is seen to be a non-linear function of the energy charge. The slope increases with increasing charge, and the curve becomes quite steep in the region of high charge. A similar experiment with a total adenylic pool of 2 mM yielded the results presented in the curve marked 2; the corresponding energy charge values are indicated on the linear scale above this curve. Clearly the reaction rate responds primarily to energy charge rather than to individual concentrations. To show this point more clearly, the experimental points of the 2 mM curve have been replotted on the charge scale of the 4 mM curve as open circles (in effect the horizontal distance of each point from the origin has merely been doubled). The normalized points coincide with the 4 mM

curve. Thus, over at least the range investigated and under the conditions of these experiments, the rate of the reaction is entirely insensitive to the absolute concentrations of the adenosine nucleotides, and depends entirely on the ratio of their concentrations (the energy charge of the system). Clearly metabolic regulation in response to energy charge will not be disrupted by minor fluctuations in the size of the total adenylate pool.

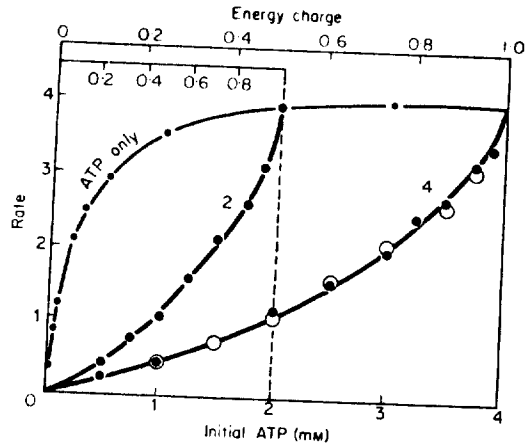


Fig. 6. Rate of the reaction catalysed by rat liver ATP citrate lyase as a function of the energy charge of the adenylate system. Assay mixtures initially contained 100 mM Tris Cl, pH 7·5, 5 mM $MgCl_2$, excess commercial adenylate kinase, ATP as indicated on the abscissa, and AMP to bring the total adenylate concentration to 2 or 4 mM, as indicated by figures identifying the curves. After equilibration of AMP, ADP and ATP, the additional components of the ATP citrate lyase assay were added: 40 μM coenzyme A, 5 mM sodium citrate, 100 μM NADH, excess malate dehydrogenase and enzyme preparation. The curve labelled 'ATP only' presents the results of assays lacking AMP. The scales at the top of the figure indicate energy charge values for the two experiments. The experimental results are plotted directly as solid circles; open circles present the results of the 2 mM experiment normalized to the 4 mM charge scale.

In one sense the curves of Fig. 6 may be considered not to be strictly comparable. Points on the control curve correspond to rates obtained at the ATP concentrations indicated on the abscissa. In the experiments with constant adenylate pool size the concentration of ATP at equilibrium is always less than its initial level. It is thus of interest to know how importantly this change in ATP concentration contributes to the results obtained. One way to answer this question is to compare the results obtained in the constant-pool experiments with the curve to be expected if the rate depended on ATP concentration alone (that is, if it were not affected by ADP or AMP). This has been done in Fig. 7. The control curve and that obtained in the 4 mM constant-pool experiment are identical with those of Fig. 6. In addition, each point of the control curve has been replotted at the point along the energy charge scale where the calculated equilibrium concentration of ATP is equal to that actually used in the control experiment. The resulting curve

thus indicates the result to be expected in the constant-pool experiment if the velocity depended only on the ATP concentration. Although displaced somewhat to the right and altered slightly in shape, this curve is basically similar to the control curve. It is clear that the decrease in ATP concentration as such contributes very little to the shape of the curve actually obtained in the energy charge experiments.

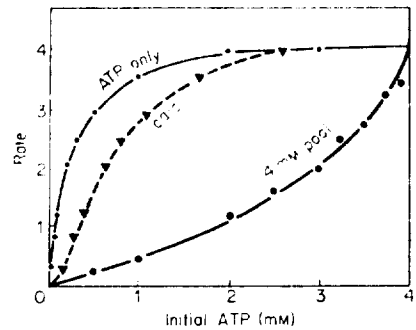


Fig. 7. Effect on the ATP citrate lyase reaction of the decrease in ATP concentration owing to the action of adenylate kinase. The curves labelled 'ATP only' and '4 mM pool' are replotted from Fig. 6. The broken curve labelled 'calc' was obtained by calculating, for each point of the 'ATP only' curve, the amount of ATP required in the energy charge experiment to provide at equilibrium the concentration of ATP actually used in the control experiment. The point was then plotted at the corresponding position along the abscissa.

In a previously published figure similar to Fig. 6 the curves were more sharply concave upward, and the apparent $K_m$ indicated by the control curve was abnormally high (Atkinson & Walton, 1967). Both of these differences might result from partial hydrolysis of ATP, and it seems probable that the enzyme preparation used in the earlier experiments may have contained contaminants capable of hydrolysing ATP. The results of both sets of experiments are, however, in good qualitative agreement and both support the conclusion that the energy charge of the adenylate system is an important factor in regulation of the reaction catalysed by ATP citrate lyase. Because of this effect, it appears that ATP will be expended for the production of extramitochondrial acetyl coenzyme A only when the cell's energy charge is high.

*Biosynthetic enzymes*

It would seem advantageous for the cell to have similar mechanisms for the conservation of ATP (that is, for limiting the rate of its expenditure when the cell's energy charge is low) at every point where ATP is used for biosynthesis or for the production of storage compounds. For initial investigation of this possibility we chose two ATP-utilizing reactions that produce intermediates of importance in a number of biosynthetic sequences. Phosphoribosylpyrophosphate synthase catalyses the formation of an intermediate that is required in the synthesis of tryptophan, histidine and all

nucleotides. In addition to weak inhibition by tryptophan and by nucleotides generally, this enzyme is strongly inhibited by ADP, and its response to the energy charge of the adenylate system resembles that of the citrate cleavage enzyme except that it is even more strongly concave upward (Atkinson & Fall, 1967). Aspartokinase catalyses the production of an intermediate needed for the synthesis of four amino acids, and it responds similarly to the energy charge (D. E. Atkinson, L. Fall & J. H. Hageman, unpublished). Thus the results so far obtained are consistent with our working hypothesis that all reactions in which ATP is used for biosynthesis or for the production of storage compounds are subject to regulation that provides for the conservation of ATP. This general control must, of course, act jointly with specific product feedback regulation, so that the rate of synthesis of any particular metabolite would be expected to depend both on need (as indicated by the concentration of the metabolite) and on the cell's current energy resources (as indicated by the energy charge of the adenylate system).

## ENERGY CHARGE AND CITRATE METABOLISM

But we have digressed from the regulation of citrate metabolism. The original suggestion that adenylates play a general role in metabolic regulation came from observations on enzymes that are involved in sequences leading to the regeneration, rather than the expenditure, of ATP. It is thus of interest to see how these enzymes respond to variations in the adenylate energy charge. The results of preliminary experiments in this direction are

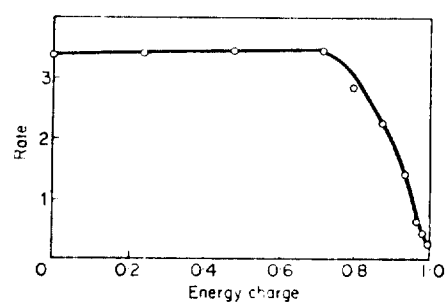


Fig. 8. Rate of the reaction catalysed by yeast NAD isocitrate dehydrogenase as a function of energy charge. Total adenylate concentration was 4 mM; desired values of the energy charge were obtained as explained in the legend to Fig. 6. In addition to AMP, ADP, ATP and adenylate kinase, assay mixtures contained 1 mM DL-isocitrate, 100 mM Tris Cl, pH 7·5, 5 mM $MgCl_2$ and 80 $\mu$M $NAD^+$.

encouraging. The mechanisms of the regulation of these enzymes by the adenylate system cannot be as simple as competitive interaction between ADP and ATP, but the type of response observed is very similar to that seen with ATP-utilizing enzymes (although, of course, oppositely directed).

Figure 8 shows the rate of the reaction catalysed by yeast NAD isocitrate dehydrogenase as a function of the energy charge of a 4 mM adenylate pool (G. M. Walton & D. E. Atkinson, unpublished). It is clear that, starting at any

point on the steep part of the curve, a decrease in the energy charge of the adenylate system would cause the rate of oxidation of isocitrate to increase. The resultant acceleration of electron transport would tend to restore the initial energy charge.

Since the primary effect of adenylates on yeast NAD isocitrate dehydrogenase is to control the affinity of the enzyme for isocitrate, a plot of velocity at a fixed concentration of isocitrate as a function of the energy charge, as in Fig. 8, does not represent the interaction ideally. A plot of $(S)_{0\cdot5}$ (the substrate concentration required for half-maximal velocity) as a function of the energy charge would be more definitive. However, the appropriate experiment has not yet been done in this case. In the case of citrate synthase, $(S)_{0\cdot5}$ values as a function of the energy charge have been measured (see Fig. 9) (D. E. Atkinson & G. M. Walton, unpublished). The $(S)_{0\cdot5}$ scale in

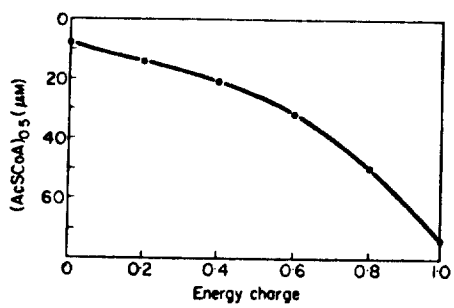


Fig. 9. Affinity of yeast citrate synthase for acetyl CoA as a function of energy charge. Values plotted are concentrations of acetyl CoA required for half-maximal velocity in the presence of 400 μM oxaloacetate. Total adenylate concentration was 4 mM.

the figure is inverted, with 0 at the top, in order that this figure will be metabolically compatible with those for ATP citrate lyase and isocitrate dehydrogenase (an increase in $(S)_{0\cdot5}$ is plotted in the same direction as a decrease in velocity).

Figure 10 brings together for comparison the three response curves that we have considered. This figure illustrates, with enzymes of citrate metabolism as examples, a general hypothesis of regulatory interactions. We predict that regulatory enzymes involved in ATP-regenerating sequences will be found to resemble isocitrate dehydrogenase in having high activity at low values of the energy charge, with a sharp decline in activity in the region of 0·8–0·9 charge. In most cases, 'activity', as used here, probably means affinity for substrates. Enzymes involved in ATP utilization should in general resemble ATP citrate lyase in having low activity over most of the energy charge range, with a steep increase in the vicinity of 0·8 charge. Curves of these two types represent ideal control behaviour from an engineering standpoint; at their intersection point both are relatively steep, so regulation can be sensitive, and they interact near the midpoints of their regulatory response ranges (the vertical scale of Fig. 10), so a strong response in either direction is possible.

We have observed the responses of six enzymes to the energy charge of the adenylate system (phosphoribosyl pyrophosphate synthase, aspartokinase, phosphofructokinase, and the three indicated in Fig. 10). In each case the response curve resembles one of the two types seen in Fig. 10, and the steep portion of each curve occurs at an energy charge of about 0·8–0·9.

If we assume that isocitrate dehydrogenase and ATP citrate lyase represent the regulated steps in the utilization of citrate for ATP regeneration and for the production of storage fat, respectively, Fig. 10 suggests how the

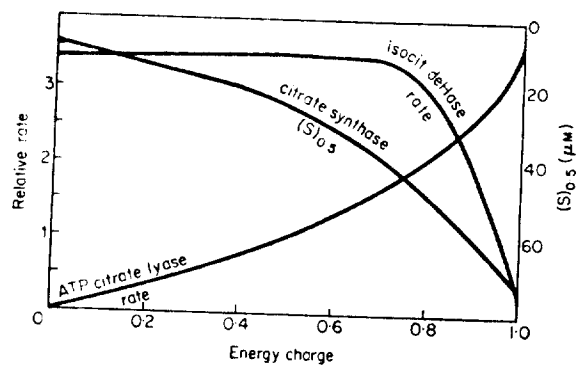


Fig. 10. Response of three enzymes of citrate metabolism to the energy charge of the adenylate system. The curves are replotted for comparison from Figs. 6, 8 and 9.

energy charge of the cell may aid in partitioning such branch point metabolites between alternate metabolic sequences. Beginning with a system in balance (at the intersection of the two curves), if the energy charges should decrease slightly, the resultant acceleration of ATP-regenerating sequences and deceleration of ATP-utilizing reactions would tend to restore the initial condition. It is clear that a change of only a few per cent in the charge of the adenylate system would cause a very sharp imbalance; this is the previously mentioned sensitivity conferred by steepness of the curves at intersection. A change in the other direction would similarly generate a restoring force—in this case the current rate of production of ATP would be depressed in favour of the production of storage compounds.

The foregoing illustration is, of course, subject to a number of reservations. The enzymes that are compared are from two very different species. Perhaps more important, it was tacitly assumed that isocitrate dehydrogenase and ATP citrate lyase compete for the same citrate-isocitrate pool, although the dehydrogenase occurs inside the mitochondrion and the lyase outside. Obviously they will compete directly only if the mitochondrial membrane is freely permeable to citrate, and we do not have adequate information in this area. It seems likely that movement of substrates into and out of mitochondria may be regulated by the energy charge of the adenylate system, as well as by the concentrations of other metabolites. If so, another control

system intervenes between the dehydrogenase and the lyase. Such a system, by regulating the relative concentrations of substrate available to the two enzymes, would modify the competition between the dehydrogenase and the lyase that we discussed above, but it seems likely that the result would be to supplement, rather than to defeat, the regulatory interactions suggested by Fig. 10.

In summary, we propose that the primary role of the adenylate system in the energy transductions of the cell is matched by an equally important function in integrating the cell's economy by regulating the balance between energy-yielding and energy-requiring processes in such a way as to conserve, or buffer, the ATP level. The central position of the Krebs citrate cycle, as the primary supplier of electrons for electron-transport phosphorylation and as an important source of biosynthetic intermediates, requires very important regulatory interactions between the cycle and the adenylate system. The effects of these controls converge at the citrate-isocitrate branch point. Although several regulatory effects have been observed, it seems certain that others remain to be discovered. It is still more certain that we have only begun to comprehend the ways in which the primary effects interact to produce a stable, yet flexible and responsive, metabolic unit.

This article was written while I was on Sabbatical leave from the University of California. I thank the John Simon Guggenheim Memorial Foundation for a fellowship and the Massachusetts Institute of Technology (Professors Boris Magasanik and J. M. Buchanan) for generous hospitality during my leave.

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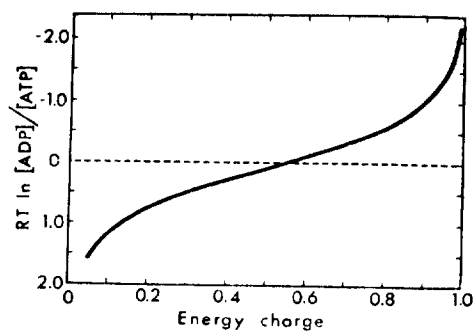
## DISCUSSION

**Klingenberg:** How is the 'charge' of the system related to its free energy?
**Atkinson:** 'Charge' is a stoicheiometric parameter—it is a direct linear function of the number of anhydride-bound phosphate groups in the adenylate pool, and thus of the amount of metabolic energy stored there. The free energy or chemical potential of the system varies, of course, in a non-linear way with charge. It is particularly with regard to this comparison between charge and potential that the electro-chemical cell seems to me to supply a useful analogy in consideration of the role of the adenylate system in metabolism.




The figure shows the relationship between the free energy of hydrolysis of ATP and the energy charge of the adenylate system. For these calculations the equilibrium constant for the adenylate kinase reaction was taken as 0·8, and a temperature of 27°C was assumed. The function plotted is the last term in the expression for the free energy of hydrolysis of ATP.

$$\Delta G = \Delta G' + \mathrm{RT} \ln (\mathrm{ADP})/(\mathrm{ATP}),$$

where $\Delta G'$ is the free energy of hydrolysis when the concentrations of ADP and ATP are equal under any specified conditions (pH, ionic strength, concentrations of orthophosphate and $Mg^{2+}$). Thus, for example, if $\Delta G'$ is taken as −7 Kcal/mole, the scale of the figure runs from −5 to about −9·4 Kcal/mole.

The figure is analogous to a plot of potential against charge for a reversible electrochemical storage cell. The point that I want to emphasize is the difference between the stoicheiometric function *charge* on the one hand, and free energy, chemical potential, or electromotive force on the other. Each type of function is useful; the choice of which to employ depends on the context. In metabolism, stoicheiometric considerations are of primary importance; hence we feel that the charge of the adenylate system is of greater immediate importance in metabolic regulation than is its potential.

**E. C. Slater:** A discussion of the role of the ATP : ADP ratio in the regulation of energy metabolism is incomplete without a consideration of the effect of this ratio on the rate of operation of the respiratory chain. The rate of respiration of isolated mitochondria is normally unaffected by the ATP : ADP ratio until a very high value is reached. I wonder then whether inhibition at NAD-linked isocitrate dehydrogenase, which sets in at much lower ATP : ADP ratios, can be an important regulatory mechanism.

**Garland:** I disagree with Professor Slater's view that mitochondrial respiration is controlled by the interaction of adenine nucleotides with the respiratory chain—at least, in the case of mitochondrial respiration that is supported by the catalytic functioning of the tricarboxylic acid cycle. For instance, maximal rates of respiration by rat liver mitochondria oxidizing palmitoyl-(–)-carnitine in the presence of L-malate are restricted by the inhibitory effects of ATP on citrate synthase. (Shepherd, D., Yates, D. W. and Garland, P. B. (1965). *Biochem. J.* **97**, 380; Shepherd, D. and Garland, P. B. (1966). *Biochem. biophys. Res. Commun.* **20**, 89.) Likewise, the inhibitory effects of palmitoyl-(–)-carnitine oxidation on isocitrate oxidation in liver mitochondria are explicable in terms of the allosteric properties of the NAD-specific isocitrate dehydrogenase, as discussed in greater detail in my paper later in this symposium. More recently, Chappell and Hansford (personal communication) have demonstrated that the tricarboxylic acid cycle in blowfly mitochondria is probably controlled at the isocitrate dehydrogenase step. Since citrate synthase and isocitrate dehydrogenase lie close to the point of entry of acetyl-CoA into the tricarboxylic acid cycle, control of these enzymes can regulate the remainder of the cycle. Studies of the control of mitochondrial respiration that may have led Professor Slater to his viewpoint usually involve substrates (e.g. succinate, 2-oxoglutarate, malate and glutamate, D-3-hydroxybutyrate) that evade the regulatory steps at the commencement of the tricarboxylic acid cycle.

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# THE DETERMINATION OF PART-PER-BILLION LEVELS OF CITRIC AND NITRILOTRIACETIC ACIDS IN TAP WATER AND SEWAGE EFFLUENTS*

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## SUMMARY

Citric and nitrilotriacetic acids can be determined at the 1–10,000 p.p.b.** levels in aqueous systems ranging from tap water to sewage effluents by use of anion-exchange clean-up, derivatization with butanol–HCl and gas chromatography. A variety of metals present at legal tolerance limits do not interfere. The two esterified acids separate well on a special gas chromatographic phase; however, citric acid can also be separated from nitrilotriacetic acid by ion exchange prior to derivatization, if so desired.

## INTRODUCTION

Both the "natural" citric and the man-made nitrilotriacetic acid (NTA) are efficacious complexing agents that can be used as "builders", *i.e.*, detergent additives that soften the water. The fate and effects of NTA have been the subject of much controversy, focusing on its biodegradability, its toxicological effects, and its role in the transport of heavy metals in natural bodies of water (see, for instance, refs. 1–13). The use of NTA in detergents is currently permitted in Canada and prohibited in the United States; however, recent U.S. Government decisions make its re-introduction under restricted conditions almost certain[14].

Analytical methods for NTA and citric acid abound[1,2,15–55]. The zinc Zincon[2], polarographic[21–29] and gas chromatographic (GC) techniques[30–33] are most often used for NTA, while citric acid is usually determined by either liquid chromatography[8–11] or GC[45–55].

The reliable determination of complexing agents present at very low levels in motley waters involves the elimination of inorganic interferences as the main problem. Soft waters are fairly easy to handle but waters with a high degree of hardness or significant levels of heavy metal ions are difficult to analyze by all the methods listed.

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** Throughout this article the American ($10^9$) billion is meant.

That metal ions may interfere with analytical methods based on chelation equilibria is fairly obvious. However, gas chromatographic methods suffer no less. The hardness of Columbia water, for instance, effectively prevents the esterification of NTA even at the relatively high 1 p.p.m. level, and ion-exchange clean-up consequently becomes necessary. Other chelating agents, such as naturally occurring carboxylic, hydroxycarboxylic or amino acids, as well as NTA metabolites, can also interfere with both types of methods. Furthermore, acidification of turbid or contaminated water samples is often necessary to release the full amount of chelating agents for analysis.

Under these circumstances, the concomitant use of two disparate techniques, e.g., polarography and GC, undoubtedly improves analytical reliability. This study is concerned with the GC method for NTA and citric acid, the improvement of its accuracy and sensitivity down to the lower p.p.b. ranges, using aqueous systems with high levels of inorganic and organic contaminants. The need for these improvements arose during an analytical project involving 300 samples of tap water, river water and sewage treatment plant influents and effluents.

For this project in particular, and for similar problems in general, the limits of detection were in need of improvement. Minute traces of NTA can occur in drinking water in regions where it is used as a detergent additive. Citric acid is a common metabolite whose presence in water is interesting with regard to biological activity. Both compounds, of course, strongly influence metal equilibria in aqueous systems. Consequently, precise concentration data are necessary to estimate the relative amounts of metals that are chelated, non-chelated, or, for that matter, may be solubilized from sediments under equilibrium conditions.

NTA is frequently monitored in the input and output of sewage treatment plants. Interest in these data is based on the bacterial degradation of NTA in the activated sludge and the role of residual NTA in the transport of heavy metals through the treatment into the plant effluent. Citric acid can be present in these effluents at fairly high levels and, unfortunately, its ester derivatives interfere with those of NTA on most GC phases.

Consequently, one of the first problems approached in this study was the separation of the NTA and citric acid esters by gas–liquid chromatography (GLC). A second problem involved the removal of materials that interfered with the derivatization. Initial experiments in our laboratory had shown that not all methods described in the literature gave satisfactory results with Columbia water, which is hardly surprising in view of its hardness (Table I); however, we felt that this very fact gave us a chance to study a particularly difficult matrix. In a third set of experiments, several metals with high chelate formation constants (Table II) were added to Columbia water and the analytical technique adjusted accordingly in order to exclude their interference. Finally, we searched for particular conditions under which most of the citric acid could be separated from NTA by ion exchange prior to GC. Although our GC separation of the two derivatives was adequate at comparable concentrations, a great excess of the earlier-eluting citric acid would have interfered with the NTA determination.

Much of the analytical technique used in this study is based on the work by CHAU AND FOX[32] and WARREN AND MALEC[33]. Quite helpful was the methodologically related knowledge on the determination of amino acids, directly available to us from GEHRKE's group (*e.g.*, ref. 56). The packing material used for GC originated from one

TABLE I

COLUMBIA TAP WATER ANALYSIS

| *Component* | *Amount (p.p.m.)* | *Component* | *Amount (p.p.m.)* |
|---|---|---|---|
| Bicarbonates | 357 | Calcium | 59 |
| Carbonates | 0 | Magnesium | 27 |
| Silica | 8 | Nitrate | 0.3 |
| Iron | 0.02 | Sulfate | 16 |
| Aluminum | 0.1 | Chloride | 33 |
| Manganese | 0 | Fluoride | 1.2 |
| Sodium | 46 | | |
| Potassium | 5.6 | | |
| | Total residue | 424 | |

TABLE II

METAL IONS ADDED TO COLUMBIA TAP WATER

| *Metal* | *Added as* | *Metal concentration (p.p.m.)* |
|---|---|---|
| Lead | $Pb(NO_3)_2$ | 0.10[a] |
| Copper | $Cu(NO_3)_2$ | 0.02[a] |
| Nickel | $Ni(NO_3)_2$ | 0.80[a] |
| Zinc | $ZnCl_2$ | 0.10[a] |
| Chromium | $CrCl_3$ | 1.00[a] |
| Cadmium | $CdCl_2$ | 0.01[a] |
| Mercury | $Hg(NO_3)_2$ | 0.005[b] |
| Iron | $Fe(NO_3)_3$ | 1.00[c] |

[a] Legal limit in Missouri water, adopted by the State Pollution Control Board, 1971.
[b] Tentative standard, U.S. Department of Health, Education and Welfare.
[c] Arbitrary value, no legal limit set.

of our own studies of non-extractable polymers on diatomaceous supports[57].

## EXPERIMENTAL

### General procedure

The water samples can be stabilized with 1% formaldehyde if they are not processed immediately. The analysis starts with the addition of 2 ml of 4 *M* formic acid to 50 ml of sample. The pH is checked and lowered to 2.5 by the addition of more formic acid if necessary. The 125 ml erlenmeyer flask containing the sample is then put into a water-bath at 60° and purified nitrogen is bubbled through the solution from a PTFE capillary for 30 min. After coming to room temperature the sample can be transferred to the reservoir of the ion-exchange column.

Bio-Rad AG1-X2, 50–100 mesh (Bio-Rad Laboratories, Richmond, Calif.) is used to pack a 2-in. resin bed into a 150 mm × 5 mm I.D. column (Fischer and Porter Co., Warminster, Pa.). (Solvent reservoirs were blown from some of these columns to speed up the handling of multiple samples.) Before runs, 10 ml of 16 *M* formic acid are passed through the resin followed by de-ionized water up to a pH of 5–6.

The sample is poured into the reservoir and allowed to flow through the resin at full speed (*ca.* 3 ml/min for tap water). It is followed by two 5 ml portions of 0.1 *M*

formic acid, and then the NTA and citric acid are eluted from the column with 16 *M* formic acid, of which 8 ml are collected in a 16 × 75 mm culture tube with a PTFE-lined screwcap. (These culture tubes are boiled for 2 h in concentrated HCl and rinsed with de-ionized water before re-use.)

The formic acid is evaporated from the culture tube in a tube-heating block under a stream of nitrogen. The block maintains the tubes at 85° for 2 h and is then allowed to cool while the evaporation continues overnight.

A 2 ml volume of dry 3 *N* HCl–butanol[56] is added to the dried samples and the culture tubes are capped tightly and placed for 25 min in an ultrasonic water-bath at 75°. The ultrasonic stirring is used only for the first 5 min of reaction time. After esterification, the tubes are cooled, opened and the excess of reagent removed by a slow stream of nitrogen in the tube-heating block. The evaporation starts at 85°, but the heat is turned off after a few minutes and the tubes are removed immediately when they appear to be dry.

Just prior to GC analysis, 100 $\mu$l of dry acetone are added to the esterified acids for a 2 $\mu$l injection into the gas chromatograph. The 5 ft. × 4 mm I.D. glass column contains a non-extractable packing derived from Carbowax 20M on Celite 545, 100–120 mesh (details of the preparation of this particular column will be published separately[57]. Its performance is roughly comparable to that of a well coated, well conditioned 0.3% Carbowax 20M on well acid-washed Chromosorb W). The Microtek 220 injection port is maintained at 230° and on-column injection is used for isothermal chromatography at 183°. Calculations are based on peak heights.

*Tap water calibration curve*

Citric acid and nitrilotriacetic acid trisodium salt were added to Columbia tap water in amounts corresponding to 1, 3, 10, 30, 100, 300, 1000, 3000 and 10,000 p.p.b. each of the free acids and the samples were analyzed as described above.

*Metal ions in water*

A number of metals likely to interfere with the analytical procedure were added to tap water (Columbia municipal water supply) at levels corresponding to legal limits, as shown in Table II. There is no legal limit for iron in Missouri and its concentration was arbitrarily chosen. The resulting water was spiked with NTA and citric acid at the 1, 3, 10, 30 and 100 p.p.b. levels and analyzed as described under *General procedure*.

*Sewage samples*

Effluent from the Columbia sewage treatment plant was spiked with NTA and citric acid at the 1, 3, 10, 30 and 100 p.p.b. levels, and analyzed as described under *General procedure*.

*Removal of citric acid by ion exchange*

Columbia tap water was spiked with 1–100 p.p.b. of NTA, accompanied by a 100-fold excess of citric acid thus ranging from 100 to 10,000 p.p.b. The general procedure was followed; however, the resin was washed with two 5 ml portions of 2 *M* formic acid after the sample had passed through. This fraction (which contains up to 99% of the citric acid) can be discarded. NTA was then eluted with 16 *M* formic acid and derivatized.

## RESULTS AND DISCUSSION

The general procedure described above has been developed in response to analytical problems that arose during a sampling project. The additional experiments on citric acid and metal interference were conducted to ensure a broader applicability of the method.

The ion-exchange and derivatization steps are modifications of the procedures described by CHAU AND FOX[32], WARREN AND MALEC[33] and GEHRKE and coworkers (*e.g.*, ref. 56). The purpose of most of the procedural details is self-evident, but two points may warrant additional discussion.

The initial sample heating at 60° for 30 min in formic acid of pH 2.5 under nitrogen serves several purposes. First, it avoids the formation of bubbles in the later ion-exchange chromatography during elution with 16 *N* formic acid, thus circumventing an upset of the resin bed. Second, a certain acid concentration is necessary to prevent heavy metals from interfering with the analytical method. This was established during initial studies of metal interferences, which involved the application of a neutral sample to the ion-exchange column. In this event, almost no acid derivatives were found by GC analysis. Third, the repeated analyses of sewage samples from different locations with varying acid concentrations in the initial step showed that often, but not always, the highest amount of NTA was released by high acid concentrations. It has not been established whether this effect arose from conjugate hydrolysis, desorption from particulates, dissolution of suspended materials, and/or release of chelated metals. It should be stressed at this point that we do not filter turbid samples as is common practice in NTA analysis. Our own choice of acid concentration clearly represents a compromise engendered by the disparity of samples. It should be optimized whenever NTA or citric acid levels are studied in one particular type of water.

The second point of interest concerns the choice of a final solvent for injection into the gas chromatograph. Initially, methylene chloride was used in analogy to amino acid analysis. However, our flame ionization detectors slowly became noisy and lost sensitivity, presumably from carbon deposits. Changing to Freon 113[34] avoided the deposits, but the NTA derivative was increasingly decomposed in the gas chromatograph, despite the on-glass column injection, when greater numbers of samples were processed in succession. Therefore we made the somewhat unlikely choice of acetone. For some time, *n*-octacosane was used as internal standard in a manner similar to literature methods, but was abandoned as its use did not result in improved precision.

Fig. 1 shows the separation of NTA and citric acid butyl ester derivatives from Columbia tap water spiked with 100 p.p.b. each of the free acids. Fig. 2 is a calibration curve of the two acids in Columbia tap water. The lower part of this calibration curve is enlarged in Fig. 3, and the results of experiments 3, 4 and 5 are superimposed. Columbia sewage effluents contained a peak corresponding to 10 p.p.b. of NTA derivative and the respective results therefore trail off at this level. All other results fall reasonably close to the two lines.

The results indicate that the method works well with Columbia tap and sewage effluent water and could be expected to do likewise with a variety of other waters. (Sewage influent, however, still presents problems in the lower concentration ranges.)

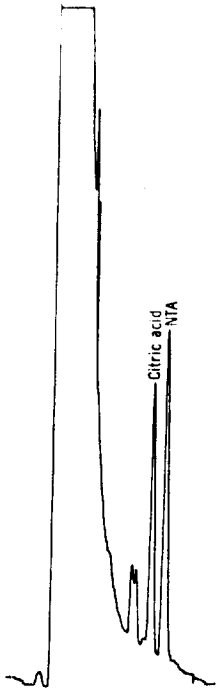


Fig. 1. Typical chromatogram from water spiked with 100 p.p.b. each of NTA and citric acid.

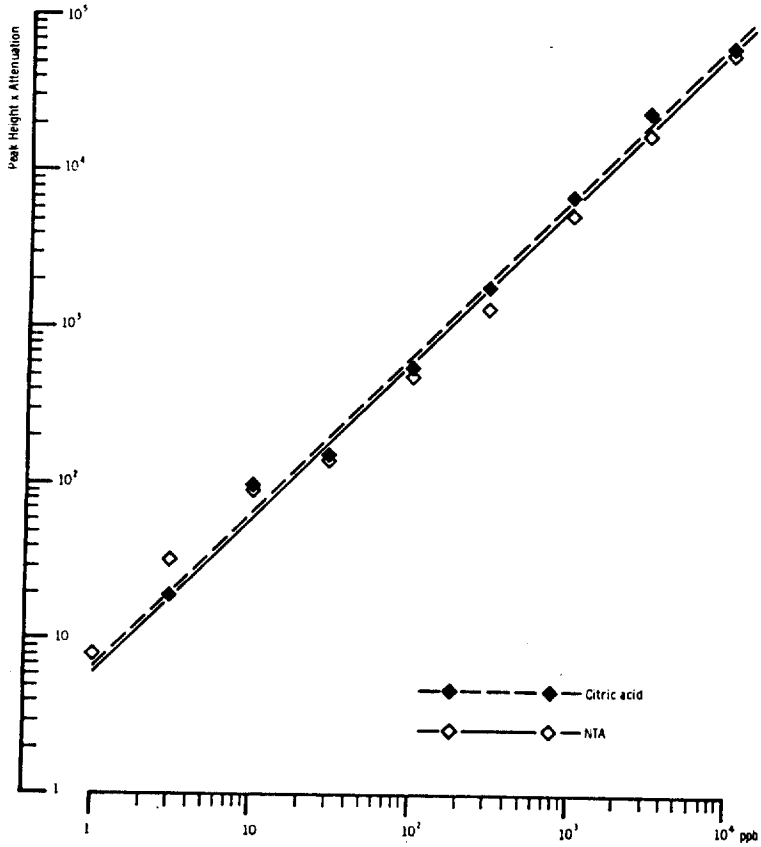


Fig. 2. Columbia tap water calibration curve.

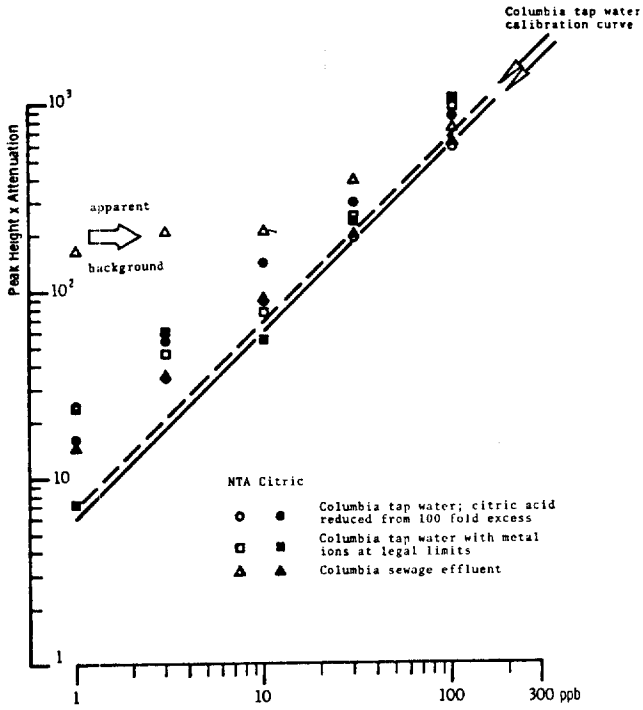


Fig. 3. Determination of NTA and citric acid under various conditions in the lower p.p.b. range.

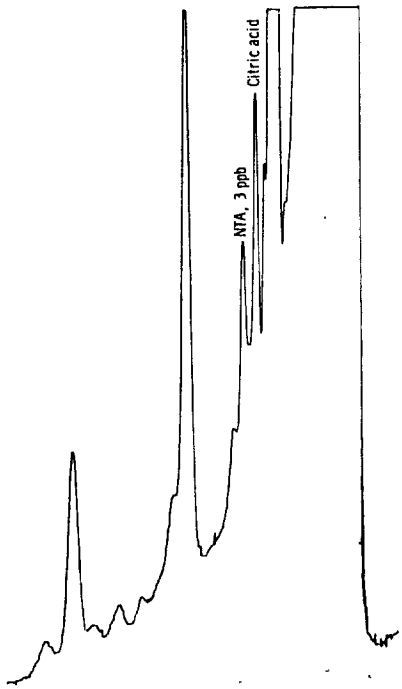


Fig. 4. Analysis of Columbia tap water containing 3 p.p.b. of NTA and 300 p.p.b. of citric acid, the latter reduced by ion exchange.

The metals selected did not interfere at the chosen conditions. The modified ion-exchange procedure worked well, reducing citric acid to approximately 1–2% of its original value. Fig. 4 shows a chromatogram of 3 p.p.b. of NTA determined in the presence of (originally) 300 p.p.b. of citric acid.

The method, as described, lowers the former limits of detection considerably, to about 1 p.p.b. of either acid. There is little interference from heavy metals and, generally, few extraneous peaks show up in the gas chromatogram. It should be noted, however, that the accuracy of the results, as is characteristic of most types of trace analyses in the p.p.b. range, could vary widely with waters of greatly different compositions. The analytical results obtained from such systems should therefore be interpreted with caution.

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0264

"The Effect of a Prolonged Intake of Phosphoric and Citric Acids in Rats" (thesis)

# CONTENTS


1. INTRODUCTION . . . 1
2. LITERATURE SURVEY . . . 3
   2.1 Regulation of acid-base equilibrium in the blood . . . 3
   2.2 Renal regulation of acid-base equilibrium . . . 4
   2.3 Other regulating mechanisms . . . 6
   2.4 Effect of acid intake on general condition . . . 7
   2.5 Effect of acid intake on mineral composition of the tissue . . . 10
   2.6 Effect of acid intake on general metabolism . . . 11
   2.7 Effect of acid intake on Ca- and P-metabolism . . . 12
   2.8 Effect of acid intake on N-metabolism . . . 13
   2.9 Conclusions . . . 14
3. TECHNIQUES . . . 16
   3.1 Experimental design . . . 16
      3.1.1 General program . . . 16
      3.1.2 Basal diet . . . 16
      3.1.3 Administration of the acids . . . 17
      3.1.4 Dietary groups . . . 18
   3.2 Biological techniques . . . 19
      3.2.1 Growth and reproduction . . . 19
      3.2.2 Blood picture . . . 20
      3.2.3 Autopsy . . . 20
      3.2.4 Investigation of the teeth . . . 20
   3.3 Chemical techniques . . . 21
      3.3.1 Blood . . . 21
         3.3.1.1 Obtaining and handling . . . 21
            2 Inorganic phosphorus . . . 21
            3 Calcium . . . 22
            4 Total base . . . 22
            5 Alkaline reserve . . . 24
            6 Chloride . . . 25
            7 Alkaline phosphatase . . . 25
      3.3.2 Liver and Muscle . . . 26
         3.3.2.1 Sampling . . . 26
            2 Total phosphorus . . . 26
            3 Total base . . . 27
            4 Potassium and sodium . . . 28
            5 Chloride . . . 29
      3.3.3 Kidneys . . . 29
         3.3.3.1 Water and ash content . . . 29
            2 Alkaline phosphatase . . . 30
      3.3.4 Bone . . . 30
         3.3.4.1 Ash content . . . 30
            2 Phosphorus . . . 30
            3 Calcium . . . 31
      3.3.5 Urine . . . 31
         3.3.5.1 Collection and preservation . . . 31
            2 pH and titratable acidity . . . 33
            3 Ammonia, urea and total nitrogen . . . 33
            4 Phosphorus . . . 34
            5 Calcium . . . 34




S. L. Bontius, Univ. Amsterdam, 1952.

6 Total base . . . 35
7 Chloride. . . . 35
8 Citric acid . . . 35
3.3.6 Feces . . . 36
3.3.6.1 Collection and preparation . . . 36
2 Phosphorus . . . 36
3 Calcium . . . 36
4 Total nitrogen . . . 37
3.3.7 Experiments with radioactive phosphorus . . . 37
3.3.7.1 General remarks . . . 37
2 Endogenous fecal phosphorus excretion . . . 38
3 Phosphorus retention of the bone . . . 39
3.4 Statistical techniques . . . 39
3.4.1 Standard deviation and standard error . . . 39
3.4.2 Wilcoxon's test for the problem of two samples . . . 40
3.4.3 Rank correlation . . . 42
3.4.4 A non-parametric test against trend . . . 43
3.4.5 Combination of bilateral tail errors . . . 44
4. BIOLOGICAL PART . . . 45
4.1 Growth . . . 45
4.2 Reproduction . . . 47
4.3 Death rate . . . 49
4.4 Blood picture . . . 49
4.5 Autopsy and histological examination . . . 53
4.6 Investigation of the teeth . . . 55
4.7 Discussion . . . 55
5. CHEMICAL PART . . . 57
5.1 Metabolism experiments . . . 57
5.1.1 Introduction . . . 57
5.1.2 Phosphorus metabolism . . . 58
5.1.3 Calcium metabolism . . . 63
5.1.4 Nitrogen metabolism . . . 66
5.1.5 Total base exretion . . . 67
5.1.6 Citric acid excretion . . . 67
5.1.7 Titratable acidity, pH, volume and chloride content of the urine . . . 69
5.1.8 Fate of the ingested acids . . . 71
5.2 Tissue analyses . . . 73
5.2.1 Introduction . . . 73
5.2.2 Blood . . . 74
5.2.3 Liver and muscle . . . 77
5.2.4 Kidney . . . 79
5.2.5 Bone . . . 80
5.3 Experiments with radioactive phosphorus . . . 82
5.3.1 Endogenous fecal phosphorus excretion . . . 82
5.3.2 Phosphorus retention of the bone . . . 83
5.4 Discussion . . . 85
6. CONCLUSIONS . . . 88
7. SAMENVATTING . . . 90
8. LITERATURE CITED . . . 93

# 1. INTRODUCTION

At present the use of orthophosphoric acid ($H_3PO_4$) as an acidulant in refreshing beverages has become rather widespread because of its agreeable acid and refreshing taste in dilute solution. In this respect it has replaced citric acid and tartaric acid to a considerable extent. Organic acids like citric acid and tartaric acid are used mostly for the preparation of lemonades containing fruit juice, while phosphoric acid is used preferentially in refreshing beverages containing extracts from leaves, roots and nuts as the flavoring component. To obtain a suitable taste a concentration of the order of 50-70 mg $H_3PO_4$ per 100 ml of beverage is required, establishing a pH of 2.4-2.8. Beer contains as an acidulant primary phosphate $NaH_2PO_4$ in a concentration of about 60 to 100 mg $PO_4$ per 100 ml, giving a pH of 4.2 to 4.5.

In view of this widespread use of phosphoric acid and primary phosphates as acidulants instead of organic acids like citric acid and tartaric acid the action of these acids needs to be more thoroughly investigated than it has been hitherto. While organic acids are oxydized more or less completely by the body, phosphoric acid is not transformed and has to be neutralized before it can be eliminated. This might cause a loss of fixed bases (Na, K, Ca, Mg) from the body, which in the long run might be injurious to the organism. Also the phosphoric acid might combine with the Ca of the food in the digestive tract to form calcium-phosphate, which would not be absorbed and therefore be lost to the organism, causing a calcium deficiency.

The capacity of the body to maintain its acid-base equilibrium notwithstanding a considerable intake of acid or base is an established fact. However the question is: will this mechanism suffice in case of a prolonged intake of acid or base? From a study of the literature we concluded that our present knowledge was not sufficient to answer this question.

We decided therefore to carry out the experiments reported in this publication. In these experiments the effect of a prolonged intake of phosphoric acid or citric acid in rats was investigated. Because we wanted to carry out tissue analyses and to investigate also the reproductive capacity and the second generation we had to use animals. The rat was chosen as an experimental animal, because of all smaller laboratory animals it resembles man most closely in matters of nutrition and metabolism.

In the next chapter a survey of the literature on this subject will be given. In chapter 3 we describe the techniques used in our investigation. Our results will be given in chapters 4 and 5, followed by a summary of our conlusions in chapter 6 and a summary in chapters 7 and 8.

We are indebted very much to Prof. Dr. E. J. Bigwood, Brussels for the advice and helpful criticism given to us throughout this investigation as well as for reading the manuscript. And also to Prof. Dr. R. Nicolaysen, Oslo for his kind interest in this work. The contributions of many others have been mentioned at the appropriate places in the text.



0264

# 2. LITERATURE SURVEY

## 2.1 Regulation of acid-base equilibrium in the blood.

It is a fact already known for more than 70 years that the maintenance of a certain pH within narrow limits in the blood and the other body fluids is essential for life. The regulation of the "acid-base equilibrium" is a complicated mechanism, which includes both functional and chemical factors. Among the former are the excretion of $CO_2$ in the lungs and the urinary excretion of cations and anions. Chemical factors include the cation-anion balance in the blood and the other fluids, the amount of buffer salts present in these fluids, the distribution of alkali among the buffers and the exchange of ions between blood and tissues.

The first investigation of acid intoxication and its relation to the bicarbonate of the blood was carried out by Walter (1877). He found that ingestion of 1 g of HCl per kg in rabbits reduced the $CO_2$ content of the blood from 27 vol % to 2.5 vol % and resulted in death. Dogs were not killed by this dose of acid, because they formed sufficient ammonia to neutralize the acid. Henderson (1908; 1909) was the first to give an explanation of the mechanisms involved in the maintenance of the acid-base equilibrium in blood. Apart from the inclusion of some additional facts concerning the role played by anions other than bicarbonate and by cations his theory is still valid.

In blood the following buffer systems exist: bicarbonate-carbonic acid, monophosphate-diphosphate, protein acid form - protein anion form (albumin, hemoglobin and oxyhemoglobin). All of these are buffering the blood in proportion to their relative effectiveness at the given pH. The major buffering action for all acids other than carbonic acid is exerted by the plasma bicarbonate and in a smaller degree by the plasma protein and phosphate. The actual efficiency of the $CO_2$ system in neutrality regulation rests in the fact that $H_2CO_3$ promptly dissociates yielding $CO_2$ which is expired; and this efficiency is therefore greater than would appear to be the case from a consideration of the buffer theory alone. (see e.g. Hasselbalch, 1912). If the amount of ingested acid remains within certain limits the blood pH can be kept practically constant, while the total $CO_2$-content of the blood is decreased (compensated acidosis). If the amount of acid exceeds these limits the blood pH decreases definitely (uncompensated acidosis). According to the definition given by Peters and Van Slyke (1932, p. 870) acidosis exists, when acids are absorbed (or formed) more rapidly than they are destroyed or eliminated, causing the bicarbonate of the blood to fall and the pH of the blood to decrease in more pronounced cases.

Properly speaking we should have used the term "metabolic acidosis" in contrast to "respiratory acidosis", existing when $CO_2$ is retained by the blood, e.g. through obstructed re-

spiration. However in our case only the first type of acidosis can occur; so we shall use the term acidosis hereafter.

Determination of total blood $CO_2$ affords the easiest way of demonstrating acidosis resulting from acid ingestion. VAN SLYKE and CULLEN (1917) introduced a gasometric method for this determination.

According to VAN SLYKE et al. (1928) the total available buffer alkali in human blood is sufficient to neutralize about 30 milliequivalents of acid per 1. When dilute acid was introduced into the veins of a dog about 83% was neutralized by alkali from sources other than the blood, since the decrease in blood bicarbonate was equivalent to about 17% of the acid injected (VAN SLYKE and CULLEN, 1917). This would mean that the total buffer capacity of the body was equivalent to about 15 meq/kg. By injecting 0.1 N HCl into the external jugular vein of a dog until coma occurred and heart action became very feeble PRENTICE et al. (1920) found for the total buffer capacity of the body 12 meq/kg, which is in good agreement with the value found by VAN SLYKE et al.

In the foregoing we described two defense mechanisms against ingested acid, viz. neutralization by buffers of the blood and tissues and removal of $CO_2$ from the blood through the lungs by hyperventilation. Another mechanism of the blood for neutralization of acid seems to consist in a migration of Cl-ions from the serum into the erythrocytes, where they combine with the proteins according to FELDMANN and ULANOVSKAJA (1935). The Na-ions remaining in the plasma would combine with the acid-anions to neutralize them. An increase of the ratio of erythrocyte-chloride to plasma-chloride therefore would be an early sign of acidosis according to the authors, who fail to explain however in which way the Cl-ions are bound to the proteins.

Probably this mechanism is the same as the well known "chloride shift" in which chloride ions compensate for the transport of bicarbonate ions from cell to plasma occurring in the respiratory process (see e.g. PETERS and VAN SLYKE, 1932, p.1020). Then the bicarbonate ions emerging from the cells would replenish the reduced bicarbonate content of the plasma and the chloride ions entering the cells would be neutralized by the potassium ions of the erythrocytes.

## 2.2 RENAL REGULATION OF ACID-BASE EQUILIBRIUM.

The kidneys play an important part in the regulation of the acid-base equilibrium by varying the composition of the urine according to the relative proportions of acid and basic products of metabolism. While the blood pH normally remains constant between 7.32 and 7.40 (BIGWOOD, 1925), the pH of the urine may vary between 4.8 and 8.2. As the urinary pH is usually lower than the blood pH, the kidneys must be excreting acid products which would, if retained, tend to lower the blood pH. These products must be buffers, as only infinitesimal quantities of strong acids, like HCl, can be present even at the lowest urinary pH.



Most important of these buffers is the acid phosphate. While in the blood at pH 7.4 80% of the inorganic phosphate is in the form of $Na_2HPO_4$ and 20% is in the form of $NaH_2PO_4$, the relative quantity of $NaH_2PO_4$ in urine of a lower pH may be much higher. This results in the partial retention of the alkali which was combined with phosphate in the body. The retained alkali may then be used for the neutralization and subsequent excretion of additional acid. HENDERSON (FITZ et al, 1907; HENDERSON, 1911; HENDERSON and PALMER, 1914) and HALDANE (HALDANE, 1921; HALDANE et al, 1923) were the first to demonstrate this mechanism. Recent investigations by PITTS and ALEXANDER (1945) have proved that in the tubules of the kidney the following process takes place:

$$Na_2HPO_4 + H_2CO_3 \rightarrow NaH_2PO_4 + NaHCO_3$$

in which renal carbonic anhydrase plays an important role by bringing about the rapid hydration of carbon dioxyde to carbonic acid. The resulting $NaHCO_3$ is reabsorbed by the kidney, as long as the plasma bicarbonate concentration is below 26 mmol/1 or 61 vol% $CO_2$ (PITTS et al, 1949). This "phosphate shift", as the above mechanism is often called, results in an increase of the titratable acidity of the urine. The titratable acidity of the urine is determined according to HENDERSON and PALMER (1914) by titrating the urine with NaOH to the blood pH 7.4. Therefore this value represents approximately the amount of alkali saved by the phosphate shift.

Even more important for the preservation of the acid-base equilibrium is the excretion of ammonia by the kidneys. WALTER (1877) for the first time found an increased urinary ammonia excretion after the administration of mineral acid. Since then this fact has been confirmed in numerous investigations (HALDANE, 1921; HALDANE et al, 1923; GAMBLE et al, 1923; FISKE and SOKHEY, 1925). NASH and BENEDICT (1921) proved the kidney to be the site of formation. This was confirmed by WASSERMEYER (1929), who believed the ammonia to be formed by enzymatic deamination of adenosinephosphoric acid. MARKERT (1933) found that the formation of ammonia takes place in the glomeruli. KREBS (1932) concluded from in vitro experiments that the ammonia is formed by oxidative deamination of amino acids in kidney tissue. But more recently VAN SLYKE et al (1943) proved glutamine to be the most important physiological precursor of ammonia, the renal glutaminase splitting glutamine into glutamic acid and ammonia. A variable and less significant fraction of ammonia is formed by deamination of amino acids. By means of in vitro experiments HANDLER et al (1949) showed that the increased ammonia excretion during acidosis is not the result of an adaptive increase in the concentration of renal glutaminase or amino acid oxidases, but rather a functional response of the kidney to the progressively increasing severity of the acidosis; earlier work of BRIGGS (1934) made it probable that the $NH_3$ excretion is not controlled by the acid-base balance in the tissues but rather by that of the glomeruli of the kidney. DENNIG et al (1929) found the



0264

mechanism of ammonia formation by the kidneys to be rather slow and to reach its maximum only after 5-7 days of acid ingestion, while it does not cease at once but drops only gradually after the acid administration is stopped.

MAINZINGER and JOFFE (1928) showed that increased ammonia excretion and increase of the titratable acidity of the urine do not necessarily mean the existence of an acidosis, but that they are the normal mechanisms by which the organism tries to avoid an acidosis. Generally the most important of these is the ammonia excretion. E.g. FÖLLING (1929) found that of the acid ingested by man during experimental $NH_4Cl$ acidosis 80 to 90% is neutralized by ammonia, while 10 to 20% could be accounted for by the phosphate shift. With consumption of weaker acids the role of the phosphate shift may become more important.

### 2.3 OTHER REGULATING MECHANISMS.

Although less important than the kidney the alimentary tract also seems to play a role in the maintenance of the acid-base equilibrium. BAKALTSCHUK (1928) found a compensatory secretion of hydrochloric acid by the gastric mucosa after the occurrence of respiratory acidosis. After administration of small doses of acid phosphate (1 g) to man VORONOVA et al (1936) could not find any effect on the gastrointestinal secretions. HERRIN (1927) however found that in base deficiency the intestine conserves fixed base either by secreting a juice of lower base content or by increased absorption of base from the intestinal contents. In a very recent paper PERSON (1950) also concludes that the gastric secretion must have a regulating function with regard to the acid-base equilibrium in blood.

The liver also exerts an accessory regulation on the acid-base equilibrium by means of the oxidative degradation of organic acids, as was shown in the case of lactic acid by MCCLURE (1932). The alkali originally bound to the lactic acid and liberated in this process is used to restore the alkaline reserve of the blood.

Under [2.1] we already found the tissues to possess an important buffer capacity. Therefore it is quite understandable that changes in blood reaction are accompanied by similar changes in the tissues (ROUS et al, 1927; OGAWA, 1928). BANUS (BANUS and KATZ, 1927; BANUS et al, 1929) investigated this for a hind leg preparation perfused by acidified blood. They found a migration of Cl-ions from the blood to the tissue to be responsible for the restoration of the alkaline reserve of the blood. GOLDBERGER (1932) stresses the importance of the tissue proteins for the maintenance of acid-base equilibrium in the tissues. According to him the inorganic bases play only a minor role in this process. It seems to be probable that the Cl-ions migrating from the blood to the tissue cells combine with the tissue proteins. This would be a mechanism comparable to that for the blood cells demonstrated by FELDMANN and ULANOVSKAYA (1935) (see under [2.1]). Bicarbonate does not seem to play a role in the maintenance of the tissue



acid-base equilibrium and the muscle cell seems to be impermeable to bicarbonate ion according to WALLACE and BAIRD HASTINGS (1944). A remarkable fact is that the mean intracellular pH was found to be lower than the blood pH ($6.93 \pm 0.12$ against 7.4) by the same authors.

Finally the Ca-P-metabolism affords a possibility to counteract the effect of an ingestion of acid. Normally most of the Ca ingested is excreted in the feces and only a small part via the kidneys because of the formation of insoluble $Ca_3(PO_4)_2$ in the intestine. After ingestion of acid (0.5 meq/kg HCl orally) however, the part of Ca excreted via the kidneys more than doubled, while the P excreted via the kidneys almost doubled in experiments done by ZUCKER (1921). As via the kidney only soluble salts can be excreted, this means that the fecal excretion of $Ca_3(PO_4)_2$ must have been partly replaced by the urinary excretion of acid calcium phosphate with a consequent saving of alkali. Furthermore the $Ca_3(PO_4)_2$ excreted in the intestine also acts as a buffering mechanism in situ, being partly transformed into $CaHPO_4$, which is in part excreted in the feces and in part in the urine (LOFFLER and KARTAGENER, 1931).

Summarizing we find the following mechanisms active in the restoration of the acid-base equilibrium after acid ingestion:

1. ammonia excretion by the kidney.
2. phosphate shift, resulting in an increase of the urinary titratable acidity.
3. excretion of acid calcium phosphate via kidney and intestine instead of $Ca_3(PO_4)_2$.
4. compensatory changes in the gastrointestinal secretion.
5. oxidative degradation of organic acids by the liver.
6. buffering capacity of the tissue and blood cells by means of a shift of Cl-ions to these cells.
7. neutralization by buffers of the blood, esp. bicarbonate-carbonic acid.
8. removal of excess $CO_2$ through the lungs by hyperventilation.

The mechanisms mentioned under 1 and 6 tend to neutralize the acid avoiding an acidotic condition. With continuous administration of acid as in our experiments the most important mechanisms will be those mentioned under 1 to 3, as these are probably able to function continuously without injurious effects to the organism.

### 2.4 EFFECT OF ACID INTAKE ON GENERAL CONDITION.

At the beginning of this section it should be stated that many of the experiments reviewed here involved unphysiologically large doses of the administrated acids. They were included, however, in order to have an indication what effects might possibly occur.

ZILZER (1930) fed rats a basic diet to which he added either acid or basic salts. He compared the growth during 5–13 weeks and found that the average



0264

weight gain of 27 base-fed animals was 8% greater than that of 27 acid-fed animals. This could not be due to a diuretic effect of the acid diet. In the abstract no particulars about the added salts and the dose are given. On the other hand MITCHELL and MILLER (1931) found no effect on the growth, reproduction or bone calcification of rats on changing the dietary acid equivalence from —20.6 to +19.4 meq/100 g dry ration by adding HCl, $H_2SO_4$, citric acid and tartaric acid. Neither did LAMB and EVVARD (1931) find any effect on growth, reproduction and bone composition of pigs, rats and rabbits by adding $H_2SO_4$ to the diet. With the rats and rabbits they continued the experiment for three generations without observing any effect. The pigs received 200–300 meq/d of acid, the rats received a diet containing 15 meq/100 g of ration, while the rabbits tolerated 5 meq/d of acid maximally. The rabbits excreted this dose of acid principally by means of the phosphate shift. In some investigations with adults the administration of acids or acid-forming substances caused a temporary loss of weight by means of the resulting diuresis (KOHLER, 1927; MASON and HELLBAUM, 1938). This is however, not an effect on the growth.

HOUSEHOLDER (1921) found spleen enlargement and anemia in experimental acidosis resulting from daily subcutaneous injection of 0.1–0.5 meq of HCl. HALDANE et al (1928) observed an injurious effect of $NH_4Cl$ acidosis in man (20 g of $NH_4Cl$ per day orally) on the blood and endothelial cells. Hyaline leucocytes were increased, erythrocytes showed an increased tendency to hemolyze resulting in a mild grade of anemia. Changes in the leucocyte picture in experimental acidosis following injection of HCl (no amount given in the abstract) into young dogs have been reported by SAKAI (1932). There was a leucocytosis, an increase in lymphocytes and a relative diminution of neutrophiles in the first hour; in the second hour there was a diminution of lymphocytes, an increase in neutrophiles and a moderate number of eosinophiles, later decreasing. KUGA (1934) also found changes in the blood picture of rabbits during experimental acidosis: a decrease in hemoglobin, red cell count and blood platelets, leucocytosis with increasing number of basophiles, decreasing number of eosinophiles and lymphocytes and increasing number of pseudoeosinophiles. He concluded that in acidosis the hematopoietic organs are irritated. There was no change in total blood volume. TERZIOGLU (1944) however found an increase of erythrocytes in acidotic rabbits and attributed this to a stimulation of the bone marrow by the decrease in the blood pH.

As the kidney is the most important organ in the maintenance of acid-base equilibrium, it is natural that many investigators looked for a possible pathological effect of acid ingestion on the kidney. MACNIDER (1923) found after two injections of 2.5 meq/kg of HCl in normal dogs a slight kidney degeneration consisting of a cloudy swelling of the epithelium esp. in the cells of the convoluted tubules. Progressive degeneration consisted of edema, occurrence



of vacuoles and more rarely of an early necrosis of the tubular epithelium. ADDIS et al (1927) however, found no marked degenerative changes in the kidneys of rats receiving a diet with 2% $CaCl_2$ (equivalent to 14.4 meq/100 g ration of HCl) during one year. Segal (1927), like MACNIDER, found in the kidneys of rabbits receiving 0.1 N HCl, 2% $CaCl_2$ or $NH_4Cl$ during from 11 days to one year a degeneration of the convoluted tubules, similar to cloudy swelling, but also accompanied by marked pyknosis of the nuclei in cases of longer duration. When the administration of acid was ceased the kidney promptly returned to normal. Dogs gave similar results but required higher doses. MACKAY and OLIVER (1935) reported marked renal lesions in rats on diets with an excess of inorganic phosphate (2.8-5.6% P against 0.62% P in the basal diet) in the form of $H_3PO_4$, $NaH_2PO_4$, $Na_2HPO_4$ or $Na_3PO_4$. These lesions, being similar with all diets, cannot be due to the acid-base balance of the diet, but must be due to the great excess of $PO_4$-ions. They consist of necrosis of the cells of the convoluted tubules, formation of atypical epithelium, calcification of the necrotic debris filling the tubules, distortion of the outer zone of the medulla as well as an increase in interstitial connective tissue, while the kidneys are markedly enlarged. Production of urinary calculi containing uric acid and urates in rats receiving acid phosphates with $NH_4Cl$ was observed in 43% of the animals by HAMMARSTEN and LUNDGREN (1940).

Liver degeneration in rabbits receiving 0.25 N HCl by stomach tube and trypan blue by injection was found by HESS and GOLDSTEIN (1925). Necrotic areas with loss of dye from the parenchymatic cells and the Kupffer cells occurred. TATSUZAWA (1933) found marked disturbance of the liver function in rabbits fed $NaH_2PO_4$. Administration of 0.5-1.0 g/kg of $NH_4Cl$ to rabbits causes necrosis of the renal tubular epithelium (mainly in the distal portion) with subsequent death (GOVAN and PARKES, 1946); while daily oral administration of $CaCl_2$ (2.5 g/kg) to rabbits caused death in four days with extensive focal necrosis of the liver and necrosis of the juxtamedullary region of the kidneys, blood ammonia rising to 6 mg% (GOVAN and PARKES, 1949).

Decalcification of the bones does not seem to occur very easily after administration of acid. Neither MITCHELL and MILLER (1931), nor LAMB and EVVARD (1931) found any effect on the mineral content of the bones in their experiments (see above). GOTO (1918) found in fact that during acidosis in rabbits caused by feeding HCl the lost phosphorus came from the organic phosphates rather than from the bone phosphates. Not even BURNS (1929) finds evidence of decalcification of the bones of rats and rabbits on feeding increasing amounts of HCl until death occurred. Prolonged feeding of large amounts of $NH_4Cl$ to rats did not alter the citrate and Ca content of the skeleton, neither did it impair the kidney function (LEONARDS and FREE, 1944). Also the possible effect of the acid-base equilibrium on dental caries incidence was investigated. PRICE (1935) found



0264

no correlation between dental caries incidence among primitive races and the acid-base balance of the diet. FORSHUFVUD (1938) could not obtain dental caries in rats after subcutaneous injection of $NH_4Cl$. Frequent alternating injections of $NH_4Cl$ and $NaHCO_3$ however produced a high incidence of dental caries. THOMAS and BODECKER (1942), repeating these experiments during 114 days, failed to produce any caries. On histological examination no abnormalities were found that could be associated with the compounds used. Dental erosion, not dental caries, occurred in rats receiving 10 ml of a solution containing 0.055% phosphoric acid and 10% of sugar according to MCCAY and WILL (1949). MULLER and GORTNER (1949) obtained the same result with rats receiving 20 ml/d of this solution during 7 days. Meanwhile the same effect has been found for fruit juices by WYNN and HALDI (1948), JARVIS et al (1949) and MILLER (1950). JARVIS et al found that lemon juice dissolves 70% of the radioactive phosphorus present in rat teeth after intraperitoneal injection of radioactive $KH_2PO_4$.

## 2.5 EFFECT OF ACID INTAKE ON MINERAL COMPOSITION OF THE TISSUES.

Daily oral intake of 25 g of $NH_4Cl$ during three days by man causes an increase in inorganic serum P, a marked decrease in non-hydrolyzable P in total blood and a slight increase in lecithine-P while hydrolyzable P (the fraction of organic P which can be hydrolyzed by a bone enzyme preparation) remains constant (KAY, 1924). KAY considers the non-hydrolyzable P to be a phosphorus reserve of the body, that is used in acidotic conditions to excrete the ingested acid via the phosphate shift. MARTLAND (1925) found hydrolysis of organic P of the blood to occur at a pH below pH 7.3 by means of an esterase. At a pH above 7.35 esterification occurred. This would explain the results of KAY. POTOP (1935) confirmed the increase in inorganic serum P during a decrease in the alkaline reserve. Later work by RAPOPORT (1937) proved the hydrolyzable P of KAY to be diphosphoglyceric acid.

As the blood chloride and bicarbonate are the most important plasma anions and the total cation concentration of the plasma is kept at a comparatively constant level through renal control in order to maintain the osmotic balance of the extra-cellular fluid, changes in the blood bicarbonate level tend to produce compensatory reciprocal changes in the chloride content (GAMBLE et al, 1923; GOLLWITZER-MEYER, 1925; PETERS et al, 1926). Under [2.1] and [2.3] we saw already the contribution towards the maintenance of the acid-base equilibrium made by the shift of Cl-ions to the cells of the blood and tissues. When anions of other acids enter the blood the plasma Cl may decrease parallel to the alkaline reserve (BLUM et al, 1925). CARERE-COMES and DE GENNARO (1941) found no change in the Cl, Na, K and Ca of the blood of rats fed from 0.5 to 6 meq/d of HCl during four months. Only the water content and K of the soft tissues decreased, while the ash content, Mg and Na increased. Feeding dogs $NH_4Cl$ did not change the concentration of fixed base and Na of blood as well as the ratio Cl+bicarbonate/fixed base of the blood, while serum Cl increased and plasma $CO_2$ decreased (HOUSTON and HENDRIX, 1944). The urinary excretion of Cl, fixed base and $NH_4$ increased, which could be explained by a decrease of body water during acidosis. In the rabbit the total base and chloride concentrations in muscle varied with the plasma concentrations, but were not significantly changed by pH changes as such (YANG and WU, 1932).

A decrease of the alkaline reserve of the blood is generally accompanied by an increase in the ultrafiltrable Ca (HALDANE, 1924; BLUM et al, 1924). Infusion of HCl and lactic acid in cats gave an increase of both total and ultrafiltrable Ca; phosphoric acid however, produced decreases in both, probably as the result of the formation of slightly soluble complexes (MEGLITSKIÎ and ZINOV'EVA, 1946).

## 2.6 EFFECT OF ACID INTAKE ON GENERAL METABOLISM.

Much has already been reviewed of the effects on general metabolism under [2.2] and [2.3] which needs not to be repeated. Some additional results of investigations in this field will be described here. A decrease in metabolic rate during acidosis, produced by oral intake of HCl, was found by FRASCARELLI and BUTTARO (1948).

SHOHL and SATO (1923) determined the total acid-base metabolism of two infants by determining Na, K, Ca, Mg, $SO_4$, $PO_4$ and Cl in food, urine and feces. Of the hydrochloric acid added to the diet 12–15% was retained, the remainder was excreted via the kidneys. The positive base balance decreased from 1.2 to 0.95 meq/kg after 25 meq of HCl, accounting for approximately 10% of the acid ingested. Therefore about 75% of the acid must have been excreted by means of $NH_4$ formation and phosphate shift. The salt retention decreased, while the excretion of ash, N, Ca and Cl in urine and feces increased. Also increased were Na and P in urine as well as Mg in feces. P in feces was decreased. It would seem that the diuretic effect of the acid also played a role here.

MICHALOWSKY (1930), ROSSI (1932; 1933) and BISCHOFF et al (1934) found no effect on the alkaline reserve of persons receiving acidogenic diets (large quantities of animal food, few vegetable products). MICHALOWSKY even found no effect after daily consumption of 2.5 g of $H_3PO_4$ during 6 weeks. After oral administration of $NH_4Cl$ however ROSSI (1932) obtained a large decrease of alkaline reserve and a urine pH of 4.4. BISCHOFF et al (1934) found a just perceptible decrease of alkaline reserve and blood pH in a diet consisting only of meat and rice, which was taken for 3 days by one person. The acid equivalence of this diet was 200 meq/d. The urine pH decreased from 5.8 to 5.3, the blood pH from 7.49 to 7.45. Urinary ammonia rose from 0.4 to 0.8 g/d of nitrogen.



0264

The effect of organic acids was investigated by BLATHERWICK and LONG (1922). Orange juice in a daily volume of 2.4 l, containing 48 g/d (or 500 meq/d) of citric acid, produced in man an increase in urine pH and urinary organic acids as well as a decrease of urinary ammonia, presumably because of the oxidative degradation of most of the citric acid. On the contrary 2 l/d of milk acidified with 2% lactic acid (440 meq/d) produced a strongly acid urine, with increased titratable acidity and P content. As the organic acid content was not increased, a considerable part of the lactic acid must have been retained and neutralized by the body. Quite different results were found by RODLER (1939), who administered 7–9 g/d of lactic acid or citric acid with the milk to two infants during 3 to 6 days. Lactic acid gave a higher organic acid excretion, but a lower P- and fixed base excretion, than citric acid. SHEA and FELLERS (1942) added to the diets of healthy men 800 to 1000 g/d of apples during 3 days and found that about 90% of the organic acids of the apples was oxidized. The urinary pH was decreased, but the alkaline reserve was not affected. There was no difference between a neutral basal diet and an acidogenic basal diet in this respect. Other authors however, found a decreased output of citric acid in acidosis (OESTERBERG, 1930; SCHROEDER and SMITH, 1943), which brings them to the conclusion that the citric acid excretion plays a role in the regulation of the acid-base equilibrium. Most probably in our opinion the extent to which the citric acid, ingested or produced in the organism, is oxidized, is determined by the acid-base equilibrium.

### 2.7 EFFECT OF ACID INTAKE ON CA- AND P-METABOLISM.

Under [2.3] we already described the influence of acid ingestion on Ca- and P-metabolism. Part of the $Ca_3(PO_4)_2$ of the intestinal contents is transformed into soluble acid phosphates, which are excreted partly via the kidneys and partly in the feces.

Dogs daily receiving 1.1 g/kg (12 meq/kg/d) of lactic acid during 5 days showed a marked decrease of fecal Ca- and Mg-excretion, sometimes accompanied by a slight increase in urinary excretion. For both elements a marked retention occurred (FLORIS, 1923). Increasing the dose to 1.7 g/kg (19 meq/kg/d) gave an increased excretion of Ca both in feces and urine with a negative Ca-balance. For Mg no change occurred in the excretion and retention. Presumably the higher dose could not be neutralized by ammonia excretion and phosphate shift, contrary to the lower dose.

FARQUHARSON et al (1931) varied the acid-base balance of some patients by means of acidogenic diets or administration of $NH_4Cl$. The Ca excretion varied with the titratable acidity and $NH_4$ content of the urine, but was more or less independent of the pH of the urine. The quantitative increase also depended on the dietary Ca content as well as on the potential amount of acid ingested.



Fecal Ca remained constant even with great changes in the potential acidity of the diet. Ingestion of $NaH_2PO_4$ produced a rise in urinary acidity, but had no effect on the excretion of $NH_4$ and Ca. In this respect the phosphate differed from the other acidogenic substances studied (FARQUHARSON et al, 1931 a). When $NaH_2PO_4$ and $Na_2HPO_4$ were fed to subjects with a constant neutral diet, 25% of the ingested phosphate was retained. Of the excess phosphate excretion about 25% was fecal. There was an increase in urinary total base excretion equivalent to the amount of base necessary to neutralize the excess phosphoric acid excretion. No influence of the potential acidity of the diet upon the absorption or retention of inorganic P was observed (SALTER et al, 1932). NICKISCH (1940) found no difference in Ca- and P-retention in pigs receiving a diet to which mono-, di- or tri-calciumphosphate had been added.

### 2.8 EFFECT OF ACID INTAKE ON NITROGEN METABOLISM.

BERG's theories (RÖSE and BERG, 1918; BERG, 1930; 1931) about the relation between the dietary acid-base balance and the N-metabolism started a great number of investigations on this subject. Some of these will be reviewed here. BERG reasoned thus: when an acid diet is ingested, ammonia is formed to neutralize the acid. As he believed the ammonia to result from degradation of protein, either tissue protein or dietary protein, he concluded that with an acid diet the minimum protein need must be higher than with an alkaline diet. An unproved dogma, he used, is that all dietary components should be oxidized as fully as possible and that for nitrogen compounds this should mean conversion in urea. Ammonia is not far enough oxidized and therefore ammonia formation is an abnormal and incomplete form of protein metabolism, namely a "hydrolytic degradation" instead of an "oxidative degradation" of protein. Retention of these nitrogen compounds that are not fully oxidized would cause a pathological condition. He tested his theories by keeping his subjects on an alkaline diet containing the minimal amount of nitrogen necessary for conserving body weight, and obtaining a minimal nitrogen balance. He then made the diet acidogenic and determined the change in the minimal nitrogen need. He found an increased need for nitrogen. Without saying more about his untenable dogmas we shall confine ourselves to this only experimental result of his laborious investigations. This result was tested by several investigators. No effect was found by STEENBOCK et al (1914), GIVENS and MENDEL (1917), LAMB and EVVARD (1931), JANSEN (1919), FUHGE (1919), BAUMGARDT (1921), LAUBENDER (1932) and ROSSI (1932a). Confirmation of the results of BERG was found by SHOHL and SATO (1923), ADDIS, MCKAY and MCKAY (1927), TERROINE and CHAMPAGNE (1932) and TENGBERGEN (1941). The most important factor causing different results is the amount of nitrogen ingested. TENGBERGEN for instance found a retardation of growth of about 13% with an acid diet



0264

containing a minimal amount of protein, but no effect with a diet rich in protein. Other causes are: too short duration of the experiments; use of food tables for calculating balances instead of chemical analyses; use of deficient diets; failing of a statistical treatment of the results. As TENGBERGEN avoided all these factors his results seem to us to be the most reliable. In the light of our present knowledge about the source of the urinary ammonia, these results seem plausible: at a minimal protein intake no excess glutamine will be available; in that case the glutamine necessary for the formation of ammonia in the kidneys will have to be obtained from tissue or food protein, resulting in a slightly negative N-balance on acid ingestion. When the protein supply is abundant, enough glutamine will be available; so the only effect will probably be a decreased urea excretion while the N-balance will remain positive and no impairment of growth will occur. The more so as glutamine is a – to a certain extent – dispensable amino acid (WOMACK and ROSE, 1947), which can be synthesized from proline (STETTEN and SCHOENHEIMER, 1944). Therefore as normal individuals under normal circumstances obtain a sufficient supply of protein and especially of glutamine, one could hardly expect a perceptible influence of the dietary acid-base balance on the protein metabolism in their case.

## 2.9 CONCLUSIONS.

From the foregoing literature survey we may conclude first of all that disturbances of the acid-base equilibrium are counteracted by several regulation mechanisms. The literature on the effects of acid intake shows that:

1. the effects are most marked with the herbivorous rabbits, who are accustomed to an alkaline diet and are not able to excrete ammonia but generally are less marked for omnivorous animals like man, dog and rat, who are able to form ammonia in the kidneys.
2. the effects of the administration of HCl, $H_2SO_4$, $CaCl_2$ or $NH_4Cl$ are generally much more marked than those with naturally acidogenic diets and also more marked than with phosphoric acid.
3. although the organic acids are oxidized to a considerable extent, facilitating the task of the regulation mechanisms, they are still able to exert an acidifying action. More should be known about their effect on the organism.
4. comparatively few experiments of long duration, involving more than one generation have been carried out to determine the capacity of the different regulation mechanisms during continuous administration of phosphoric acid and organic acids as may occur in the regular use of foods containing these compounds.
5. investigations meant to supplement or eventually correct our knowledge of the effect of a continuous intake of acids should cover a broad field, including metabolism, tissue composition, growth, reproduction, blood picture and autopsy. Use of statistical methods should be made in drawing conclusions from the observations obtained.



In discussing the results of our own investigations we shall find occasion to treat more fully several of the results obtained by other investigators, reviewed briefly in this literature survey.



0264

# 3. TECHNIQUES

## 3.1 Experimental design.

### *3.1.1. General program.*

The object of our investigation has been stated in the Introduction (see under [1]). From a survey of the pertinent literature we concluded that an investigation regarding the effect of a prolonged intake of acid on more than one generation of experimental animals covering a broad field was needed (see under [2.9]). And also that too little was known about the effect of organic acids under similar circumstances. This determined the design of our experiments.

We decided to investigate metabolism, tissue composition, growth, reproduction, blood picture, make autopsies, rear the young resulting from the reproduction and investigate them also. This set-up necessitated the use of experimental animals. To obtain results that could be applied to man at least with some degree of accuracy the animal resembling man most closely in matters of nutrition and metabolism should be chosen. As relatively large groups had to be used, in view of the biological variation, the animals should not be too large and reproduce rapidly. Therefore the albino rat *(Mus norvegicus)* was chosen. All animals were born and bred in our own animal department and belonged to the same strain, which was obtained from the Wistar Institute (U.S.A.) and has been bred here for 20 years. The animals possessed individual cages with an automatic drinking-water supply. Food was given ad libitum every other day. Every week the weights were taken. A constant temperature of 25° C. and a more or less constant humidity were maintained.

### *3.1.2. Basal diet.*

All animals received the same basal diet, which was designed to resemble the average Dutch diet as closely as possible, especially with respect to the acid-base balance. The composition is given in table 1. The linseed oil was added four months after the beginning of the experiments, when a slight dermatosis of the tail was observed in a considerable number of the rats grown on this diet, which we ascribed to a linolenic acid deficiency (Burr and Burr, 1929, 1930). After this addition the symptom disappeared gradually. Apart from this addition of linseed oil no changes in the basal diet have been made.

This diet is slightly acidogenic: we obtained urine with a mean pH of 5.87 (standard error 0.03) from the rats.



**TABLE 1**

*Composition of basal diet*

| | |
|---|---|
| Casein | 6.0 % |
| Dried full milk | 10.0 % |
| Whole wheat flour | 34.5 % |
| Potato flour | 33.0 % |
| Peanut oil | 6.5 % |
| Cod liver oil | 0.5 % |
| Linseed oil | 0.5 % |
| Brewers' yeast | 5.0 % |
| Salt mixture | 4.0 % |
| | 100.0 % |

Salt mixture:

| | | | |
|---|---|---|---|
| $Ca_3(PO_4)_2$ | 20.0 g | $MgCO_3$ | 2.0 g |
| $Na_2HPO_4 . 2$ aq. | 8.0 g | Na-citrate | 1.0 g |
| KCl | 10.0 g | various salts [1]) | 1.0 g |

[1]) according to Jansen, Westenbrink and Boeke (1935)

### *3.1.3. Administration of the acids.*

While phosphoric acid is the only inorganic acid used for the acidulating of foodstuffs, there are three organic acids used for this purpose: lactic acid, citric acid and tartaric acid. As we wanted to run parallel experiments with one and the same of these organic acids beside all experiments with phosphoric acid, we had to choose one organic acid for our investigations Phosphoric acid is peculiar in two respects: its salts are naturally occurring in our body and it is a tri-basic acid, resulting in its typical buffer capacities we noted in the renal regulation of the acid-base equilibrium. Therefore we chose citric acid, the salts of which also occur naturally in our body, which is also tri-basic and which is not so very much weaker than phosphoric acid.

The dissociation constants of the acids mentioned above are at a temperature of 20° C.:

| | | |
|---|---|---|
| phosphoric acid: | $k_1 = 8.0 \; .10^{-3}$ | $pk_1 = 2.10$ |
| | $k_2 = 7.5 \; .10^{-8}$ | $pk_2 = 7.13$ |
| | $k_3 = 5 \; .10^{-13}$ | $pk_3 = 12.30$ |
| citric acid: | $k_1 = 8.4 \; .10^{-4}$ | $pk_1 = 3.08$ |
| | $k_2 = 1.77 \; .10^{-5}$ | $pk_2 = 4.75$ |
| | $k_3 = 3.9 \; .10^{-6}$ | $pk_3 = 6.41$ |
| tartaric acid: | $k_1 = 9.7 \; .10^{-4}$ | $pk_1 = 3.01$ |
| | $k_2 = 2.8 \; .10^{-5}$ | $pk_2 = 4.55$ |
| lactic acid: | $k = 1.5 \; .10^{-4}$ | $pk = 3.82$ |

Next arose the question of how to administer the phosphoric acid and the citric acid to the animals. Because of the automatic drinking water supply system used in our animal house, it was impractical to give the acids with the



02964

drinking water. Moreover there was a good chance that the animals would not take it in this way continuously. Giving it by stomach tube would certainly have led to injuries of the oesophagus after several times and would moreover have been a technique requiring too much time with several hundreds of experimental animals. Therefore we added the acids to the basal diet in the required amounts. The mixture was homogenized by rubbing it through a sieve. Phosphoric acid 89%, sp.gr. 1.75, diluted to a solution containing 500 mg/ml $H_3PO_4$ before use, and anhydrous citric acid, both of pharmacopoeia quality, were used throughout the investigation.

*3.1.4. Dietary groups.*

In some preliminary experiments we found that young animals would take diets containing up to 0.75% by weight of phosphoric acid and 3.1% by weight of citric acid without impairment of growth. According to analysis one of the most popular soft drinks contains per bottle of 185 ml about 100 mg of phosphoric acid as well as 20 g of sugar being equivalent to 82 cal. If consumed regularly in large quantities the maximum intake will be determined by the human caloric need. Assuming this to be 3000 cal/d, we arrive at an absolute daily maximum of 37 bottles of this beverage, representing a phosphoric acid intake of 3.70 g/d or 62 mg/d/kg. To obtain about the same daily intake per kg body weight for an adult rat, the diet should contain about 0.1% of phosphoric acid. When calculated on the average caloric intake of an adult rat, this content should be about 0.5%. We used diets containing 0.05; 0.15 and 0.40%. For the diets containing citric acid we used additions of 0.15; 0.45 and 1.20% of this acid. We based this 1 : 3 weight ratio between the acids (1 : 1.5 molar ratio) on the following observations. Solutions containing 55 mg/100 ml of phosphoric acid and 165 mg/100 ml of citric acid in distilled water possessed a pH of 2.5 and 2.7 respectively. Using drinking water for these solutions we found a pH of 2.7 and 2.9 respectively. Tasted by several persons, who did not know the composition, the citric acid solutions were found to taste slightly more acid than the corresponding phosphoric acid solutions.

The dietary groups we used in our experiments are given in table 2. Group PA served as a control group. The animals given the diet PBB during our preliminary experiment were kept at this diet and a second and third generation obtained. As the diet PBB was the most extreme one, we thought it interesting to include the results obtained with this group in this report. In the beginning we made our experiments with all dietary groups. When we found no important effects during the first 6 months in either of the dietary groups, we decided to confine ourselves to the extreme diets: PA, PD, (PBB) and PG, enabling us to have larger groups of animals in the experiments.

As our investigations covered a broad field entailing a very large number of



TABLE 2

*Dietary groups*

| GROUP | ACID ADDED | | AV. INTAKE in mg/d/kg | FOR ADULT RAT in mcq/d/kg [1] |
|---|---|---|---|---|
| PA | — | — | — | — |
| PB | $H_3PO_4$ | 0.05% | 34 | 0.6 |
| PC | ,, | 0.15% | 100 | 1.8 |
| PD | ,, | 0.40% | 270 | 5.0 |
| PBB | ,, | 0.75% | 500 | 9.2 |
| PE | citric acid | 0.15% | 100 | 1.0 |
| PF | ,, | 0.45% | 300 | 3.1 |
| PG | ,, | 1.20% | 800 | 8.3 |

[1]) at pH 7.4 phosphoric acid has bound 1.8 meq/mM alkali; citric acid 2.0 meq/mM.

analyses of all types, it was necessary to employ some technical assistants. Therefore simple techniques were needed, suitable for routine use. On the other hand it was often necessary in view of the limited sample size – especially in the blood analyses – to employ semimicromethods. The choice of the techniques to be described in the next parts of this chapter was governed by these principles.

3.2 BIOLOGICAL TECHNIQUES.

*3.2.1. Growth and reproduction.*

The determination of the growth in the different dietary groups was a simple matter. Weekly the body weights of all rats were taken. The weekly averages for each group were compared during the first 20 weeks. The animals received the diet for the first time at the age of 3 weeks, immediately after they were taken from the mother.

In order to study the reproduction we brought together 8 females with 3 males of the same dietary group for two weeks. After that period the females were kept in special, individual cages until three weeks after the delivery or until it was certain that no fertilization had occurred. Of each female the weight before and after pregnancy was recorded as well as the total number of young, the number of stillborn animals, the total weight of the living young, the average weight of the young of each litter after three weeks and finally the number of young missing after that period (having died from natural causes or being killed by the mother). As we wanted to keep the young for further investigation, we reduced the number of animals in each litter to a maximum of seven. The same females and males were used for a second reproduction, as often the harmful effect of a certain diet manifests itself only during the second pregnancy.



0264

*3.2.2. Blood picture.*

Taking blood samples for the determination of the blood picture was accomplished most easily by amputating the tip of the tail (GRIFFITH and FARRIS, 1942, p. 351). Dilatation of the tail vein was obtained by rubbing the tail with toluene. We simplified this method by using a small box, fitting more or less tightly around the rat, with a lid that could be closed and a hole in one of the small sides for the tail. The erythrocyte number was determined by diluting the blood about 20-fold with NaCl 2.5% and measuring the extinction at 650 mμ (JONXIS, 1948)

Experimental procedure.

After discarding the first drop of blood emerging from the tail a large drop of blood was drawn up quickly into a capillary pipet, which had first been rinsed with a 1% solution of Liquoid-"ROCHE" as an anti-coagulant and which was calibrated to contain 30 μl (1 μl = 1 micro liter = $10^{-3}$ ml). When the meniscus had been adjusted, the pipet was blown out into 6 ml of NaCl 2.5%. The mixture was shaken well and used for a determination only when no clots were present. All determinations were carried out in duplicate. The extinction was measured in a Coleman Junior spectrophotometer at 650 mμ. As only comparative measurements were needed, the results were expressed as extinctions. The relative standard error of the means amounted to 3.7% (for the calculation of the standard error see under [3.4.1]).

When the extinction was plotted against the erythrocyte count for human blood a perfectly straight line was obtained.

The leucocyte number was determined in the ordinary way by diluting a blood sample 1 : 11 with TÜRK solution (10 mg gentian violet dissolved in 100 ml 1% acetic acid) in a counting pipet and counting the number of leucocytes under the microscope by means of a hemocytometer. All determinations were carried out in duplicate. The relative standard error of the means amounted to 4.5%.

The differential count of the leucocytes was made with a blood smear after staining with GIEMSA solution. In each sample 100 leucocytes were differentiated into lymphocytes, neutrophiles, eosinophiles, basophiles, young leucocytes and monocytes.

*3.2.3. Autopsy.*

Investigated were liver, kidneys, adrenals, spleen, skeletal muscle, testicles and femur. The animals were killed by decapitation without previous narcosis. The organs were fixed immediately in formaldehyde 12%, the testicles in PFUHL's solution. The organs were imbedded in paraffin and cut by means of a microtome. The femur was decalcified with 5% trichloroacetic acid. Staining was done with hematoxylin-eosine.

*3.2.4. Investigation of the teeth.*

From the heads of the decapitated animals the maxilla and the mandibula were dissected free and fixed in 10% formaldehyde. The molars were studied by means of a binocular microscope at a magnification of 15 ×. For the quantitative



appraisal of the caries the method worked out by KEYES (1944) as modified by JANSEN and LUYKEN et-al (1951) was used.

The "caries score" gives an estimate of the amount of dental tissue that has been destroyed. KEYES divided each molar into a number of unit areas. By multiplying these with either 1, 2 or 3, according to the depth of the cavity, and adding the results for all molars, he obtained a "caries score". JANSEN et al multiplied the number of unit areas with the depth of the cavity in mm. In this way a somewhat more accurate estimate of the amount of dental tissue, destroyed in the carious process, was obtained. The average height of the molars being about 2 mm, the maximal score obtained in this way (completely destroyed molar) amounted to 90. Dividing the actual score by 90 gives the "caries percentage". The incisors are not used in this calculation, as they are growing permanently and do not show caries.

The caries percentages for the different dietary groups were determined. Also the degree of dental erosion was observed. Finally histological preparations were made of some of the molars by the method of MANN as modified by DOBELL (ROMEIS, 1928).

3.3 CHEMICAL TECHNIQUES.

*3.3.1. Blood.*

1. Obtaining and handling of the blood.

Under [3.2.2] we already described the method we used to obtain a few drops of blood for studying the blood picture. The animals used for the analysis of blood and tissues were killed by decapitation and the blood collected in centrifuge tubes. We did not anesthesize the animals before decapitation, as this may cause marked changes in the blood composition. (AUSTIN, CULLEN et al 1924; MARTLAND and ROBISON, 1924).

Some experiments were carried out by ourselves to verify this. Three rats were brought under light ether anesthesia; the sinus cavernosis behind the eye was pierced by means of a glass capillary and some blood was collected. Hereafter some blood was obtained in the same way from the same rats without ether narcosis. The inorganic phosphorus content of the serum was considerably increased in the animals under narcosis (20 to 53%), while the chloride content was increased in one case only (3.4%). This is in agreement with the findings by AUSTIN, et al and MARTLAND and ROBISON.

The blood was centrifuged within 30 minutes and the serum transferred by means of a pipet to a tube that could be closed with a stopper. The rapid separation of the serum was necessary in order to prevent changes in the chloride content (VAN SLYKE, WU and MCLEAN, 1923) through the chloride shift taking place when $CO_2$ is lost as wel as in the inorganic phosphorus content (ZUCKER and GUTMAN, 1921). The serum was kept in the refrigerator and all determinations were carried out within 10 days.

2. Inorganic phosphorus.

The colorimetric method developed by SUMNER (1944), employing $FeSO_4$ as reducing agent, was used. A micromethod was worked out, using 25 μl of blood and giving a final volume of 275 μl. The measurements were made in micro-



0264

cuvettes with the Beckman spectrophotometer. Readings were taken at 700 mμ. As a routine method this procedure was not very satisfactory and was therefore abandoned later for a similar procedure employing more serum in order that a Coleman Junior spectrophotometer could be used.

Experimental procedure.

0.14 ml of serum was deproteinized with 0.50 ml of 5% trichloroacetic acid. After centrifuging for 15 min. 0.30 ml of the supernatant liquid was transferred to a colorimeter tube (8 mm diameter). Finally 0.50 ml of a 2.5% ammonium molybdate solution in 8% $H_2SO_4$ and 0.50 ml of a 10% $FeSO_4$ solution in 0.1 N-$H_2SO_4$ were added. The extinction of the resulting solution was measured in the Coleman Junior spectrophotometer against a blank containing only the reagents at 700 mμ after 30 min. At the same time some standard solutions were measured. Standard deviation of the single determination: 1.3% (see for definition and calculation under [3.4.1]). The standard deviation of the micromethod was 6.5%. Recovery: 99.8%.

*Note*: we found trichloroacetic acid to delay the formation of the colour if present in a final concentration higher than 1%. The concentration used in this procedure was just low enough to avoid a marked retardation.

3. Calcium.

The calcium content of the serum was determined according to the method of WANG (1935). After deproteinizing with trichloroacetic acid the calcium was precipitated with ammonium oxalate. The calcium oxalate was dissolved in sulfuric acid and titrated with $KMnO_4$.

Experimental procedure.

0.50 ml of serum was deproteinized with 0.50 ml of 20% trichloroacetic acid. After centrifuging for 5 min at 3000 r.p.m. 0.50 ml of the supernatant liquid was transferred to a small centrifuge tube. Then were added 0.25 ml 0.2 M acetate buffer of pH 5.0, 2 drops of 0.02% bromocresol green in 50% alcohol and 0.25 ml of 1.4% ammonium oxalate. Then 4% ammonia was added dropwise until the solution obtained a light green colour and then the tubes were stored during the night. After centrifugation (5 min. at 3000 r.p.m.) the supernatant was removed by suction. The precipitate was washed with a solution containing 20 ml concentrated ammonia in 980 ml of a mixture of equal parts of alcohol, ether and distilled water. This process was carried out three times. The precipitate was dried at 105°C during 1 hr. Finally it was dissolved in 0.50 ml of 1 N-$H_2SO_4$ by heating in a waterbath and the resulting solution titrated with 0.0025 N-$KMnO_4$ from a 2 ml BANG burette. In each series two blanks were run. The $KMnO_4$ was standardized both before and after a series of determinations against 0.05 N oxalic acid in 3% $H_2SO_4$. Standard deviation: 0.9% Recovery: 99.3%.

4. Total base.

To determine the total base content of the serum we used the ion exchange method worked out by POLIS and REINHOLD (1944). In this method a known amount of serum is absorbed in a column of a cation exchange resin (hydrogen-form). The resin is eluted with water and the eluate, containing an amount of hydrogen ions equivalent to the total base content, is titrated with alkali to a pH 7.4 against phenol red or bromothymol blue after removal of carbon dioxide by aerating. A standard consisting of a 0.15 molar NaCl solution is included in each set of determinations.

In trying out this method ourselves we found it necessary to introduce some modifications in order to obtain reliable results. When we ran blank determinations, like GORTER and VAN ROYEN (1948) did, an appreciable acidity was found in the effluent, even after prolonged washing of the columns. Therefore we preceded and followed each determination by a blank. Especially after standing overnight the blank value was high and therefore the columns were rinsed with water before a series of determinations was started until the blank value was low enough. The ion exchange resin was not discarded after each determination, as this proved to be time-consuming and needlessly costly. Each column of 140 mm with a diameter of 10 mm could be used for 30 determinations at least without regeneration. For regeneration it proved to be easiest to collect the resin of all columns (twenty in our case) and regenerate it in one batch.

In a number of control experiments we determined the accuracy of the ion exchange method for different cations and different resins. The columns were of the type described by POLIS and REINHOLD. With Dusarit[1]), a sulfonated coal, which was employed by GORTER and VAN ROYEN, we obtained high and varying blank values. With AMBERLITE[2]) IR 105 and IR 120, after sufficient rinsing, the blank values were constant and low. We therefore used only the Amberlite resins in our further experiments. First of all we found the great importance of a slow percolation rate. At a rate of 3 to 6 ml/min the result for a serum sample was 3% lower than at a rate of 0.8 ml/min (1 drop per 2 sec). We always maintained a percolation rate of 0.8 ml/min. About the same decrease occurred in the first determination after a column had run dry. Recovery for Na, K and Ca with the IR 105 and IR 120 resins:

| | | | | |
|---|---|---|---|---|
| NaCl, | 0.15 N | IR 105 | 99.8% | (standard error of the mean 0.2) |
| | | IR 120 | 97.8% | ( ,, ,, ,, ,, ,, 0.2) |
| KCl, | 0.15 N | IR 105 | 99.3% | ( ,, ,, ,, ,, ,, 0.3) |
| | | IR 120 | 98.9% | ( ,, ,, ,, ,, ,, 0.3) |
| $CaCl_2$, | 0.15 N | IR 105 | 99.5% | ( ,, ,, ,, ,, ,, 1.4) |
| | | IR 120 | 100.5% | ( ,, ,, ,, ,, ,, 1.6) |

For our further experiments we chose the IR 120 resin, which has double the capacity of the IR 105 resin and does not adsorb iron irreversibly like the latter. For some serum samples the amounts of Na and K that were not exchanged, were determined in the effluent by means of the flame photometer. This amount was for Na: 1.4% (standard error of the mean 0.2). For K and Ca the amounts were too small for determination (below 10 mg/l). These experiments proved the method to be accurate and reliable, if carried out carefully.

POLIS and REINHOLD did not determine the alkali bound to bicarbonate, because they removed all carbon dioxide before titrating. The bicarbonate was determined separately by the VAN SLYKE method. GORTER and VAN ROYEN however collected the effluent in excess alkali and titrated back with HCl against bromothymol blue. They obtained results that were in agreement with those obtained with the electrodialysis method of KEYS (ADAIR and KEYS, 1934; KEYS, 1936). When we repeated these experiments, we found that condiserable losses in carbon dioxide, varying from 3% to 40% with a solution 0.120 N in

[1]) Of the Industrieele Maatschappij Activit N.V., Amsterdam.

[2]) Of the Rohm and Haas Company, The Resinous Products Division, Philadelphia, U.S.A. We should like to express our thanks to this firm for the kind advice given and the samples placed at our disposal.





0264

NaCl and 0.030 N in $NaHCO_3$ occurred. Even the use of closed flasks, with a sodium hydroxide tube, to receive the effluent and stirring with nitrogen during the titration could not prevent this. This is probably due to adsorption of carbon dioxide by the resin, as suggested by KUNIN (1949). Probably in the experiments of GORTER and VAN ROYEN the losses of carbon dioxide were more or less compensated by the absorption of carbon dioxide by the alkali during collection of the effluent and subsequent titration. We therefore decided to titrate the effluent directly after removal of $CO_2$ by heating to the boiling point in order to obtain unequivocal results.

In a control experiment this was found to remove all carbon dioxide present. We determined the base content of a solution 0.120 N-NaCl and a solution 0.120 N in NaCl and 0.030 N in $NaHCO_3$. The result was:

0.120 N-NaCl — 0.119 N (S.E.: 0.6%)
0.120 N-NaCl, 0.030 N-$NaHCO_3$ — 0.119 N (S.E.: 0.8%).

So in our experiments only the cations bound to non-volatile acids were determined.

Experimental procedure.
0.15 ml of serum was diluted with distilled water to 3 ml. A column of Amberlite IR 120 of 140 mm length and 10 mm diameter was washed with distilled water until the effluent had an acidity below $6.10^{-5}$ N. Titrations were carried out with 0.02 N NaOH in a 2 ml. Bang burette against bromothymol blue. Before titration all solutions were heated to the boiling point. The water level in the column was brought at a mark 1 cm above the resin surface. Then the diluted serum was added quantitatively and percolated at a rate of 0.8 ml/min (1 drop in 2 sec) until the water level reached the mark; 5 ml of distilled water were added, followed by 10 and 15 ml of water. The effluent was collected in a 50 ml ERLENMEYER flask, heated to remove carbon dioxide and titrated. Again 10 ml of water were percolated and titrated. Both blank values were used to correct the titration value for the serum sample. In each set of determinations some 0.15 ml samples of 0.150 N-NaCl were run as a standard. The results were used to correct the calculated total base content of the serum samples. With a rack of 20 columns one analyst could analyze about 60 samples daily. Standard deviation: 1.2%. Recovery: 100.5%. Regeneration: each column could be used at least 30 times without regeneration. For regeneration the resin was collected and washed with water to remove some precipitated serum protein. The resin was left standing overnight with 10% HCl. The next morning it was washed and left again in contact for some hours with 10% HCl. Then it was washed again until the effluent would be neutral to bromothymol blue.

5. Alkaline reserve.

The total carbon dioxide content of the serum was determined with the manometric method of VAN SLYKE (VAN SLYKE and CULLEN, 1917; PETERS and VAN SLYKE, 1932a, p.267-286). In our experiments only a metabolic acidosis could occur, resulting in a decreased total $CO_2$ content. In that case the total carbon dioxide content of the serum is the sharpest indication for an acidotic condition. We therefore did not make determinations of the blood pH, but only carried out the manometric $CO_2$ determination of VAN SLYKE.

Experimental procedure.
The animals were decapitated and the blood was collected in centrifuge tubes, that were



closed immediately. The tubes were centrifuged for 10 min at 3500 r.p.m. and the serum was transferred to paraffined tubes with rubber stoppers. These were stored at 0°C until needed for a determination. Then the serum was saturated in the tubes with a mixture of 95% air and 5% $CO_2$ (from a high-pressure gas container) at room temperature. Of this serum 0.2 ml was used for each determination, which was carried out in the ordinary way.
Standard deviation: 2.7%.

6. Chloride.

The iodometric determination worked out by VAN SLYKE and HILLER (1947) was used. In this method an excess of silver iodate is added. The chloride ions exchange against an equivalent number of iodate ions because the solubility product of silver chloride is lower than that of silver iodate. Addition of excess potassium iodide to the acidified solution liberates iodine according to the reaction equation:

$$IO_3^- + 5\,I^- + 6\,H^+ \rightarrow 3I_2 + 3H_2O.$$

The iodine is titrated with sodium thiosulfate. The advantage of this method is that each equivalent of chloride uses 6 equivalents of thiosulfate, which makes it a sensitive and accurate determination of chloride.

Experimental procedure.
To 50 µl of serum in a small centrifuge tube 1 ml of phosphotungstic acid (6 g $Na_2Wo_4.2H_2O$ dissolved in 1 l of 0.15 M-$H_3PO_4$) and 15 mg of $AgIO_3$ were added. The tube was closed and shaken by hand for 40 sec and centrifuged for 5 min at 3500 r.p.m. Of the supernatant 0.5 ml was transferred by pipet to a tube of 90 mm length and 12 mm diameter. After adding 50 mg KI and two glass beads the solution was immediately titrated with 0.01 N thiosulfate from a 2 ml Bang burette. When the yellow colour was almost gone, two drops of a 1% starch solution were added and the titration was continued until the blue colour just disappeared. Blanks were run in each series, while as a standard a 50 µl sample of 0.1 N-KCl was determined.
Standard error: 0.4%. Recovery: 98.2%.

7. Alkaline phosphatase.

The alkaline phosphatase which decomposes organic phosphorus compounds like hexose phosphate into inorganic phosphate, is supposed to play an important role in the ossification. In rickets the serum alkaline phosphatase activity for example is increased markedly. We thought it desirable therefore to include the determination of alkaline phosphatase in our program. The rapid and elegant method of BESSEY, LOWRY and BROCK (1946) was used. In this method the amount of p-nitrophenolate formed from p-nitrophenylphosphate by the enzyme in a certain time at a specified temperature and pH is measured. The substrate, p-nitrophenylphosphate, is colourless, but the nitrophenolate is yellow (absorption maximum at 400 mµ). So it is only necessary to incubate the serum with the buffered reagent, stop the reaction by addition of alkali and measure the extinction. To correct for the colour of the serum a drop of acid is added – which converts the yellow nitrophenolate into colourless nitrophenol – and the extinction is measured again. The phosphatase activity is expressed in "millimole units". A millimole unit is the phosphatase activity which will



0264

liberate 1 mM of nitrophenol per l of serum per hour. As the serum of rats has a considerably higher phosphatase activity than human serum, it was found desirable to dilute the serum three times.

Experimental procedure.

Of 1:3 diluted serum 20 µl was pipetted into a small colorimeter tube. The tubes were cooled to 0°C by immersion in ice water and to each tube was rapidly added 0.2 ml of the reagent. The reagent consisted of equal parts of a solution 0.1 M in glycine, 0.001 M in $MgCl_2$ and 0.085 M in NaOH and a solution of 0.4% disodium p-nitrophenyl phosphate in 0.001 N-HCl, both stored at 1°C. The pH of the reagent was adjusted at 10.3 before use. The tubes were then immersed in a water bath at 38°C and remained there for 30 min, when they were put back in ice water. To each tube 2 ml of 0.02 N-NaOH were added and the extinction at 415 mµ measured with the COLEMAN Junior spectrophotometer. Then one drop of 5 N-HCl is added to each and a second reading taken. In each series of determinations standards containing 20 µl of p-nitrophenol solutions containing 1, 2, 4 and 6 mM per l (equivalent to activities of 2, 4, 8 and 12 mM-units) and blanks with distilled water instead of serum were run.

Standard deviation: 10%.

### 3.3.2. *Liver and muscle.*

1. Sampling.

Liver and muscle are the soft tissues that were investigated for possible changes in mineral content. The complete liver as well as about 3 to 4 g of the skeletal muscle of the hind leg were dissected from the decapitated animal and stored in closed bottles at 1°C. The fresh weight of the liver was determined in each case. All samples used in the determinations were weighed within three days after death in order to minimize the effects of post-mortem changes. The samples for the total phosphorus, total base, potassium and sodium determinations were dried overnight at 105°C. and could then be kept indefinitely. Those for the chloride determination had to be ground and extracted and were therefore used immediately.

2. Total phosphorus.

In order to determine total phosphorus in tissues it is necessary to destroy the organic matter and bring the phosphorus into solution as inorganic phosphate. Dry ashing leads to considerable losses by formation of pyrophosphate and reactions with the enamel of the porcelain crucibles, which would have had to be used in our large series of analyses. Therefore a wet-ashing method was chosen. A great drawback of the NEUMANN (1902) method of digestion, employing sulfuric acid and nitric acid, is the fact that every sample has to be handled individually and this takes at least twenty minutes. As we would have to carry out 50 to 60 ashings at one time, this method could not be used. Moreover there is always the danger of dehydration of phosphoric acid to pyrophosphoric acid at the rather high temperature of digestion (up to 300°C.). The nitric acid-perchloric acid digestion has neither of these drawbacks. There is however the possibility of explosions. We therefore kept the following rules:



the digestion was carried through as far as possible with the fuming nitric acid alone before adding the perchloric acid; the samples were dried before use and the perchloric acid used had a $HClO_4$ content of at least 60% by weight. In over 1200 digestions we did not have a single accident. As a precaution every week the hood used was rinsed out completely with water to remove traces of condensed perchloric acid, which otherwise may give explosions, when absorbed by wood and other organic materials. The resulting digest could be used without previous neutralization for the colorimetric phosphorus determination according to SUMNER (1944).

Experimental procedure.

A sample of 0.7 to 0.8 g of tissue was dried overnight at 105°C. on a watch-glass. It was then transferred quantitatively to a 25 ml KJELDAHL flask and 15 ml of fuming nitric acid as well as two glass beads were added. Preferably the mixture was left standing overnight. Then the flasks were placed on racks with micro-burners and heated. The mixture was brought to boiling, which was continued until an almost clear solution of about 10 ml was obtained. After cooling 2 ml of $HClO_4$ 60% were added. The solution was boiled again until about 1.5 to 2 ml of a colorless solution remained. This was transferred quantitatively to a 25 ml volumetric flask and was diluted to the mark. Of this solution 0.25 ml was brought together with 2 ml of a 1.6% ammonium molybdate solution in 5% $H_2SO_4$ and 2 ml of a 7% $FeSO_4$ solution in 0.07 N-$H_2SO_4$ in a colorimetric tube. After 5 min the extinction at 700 mµ was read. With each set of determinations some standard solutions were measured.

Standard deviation of the phosphorus determination alone: 2.0%. Including digestion: 3.9%

3. Total base.

Employing the same ion exchange method used for the total base determination in serum, we worked out a total base determination for tissues. A tissue sample of about 1 g was ashed, the ash dissolved in dilute HCl and the excess acid neutralized with a known amount of NaOH against bromothymol blue. Neutralization of the sample is necessary for two reasons: *1* the ion exchange process is reversible and therefore more or less complete only when the solution fed to the column is about neutral; *2* the acidity of the effluent should be due entirely to the hydrogen ions given off by the resin in exchange for cations. The total base content of the neutralized solution was determined by ion exchange. In calculating the total base content of the ash the amount of Na ions added during neutralization was subtracted.

Experimental procedure.

A fresh tissue sample of about 1 g was dried in a porcelain crucible at 105°C during the night. The crucible was placed in an electric furnace at about 500°C for 5 to 7 hours. Having been taken out of the furnace, the crucible was left to cool in an desiccator. Then 4 ml of 0.075 N-HCl were added and the ash — still containing particles of carbon — was made to dissolve by means of stirring with a glass rod having a flat end. The crucible was left for 1 to 2 hours in an desiccator containing 0.075 N-HCl. Then 0.25 ml of the solution was transferred to a tube and titrated with 0.02 N-NaOH against bromothymol blue from a 2 ml BANG burette. After dilution to 3 ml with distilled water the solution was percolated over a cation exchange resin as described for serum (see under [3.3.1.4]). The effluent was titrated again with 0.02 N-NaOH and the difference between the two titration values used as a measure of the total



0264

base content of the ash. Samples of 0.150 N-NaCl served as a standard. Standard deviation for determinations of the same ash solution: 1.3%. For different samples of the same tissue: 6.9%.

The difficult part of this determination was the dry ashing of the sample and the solution of the resulting ash. If the temperature of ashing had been too high, the resulting ash adhered to the crucible and did not dissolve easily. If the temperature was too low, the particles of carbon remaining may occlude a marked amount of ash.

The large series of determinations, we had to carry out, made it impossible to use platinum crucibles. Silica crucibles did not improve the accuracy. Addition of a drop of sulfuric acid to the ash in order to oxidize the carbon and heating on the BUNSEN flame gave no improvement either. Wet ashing with nitric acid and perchloric acid was tried next. The solutions used for the phosphorus determination (see preceding section) were about 0.006 N in cations and 0.5 N in hydrogen ions (remnants of the acids used for digestion). Therefore it was imperative to get rid of the acid. Evaporation to dryness without spattering proved to be possible only in the following way: 5 ml of the solution were transferred to a crucible and were heated from above by an inverted BUNSEN burner. In this way the solution evaporated without boiling. The dry residue was dissolved in 2 ml of distilled water, neutralized with 0.02 N-NaOH and used for a total base determination. With porcelain crucibles results several times too high were obtained; with silica crucibles results that were sometimes too high, sometimes too low. Therefore also this method had to be abandoned.

In order to obtain more reliable results we changed the total base determination for a flame photometric determination of K and Na in the solution obtained by digestion with nitric acid and perchloric acid (see next section, [3.3.2.4] in the later part of our investigation.

4. Potassium and sodium[1]).

The solution of a tissue sample obtained by digestion with nitric acid and perchloric acid was used for the flame photometric determination of sodium and potassium. For the principle of the flame photometer we may refer to the review by BARNES et al (1945).

The instrument used in our determinations comprised a flame and sample atomizing system and two separate optical systems for simultaneous measurement of the Na and the K content. Sets of two CORNING glass filters were used (green + orange for Na and red + violet for K). Both optical systems contained a barrier-layer photocell connected to a light-spot galvanometer. The flame was fed by buta gas and compressed air. Samples of 5 to 10 ml, containing 2 to 3 meq/l of potassium and 0.7 to 1.0 meq/l of sodium, were used. About the same amounts of nitric and perchloric acid as were present in the samples were added to the blank and the standard solution which contained Na and K in approximately the same ratio as in the samples. Measurements were made on samples and standard alternatively, so as to eliminate the effects of possible changes in the sensibility of the apparatus. The concentrations of the samples were read from graphs constructed from the readings for the standards. Standard deviation for potassium: 2.8%. For sodium: 5.6%.

[1]) The flame photometric determinations were carried out by Mr. L. A. DE VRIES, biochemist at the Biochemical Laboratory of the University Clinic of Internal Diseases (director: Prof. Dr. J. G. G. BORST), Binnengasthuis, Amsterdam, to whom our sincere thanks are due.



5. Chloride.

In view of the findings of BANUS and KATZ (1927) and BANUS et al (1929) (see under [2.3]) we thought it desirable to determine the chloride of the soft tissues beside the serum chloride. We wanted to use the iodometric method of VAN SLYKE and HILLER (1947) already described for serum (see under [3.3.1.6]). Therefore it was necessary to obtain a solution containing all of the chloride.

We tried the dry ashing method described by STOLTE (1911), using a porcelain crucible and an addition of sodium carbonate as well as an ethanolic magnesium acetate solution (WINTON and WINTON, 1945). In all cases we found considerable losses of chloride. Then we tried to dissolve the sample by boiling with 3 to 10% KOH. The resulting solution was neutralized with sulfuric acid and used for a chloride determination. Results obtained in this way were too high, the more so the higher the concentration of the KOH used. Neutralization with nitric acid gave no improvement. Finally, extraction of the ground tissue with 20% trichloroacetic acid was attempted and this method gave reliable results. A blendor according to POTTER and ELVEJEHM (1936) driven by an electric motor was used for grinding the tissue sample.

Employing the trichloroacetic acid extraction method an experimental procedure could be worked out. The phosphotungstic acid used by VAN SLYKE and HILLER to precipitate the serum proteins gave no complete precipitation of the tissue proteins and therefore could not be used. The trichloroacetic acid did not give off an appreciable amount of chloride ions as shown by blank determinations. The blank values were reproducible and not too high. Therefore trichloroacetic acid could be used instead of phosphotungstic acid.

Experimental procedure.

A fresh tissue sample of about 1 g was ground in a motor-driven POTTER-ELVEJEHM blendor with 5 ml of 20% trichloroacetic acid. The resulting suspension was washed into a 25 ml centrifuge tube with distilled water and was centrifuged for 5 min at 3500 r.p.m. The supernatant fluid was transferred completely to a 25 ml volumetric flask and diluted to the mark. Of the resulting solution 2 ml were shaken with 15 mg silver iodate in a small centrifuge tube during 40 seconds. After centrifugation 1 ml of the supernatant fluid was transferred to a tube of 100 mm length and 12 mm diameter. A few drops of 2 N-$H_2SO_4$, 50 mg of KI and two glass beads were added. Then the solution was titrated with 0.01 N thiosulfate against starch solution. For the blank determination 5 ml of 20% trichloroacetic acid were diluted to 25 ml and the resulting solution treated as described above for the extract.

Standard deviation: 3.2%.

### 3.3.3 *Kidneys.*

1. Water and ash content.

After decapitation of the rat both kidneys were taken out and kept in stoppered bottles in the refrigerator. One of them was used for the alkaline phosphatase determination. The other one for the determination of the water and ash content.

Experimental procedure.

The kidney was put in a porcelain crucible that had been heated at 500 to 600°C for some



0264

hours and weighed to 0.1 mg. The crucible was weighed again. Then it was heated at 105°C during the night and weighed again after cooling in a desiccator. Finally the dried kidney was ashed in the electric furnace at 550°C during the night. After cooling in a desiccator the crucible was weighed again.

In this way were obtained the fresh weight, the water content and the ash content of the kidney.

2. Alkaline phosphatase.

The kidney shows a high alkaline phosphatase content, higher than most other tissues. The function of this enzyme is the conversion of organic phosphates into inorganic phosphates and is probably related to the excretory function of the kidney. It therefore seemed desirable to us to investigate the possible effect of the acid diet upon the renal phosphatase activity. This activity was determined in an extract of the kidney with the method described by BESSEY, LOWRY and BROCK (1946), which was also used for the serum phosphatase activity. The extract was prepared according to directions given by NEUMANN (1948) with some modifications.

Experimental procedure.

The fresh kidney was weighed and ground in a motor-driven POTTER-ELVEJEHM blendor with 5 ml of distilled water. The tissue suspension was washed into a 25 ml centrifuge tube and kept for some hours at room temperature after addition of 1 ml of toluene. After centrifugation the supernatant liquid was diluted to 40 ml and stored in the refrigerator until needed for the determination. The same procedure as described for serum (see under [3.3.1.7]) was used. Of the extract 20 μl were taken for each determination.

Standard deviation: 14.3%.

*3.3.4 Bone.*

1. Ash content.

In our experiments we also wanted to investigate the bones of the rats in order to notice a possible decalcifying action of the acid diets. ROCHE and MORGUE (1939) in their investigations found the skeleton to act as one unified structure in its metabolism. Therefore we decided to investigate the ash content as well as the Ca and P content of one bone only. We used the tibia, this being one of the largest bones and easily accessible. Before ashing it was extracted with an ether-ethanol mixture to remove the phospholipids.

Experimental procedure.

The tibia was cleaned of adhering muscle and tendons as far as possible and was then extracted with a boiling mixture of 1 part of ether and 3 parts of ethanol during 24 hours. After drying for 1 hour at 105°C the tibia was weighed in a porcelain crucible that had been heated at 600°C for some hours and weighed. It was then ashed during the night in an electric furnace at 600°C. After cooling in a desiccator the crucible was weighed again. The resulting ash was used for the determination of calcium and phosphorus.

2. Phosphorus.

By dissolving the bone ash in hydrochloric acid a solution was obtained, which could be used for the determination of the phosphorus content of the bone. For this determination again the method of SUMNER (1944) was used.



Experimental procedure.

The ash of the tibia was transferred quantitatively to a 50 ml volumetric flask and 5 ml of concentrated hydrochloric acid were added. After complete solution of the ash enough water was added to bring the volume to 50 ml. Of this solution 20 to 50 μl, according to the weight of the bone, were brought together with 2 ml of a 1.6% ammonium molybdate solution in 5% $H_2SO_4$ and 2 ml of a 7% $FeSO_4$ solution in 0.07 N $H_2SO_4$ in a colorimeter tube. The extinction was read in a COLEMAN Junior spectrophotometer at 700 mμ after 5 min.

Standard deviation: 1.9%. Recovery: 97.0%.

3. Calcium.

For the determination of the calcium content of the bone the same ash solution as used for the estimation of phosphorus could be employed. The method of WANG (1935) was used, simplified insofar as deproteinization was not necessary here.

Experimental procedure.

0.15 ml of the ash solution (see preceding section) was transferred to a small centrifuge tube. Added were 0.25 ml of a 0.4 M acetate buffer of pH 5.0, 2 drops of bromocresol green and 0.25 ml of 1.4% ammonium oxalate. Finally 4% ammonia was added dropwise until the color turned to green. After this the same procedure as described for serum (see under [3.3.1.3] was used. In each series of determinations two blanks were included.

Standard deviation: 1.1%. Recovery: 98.1%.

*3.3.5 Urine.*

1. Collection and preservation.

In the metabolism experiments we had to collect the urine as completely as possible and with as little changes in the composition as possible. The large cecum of the rat brings the maximal interval between intake and fecal excretion of the food to seven days, although the greatest part is excreted within 24 hours. Therefore it was desirable to have collection periods of seven days. The rats were kept in special metabolism cages (fig. 1). Separation of feces and urine was obtained by means of the glass bulb. A thin layer of paraffin and a few crystals of thymol were used to prevent evaporation and growth of microorganisms in the urine.

We found this to be a very satisfactory way of preservation of the urine. In special experiments we found no change in the pH and the titratable acidity and a loss of ammonia of less than 2% in urine treated in this way and standing for a week.

Notwithstanding the use of food containers, specially designed to avoid spilling of food, some of the rats were still able to scatter some food through the bottom of the cage by taking a mouthful of food and eating it in the middle of the cage. The amounts were too small to cause a considerable error in the measurement of the food intake (below 0.1 g per day generally). Another possibility however would be contamination of the urine by solution of the salts contained in the food. This would increase the mineral content of the urine. But it could be proved by calculation that, even assuming complete solution of the salts, the error in all determinations but the Ca determination



0264

FIG.1

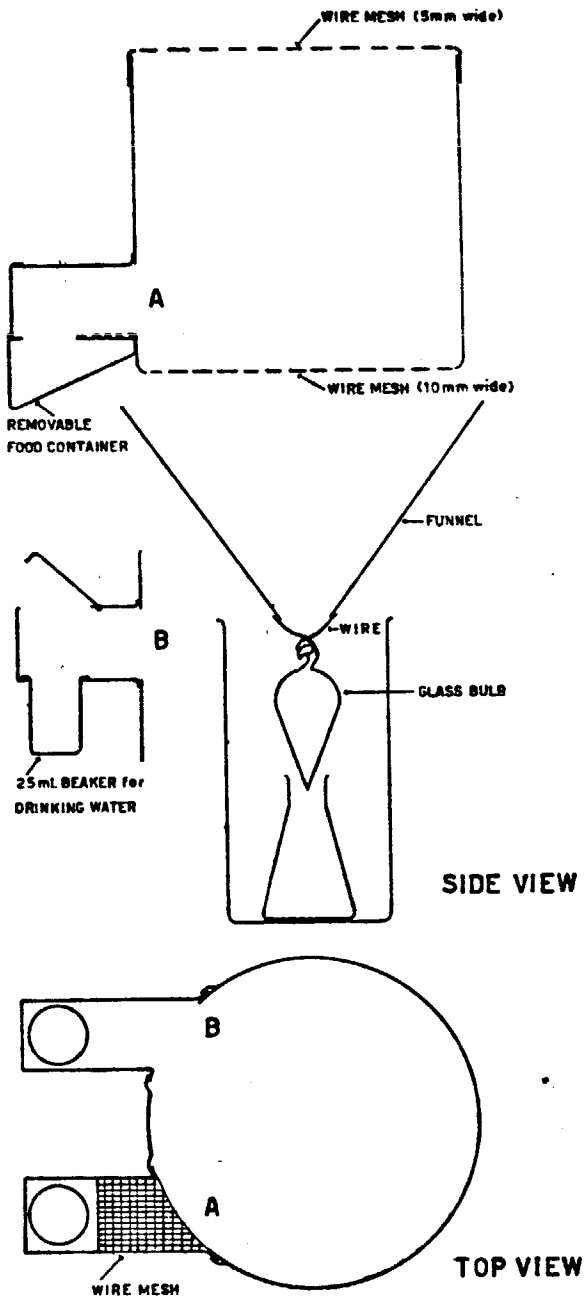


DIAGRAM OF METABOLISM CAGE

would be unimportant. As the Ca content of the urine was very low (about 0.15 mg/ml or 1.1 mg/d), the increase in this case could be considerable.

We could confirm this in a model experiment, in which 10 ml urine of pH 5.4 were filtered over 200 mg of food placed on cottonwool in a glass funnel. From 35 to 65% of the calcium present in the food was dissolved by the urine, resulting in an increase of the urinary calcium content from 25 to 50%.

For Ca-balance experiments this was not important however, as about 98% of the calcium excretion was fecal. However the results for the urinary Ca excretion should be treated with some reserve.

Experimental procedure.

A rack, bearing 40 of the metabolism cages shown in fig. 1, was used. The animals were put on the cages after being weighed and the food intake was recorded daily (including Sundays). When after some days both food intake and weight were normal the collection of the excreta was started. The funnels were covered with a thin layer of solid paraffin on the inner side to shorten the time between shedding and receiving the urine and to minimize the loss of urine. 100 ml erlenmeyer flasks containing 3 to 5 ml of liquid paraffin and a few crystals of thymol were used for the collection of the urine, while 1 l beakers served for the collection of the feces. After a period of 7 days the collected excreta were removed and clean funnels, bulbs, flasks and beakers substituted. The urine was centrifuged to remove the insoluble material. The supernatant liquid was transferred to stoppered flasks after determination of the volume. The samples were stored in the refrigerator.

2. pH and titratable acidity.

Of each urine sample we determined the pH and the titratable acidity. For the first determination a BECKMAN pH meter was used, which was standardized before each series of determinations against a buffer of pH 7.0 It was found possible to attain a reproducibility of 0.02 pH units. The titratable acidity was determined according to the method of HENDERSON and PALMER (1914), which was modified by us. In view of the low Ca content of rat urine it was not necessary to use the method of FOLIN (1903), which removes the Ca first.

Experimental procedure.

0.1 ml of urine was transferred to a tube and diluted to about 1 ml. After adding 1 drop of a 2% neutral red solution in 50% ethanol the solution is heated to the boiling point and titrated with 0.01 N-NaOH from a 2 ml BANG burette. A color standard of pH 7.4 was used for comparison, containing 0.1 ml of a mixture of 9.15 ml of 0.1 M citric acid and 90.85 ml of 0.2 M-$Na_2HPO_4 \cdot 2H_2O$, which was diluted to 1 ml and to which 1 drop of neutral red was added.

Standard deviation: 1.5%.

3. Ammonia, urea and total nitrogen.

Ammonia and urea were determined with the microdiffusion technique developed by CONWAY (1947). Instead of the pyrex glass unit we used a porcelain unit[1]) of the same dimensions as the standard unit (no. 1) described by CONWAY. This unit enabled us to observe better the endpoint of the titration and moreover it could be made more cheaply than the pyrex glass unit. A water

[1]) This unit was manufactured according to our directions by N.V. Ramie Union, Enschede, Netherlands.



0264

soluble fixative on the basis of carboxymethylcellulose (BONTING, 1951) was used, which can be prepared more easily than the gum tragacanth or gum arabic fixatives described by CONWAY. BORIC acid was used as an ammonia adsorbent.

Experimental procedure.

Into the inner chamber of the unit 1 ml of boric acid absorbent was pipetted. The absorbent was prepared by dissolving 20 g of boric acid into 200 ml of ethanol and 700 ml of distilled water, to which 50 ml of a mixed indicator containing 0.033% of bromocresol green and 0.066% of methyl red in ethanol, were added. Then 0.1 ml of urine were pipetted into the outer chamber. The lid smeared with fixative was placed in position and 0.5 ml of saturated $K_2CO_3$ pipetted into the outer chamber. The unit was closed quickly and the urine and $K_2CO_3$ mixed by tilting. It was then kept in an incubator at 37°C during 1 hour. One blank determination was included in each series. Finally the contents of the central chamber were titrated with 0.01 N-$H_2SO_4$ from a 2 ml BANG burette, the blank serving as a comparison. Cleaning of the units was done by washing with tap water and subsequently soaking them for 15 min. in 0.005 N-$H_2SO_4$. Then they were rinsed with distilled water and left to dry.

Standard deviation: 2.1%.

To determine the urea content of the urine this was incubated with urease and the resulting ammonia was determined as described above. In this way the sum of urea and ammonia was found.

Experimental procedure.

Into the inner chamber 1 ml of boric acid absorbent was pipetted. Into the outer chamber 25 $\mu$l of urine and 0.2 ml of urease solution (freshly prepared by dissolving 50 mg of a commercial urease preparation into 10 ml of distilled water) were pipetted. After closing the unit it was placed for 30 min in an incubator at 37°C. Then 1 ml of saturated $K_2CO_3$ was added and the unit kept at 37°C for 1 hour. Titration was carried out with 0.02 N-$H_2SO_4$.

Standard deviation: 0.8%.

Total urinary nitrogen was determined by means of the KJELDAHL method. Standard deviation: 1.0%.

4. Phosphorus.

The phosphorus content of the urine was determined by means of the method of SUMNER (1944) with previous deproteinization by means of trichloroacetic acid. In this way only inorganic phosphorus was determined. But as this comprises in general 95 to 99% of the total urinary phosphorus, this made only a small difference for the balance experiments. On the other hand the determination took much less time and the results could tell us more about the phosphoric acid excretion, by means of the phosphate shift. Deproteinization was necessary as rat urine always contains a certain amount of protein.

Experimental procedure.

50 $\mu$l of urine were deproteinized with 1 ml of 20% trichloroacetic acid in a small centrifuge tube. After centrifugation 50 $\mu$l of the supernatant liquid was transferred to a colorimeter tube; 2 ml of 1.6% ammonium molybdate in 5% $H_2SO_4$ and 2 ml of a 7% $FeSO_4$ solution in in 0.07 N-$H_2SO_4$ were added and after 5 min. the extinction was measured in the COLEMAN Junior spectrophotometer at 700 m$\mu$.

Standard deviation: 1.4%. Recovery: 100.7%.

5. Calcium.

Although rats excrete only a very small amount of calcium through the kidneys (about 2% of the total calcium excretion), we found it desirable to determine urinary calcium in order to see if the acid diet would increase the ratio urine Ca: fecal Ca markedly. Before precipitation of calcium oxalate according to WANG (1935) the urine was deproteinized.



Experimental procedure.

0.5 ml of the urine was deproteinized with 0.5 ml of 20% trichloroacetic acid in a small centrifuge tube. After centrifugation during 5 min at 3500 r.p.m. 0.5 ml of the supernatant liquid was transferred to another centrifuge tube. Then the reagents were added as described for the determination of calcium in bone ash (see under [3.3.4.3]). The usual procedure was followed. Titration was carried out with 0.0025 N-$KMnO_4$.

Standard deviation: 2.1%. Recovery: 102.9%.

6. Total base.

The total base content of the urine was determined by means of ion exchange. As the urinary pH was generally between 5.4 to 6.0, it was necessary to neutralize the urine first. The amount of Na added for neutralization was subtracted from the result to find the original total base content.

Experimental procedure.

50 $\mu$l of urine, after addition of one drop of bromothymol blue, were neutralized with 0.02 N-NaOH from a 2 ml BANG burette. After dilution to 3 ml this solution was handled as described for serum (see under [3.3.1.4]).

Standard deviation: 1.7%.

7. Chloride.

The chloride content of the urine was determined in some experiments in order to see if the acid diets would have any effect on the excretion of chloride. The method of VAN SLYKE and HILLER (1947) was used.

Experimental procedure.

This was exactly the same as described for serum (see under [3.3.1.6]), 20 $\mu$l of urine were used.

Standard deviation: 1.6%. Recovery: 95.5%.

8. Citric acid.

The citric acid content of the urine was determined for the animals receiving added citric acid as well as for the animals of the control group in order to see how much of the citric acid was excreted unchanged in each case. The method of WEIL-MALHERBE and BONE (1949) was used. The citric acid was converted to pentabromoacetone by means of bromine and ammonium vanadate and was subsequently extracted with heptane. The heptane extract was shaken with a sodium sulfide solution resulting in a yellow colored water layer of which the extinction was measured.

Experimental procedure.

0.25 to 1.0 ml of urine (dependent on the dietary group) were pipetted into 25 ml centrifuge tubes with glass stoppers and diluted to 5 ml. After addition of 5 ml of 27 N-$H_2SO_4$ from a buret the solutions were shaken and cooled to room temperature. Then 2 ml of a saturated bromine solution in water and 3 ml of a 2% ammonium vanadate solution (heated before use to dissolve all of the salt) were added from burets. Then the tubes were closed, shaken and kept for 20



0264

min at 50°C in a water bath. The formation of pentabromoacetone was visible by a slowly increasing turbidity. The tubes were cooled to room temperature. To the blank as many (about 6) drops of 5% sodium thiosulfate were added as needed to make the smell of bromine disappear. One drop was added in excess. The same number of drops was added to the other tubes. The color turned to green. After shaking and cooling the tubes again, 5 ml of heptane (b.p. 97-98°C) were added. The tubes were closed again and shaken for 3 min. in a rocking apparatus. The layers were separated by means of centrifugation for 5 min. at 3500 r.p.m. Then 2 ml of the heptane layer were pipetted carefully into clean tubes of the same design, containing already 5 ml of a freshly prepared solution of 4 g of $Na_2S.\ 9H_2O$ in 100 ml of distilled water. The tubes were rocked during 1 min., the upper layer was removed quickly by suction and about 3 ml of the water layer were pipetted into colorimeter tubes. The extinction was read in a COLEMAN Junior spectrophotometer at 450 m$\mu$. against the blank, which should have the same extinction as the sodium sulfide solution. From the results of determinations with known amounts of citric acid, run with each series of analyses, a graph was constructed, from which the concentrations of the urine samples were read.

Standard deviation: 3.2%. Recovery: 102.5%.

### *3.3.6 Feces.*

1. Collection and preparation.

The collection of the feces and the separation from the urine was accomplished by means of the metabolism cage shown in fig. 1 and discussed under [3.3.5.1]. The feces collected in this way in periods of 7 days were transferred to 250 ml glass beakers and were dried overnight at 105°C. The dried feces were weighed and ground to a fine powder.

2. Phosphorus.

The total phosphorus content of the feces was determined by ashing the feces with nitric acid and perchloric acid, as described for liver and muscle under [3.3.2.2], and using the digest for the phosphorus determination according to SUMNER (1944).

Experimental procedure.

250 mg of the dried and powdered feces were transferred to a 25 ml KJELDAHL flask by means of a dry funnel and digested with 15 ml of fuming nitric acid and 1.5 ml of 60% perchloric acid, as described under [3.3.2.2]. The digest was transferred to a 25 ml volumetric flask and diluted to the mark. Of the resulting solution 50 $\mu$l were used for the phosphorus determination, as described for liver and muscle (see [3.3.2.2]).

Standard deviation for the phosphorus determination alone: 2.4%. Including digestion: 4.3%. Recovery: 103.1%.

The total phosphorus content of the diets was determined according to the same procedure, 1 g samples being used.

3. Calcium.

In the feces digest used for the phosphorus determination calcium was also determined, employing the procedure of WANG (1935). Notwithstanding the content of oxidizing acids no difficulty was found in bringing the pH to 5.0 on bromocresol green, as was encountered by HITES and ACKERSON (1950).



Experimental procedure.

0.5 ml of the diluted feces digest (see under [3.3.6.2]) were pipetted into a small centrifuge tube. Then there were added 0.25 ml of the acetate buffer of pH 5.0, 2 drops of bromocresol green and 0.25 ml of 1.4% ammonium oxalate. 4% ammonia was added dropwise until a green color resulted. The procedure after this was the same as used for urine [3.3.5.5] and bone [3.3.4.3]. Titration was carried out with 0.005 N-$KMnO_4$.

Standard deviation for the calcium determination alone: 0.6%. Including digestion: 3.9%. Recovery: 102.1%.

The calcium content of the diets was determined according to the same procedure.

4. Total nitrogen.

The total nitrogen content of the feces was determined with the KJELDAHL method. In view of the fecal ammonia content it would have been necessary to digest the feces without drying. The amount of feces collected in 7 days was too large to be digested entirely .Moreover part of the feces had to be used for the determination of calcium and phosphorus. Taking a homogeneous sample of the wet feces was not possible, the more so as the consistency of the feces (dry and hard granules) was not suitable to make a paste by addition of water. Drying the feces with some hydrochloric acid resulted in a resinous mass, which could not be ground and of which no representative samples could be obtained. Through analysis of a dry and a wet sample we found the loss of nitrogen on drying of the feces to be to 5 to 7% of the total nitrogen excretion. We decided to accept this inaccuracy in the determination of the nitrogen balances. So the total nitrogen content of the dried and powdered feces was determined.

Standard deviation: 1.1%.

### *3.3.7 Experiments with radioactive phosphorus.*

1. General remarks.

In the experiments with radioactive phosphorus ($P^{32}$) a carrier-free preparation of $Na_2HP^{32}O_4$, obtained from the British Atomic Energy Research Establishment, Isotope Division at Harwell, England was used. It was diluted with physiological saline to contain 200 microcuries ($\mu$C) per ml in the first experiment and 500$\mu$C per ml in the last experiment. Of these solutions 0.1 ml was injected intraperitoneally into each of the animals. 0.1 ml dissolved in 100 ml of a 0.1% $Na_2HPO_4$ solution served as a standard.

Activity measurements were carried out using a GEIGER MULLER counting tube and an electronic scaling circuit with a scaling factor of 32 followed by a mechanical register, both manufactured by PHILIPS, Netherlands. The self-quenching counting tube was shielded with lead. The background counting rate amounted to 13 counts per min (cpm). The tube was operated at 1000 V. and had a recovery time of less than 100 microsec.



0264

Before and at the end of each series of measurements the background counting rate was determined. The samples were prepared to have an activity below 2000 cpm in order to avoid coincidence losses. The counting time was chosen in such a way that between 1000 and 5000 counts were registered. All counts were made in duplicate. The standard error of the mean was equal to or below 2.7% (including the standard deviation of the background counting rate).

2. Endogenous fecal phosphorus excretion.

The first experiment with radioactive phosphorus we carried out was meant to see if there were any differences in the endogenous fecal phosphorus excretion on the different diets. The procedure, first used by HEVESY, HAHN and REBBE (1939) and further investigated by KJERULF-JENSEN (1941) was employed. After intraperitoneal injection of 20 $\mu$C of radioactive phosphate we waited seven days and then urine and feces were collected in two periods of seven days. For each period the specific activities (activity per mg of phosphorus) of the feces and of the urine were determined, their ratio giving the percentage of endogenous phosphorus in the total fecal phosphorus excretion.

The phosphorus contents of urine and feces were determined as described under [3.3.5.4] and [3.3.6.2.] Only 10% instead of 20% trichloroacetic acid was used to deproteinize the urine, the final trichloroacetic acid concentration of the extract then being about the same as in the extract used for radioactive assay. The latter extract was prepared by mixing equal volumes of urine and 20% trichloroacetic acid. To the supernatant liquid enough $Na_2HPO_4$ solution was added to bring the total phosphorus content to 28 mg. Then magnesia mixture, ammonia and ammonium chloride were added to precipitate magnesium ammonium phosphate. The precipitate was filtered, washed, dried and ashed. The resulting magnesium pyrophosphate was assayed.

Experimental procedure.

To 1 ml of urine 1 ml of 20% trichloroacetic acid was added. After centrifugation 2.0 ml of 6.4% $Na_2HPO_4$, 2 ml concentrated ammonia, 5 ml of saturated ammonium chloride and 5 ml of magnesia mixture containing 5.5 g $MgCl_2$. $6H_2O$, 10.5 g $NH_4Cl$ and 0.2 ml concentrated HCl were added to 1 ml of the supermatant liquid. After standing for at least an hour the precipitate was filtered, washed with 2.5% ammonia, dried and ashed overnight in a porcelain crucible in a furnace at 600°C. The ash was transferred to a special aluminium planchet, fitting in the manual sample changer of the counting tube. Two samples of 0.16 ml of the standard solution were prepared in the same way. Samples and standards were counted in duplicate.

In the second period 4 ml of urine with 1.2 ml of 6.4% $Na_2HPO_4$ were used for precipitation of phosphate.

Standard deviation: 6.1%

The feces were digested with nitric acid and perchloric acid. The digest was used both for the phosphorus determination and for the radioactive assay.

Experimental procedure.

1 g of the dried and powdered feces were digested with 15 ml of fuming nitric acid and 2 ml



of 60% perchloric acid. The digest was transferred to a 50 ml volumetric flask and diluted to the mark. To 10 ml of this solution were added 1.5 ml of 6.4% $Na_2HPO_4$, 4 ml of ammonia, 5 ml of ammonium chloride and 5 ml of magnesia mixture. The precipitate was treated as described for urine.

In the second period 20 ml of the digest with 1.0 ml of 6.4% $Na_2HPO_4$ were used.

Standard deviation: 5.7%.

3. Phosphorus retention of the bone.

The phosphorus retention of the tibia in relation to the diet was determined in the second investigation with radioactive phosphorus. A procedure worked out by ARMSTRONG (1947) was used in which one hindleg was amputated at the beginning of the experiment, 24 hours after injection of the radioactive phosphate, to serve as an internal reference standard. After six weeks the animal was killed and the other tibia obtained. By comparison of the specific activities of both tibiae the retention of phosphorus by the bone was found.

Experimental procedure.

24 hours after intraperitoneal injection of 50 $\mu$C of radioactive phosphate a urine sample was obtained by means of light ether anesthesia (ADDIS, MCKAY and MCKAY, 1927). Of this sample the specific activity was determined (see under [3.3.7.2]) in order to have a check on normal absorption of the injected phosphate. Then one hindleg was amputated under ether anesthesia and with ordinary surgical precautions. The animal was kept on the same diet for six weeks and was then killed by decapitation. Then the other tibia was taken out for analysis.

The tibia was extracted with a mixture of 1 part ether and 3 parts ethanol by refluxing during 24 hours. After drying at 105°C and ashing at 600°C the ash was dissolved in 5 ml concentrated HCl and diluted to 50 ml. Phosphorus was determined in this solution as described under [3.3.4.2]. To 5 ml of the solution (of the first tibia; 20 ml for the second tibia) were added: 1.0 ml of 6.4% $Na_2HPO_4$ (2nd tibia : 0.30 ml), 2 ml of concentrated ammonia, 5 ml of saturated ammonium chloride and 5 ml of magnesia mixture. The further procedure was the same as described for urine and feces under [3.3.7.2]. Of the standard solution 50 $\mu$l with 2.0 ml of 6.4% $Na_2HPO_4$ (2nd tibia 0.4 ml with 2.0 ml of 6.4% $Na_2HPO_4$) were used for precipitation.

Standard deviation: 5.1%.

## 3.4 STATISTICAL TECHNIQUES.[1])

### *3.4.1 Standard deviation and standard error.*

The accuracy of a single observation of a random variable may be expressed (to a certain extent) by its standard deviation $\sigma$. An estimate of $\sigma$ may be obtained from a series of observations of this random variable by computing:

$$s = \sqrt{\frac{\Sigma (x - \bar{x})^2}{n - 1}} \cdot$$

where x is an observation, $\bar{x}$ the mean of the series and n the number of observations. By multiplying with $\frac{100}{x}$ an estimate of the relative standard deviation (or coefficient of variation) is obtained.

In our own experiments, unless otherwise indicated, we generally carried out

[1]) It is a great pleasure to acknowledge the kind assistance given to me in the writing of this chapter by Dr J. HEMELRIJK of the Mathematical Centre, Statistical Department (Director: Prof. Dr D. VAN DANTZIG), Amsterdam.



0264

some of the analyses in a series of experiments in duplicate, while the others were single determinations. Supposing that the standard deviations of all of the analyses are the same, an estimate of this standard deviation may be obtained with the formula given by DAVIES and PEARSON (1934):

$$s = 0.89 \cdot \frac{\Sigma (x_1 - x_2)}{m}$$

where $x_1$ and $x_2$ represent the pairs of duplicate observations and m is the number of these duplicate analyses.[1]) Multiplication with $\frac{100}{\bar{x}}$ gives an estimate of the relative standard deviation:

$$s(\%) = \frac{89 \cdot \Sigma (x_1 - x_2)}{m \cdot \bar{x}}$$

This formula was used in calculating the relative standard deviations given for the experimental techniques described under [3.2] and [3.3].

The standard error gives an estimate of the standard deviation of the mean of a series of observations of a random variable and is represented by the formula:

$$s_{\bar{x}} = \sqrt{\frac{\Sigma (x - \bar{x})^2}{n (n - 1)}}$$

where n is the number of observations (x) from which the mean ($\bar{x}$) was calculated. The standard error was used, wherever each determination was carried out in duplicate (erythrocyte and leucocyte number and alkaline reserve). The confidential limits of a single observation of a random variable x are the values $x + 2s$ and $x - 2s$. Any transgression of these limits has a probability smaller than the level of sifnificance 0.05. The confidential limits for the mean of a series of observations are given by $\bar{x} \pm 2\, s_{\bar{x}}$.

### *3.4.2 Wilcoxon's test for the problem of two samples.*

In our investigation there arose several times the question: is there a systematic difference between two series of observations from two different dietary groups? Or, expressed in statistical language, are these two series samples from different distributions (or populations) or from one and the same distribution?

In the case of normally distributed random variables Student's test (FISHER, 1948) may be applied. In our experiments, however, we were not at all sure of the normality of the variables, and in some cases the assumption of normality would have been very hazardous indeed. To avoid this assumption we used the non-parametric test of WILCOXON, which was given by WILCOXON (1945) and subsequently developed by MANN and WHITNEY (1947) and others. The only condition for this test is, that all observations are independent. The hypothesis tested is, that the two samples are from the same population.

[1]) Unlike the above mentioned estimate of the standard deviation this estimate is unbiased.



As an example the application of this test to the results of the leucocyte counts in one of our experiments may serve. Blood samples of 10 rats of dietary group PA and of 10 rats of dietary group PD were counted. The results are given below:

PA: 154, 100, 93, 99, 119, 93, 56, 81, 98, 71 — x (m=10)

PD: 70, 92, 68, 111, 106, 104, 63, 72, 66, 93 — y (n=10)

First we count for each observation of x how many observations of y are smaller. For $x_1 = 154$ this is 10; for $x_2 = 100$ 7 etc. These numbers are added, giving u = 63 (when a value x is equal to a value y $\frac{1}{2}$ is added to the score). It is clear that the maximal value of u could be $m \cdot n = 100$; then all values of x would have to be higher than those of y. The minimal value would be 0; in that case the reverse would have to be true. If the hypothesis tested is true (i.e. if x and y are samples of the same population), then the most probable value of $u$ is $\frac{mn}{2}$ (or $\frac{mn+1}{2}$, if mn is odd). From the theoretical treatment, which will not be given here, it follows that $u$ has a distribution which is approximately normal with mean $\frac{mn}{2}$ and standard deviation $\sigma = \sqrt{\frac{1}{12} mn (m+n+1)}$. The fact that $u$ possesses a probability distribution, or that $u$ represents a random variable, is expressed by italicizing the symbol u. To find the approximate size of the smallest critical region of this test, which just contains the observed value u of $u$, we have to calculate $\frac{\left| u - \frac{mn}{2} \right| - \frac{1}{2}}{\sigma}$ and find the corresponding bilateral tail error k from a table of the normal distribution. For values of m and n up to and including 10 tables of u and the exact value of k have been computed (VAN DER VAART, 1950). For larger values of m and n the normal approximation is very close. In our example we find by calculation: k = *0.346* and from the tables: k = *0.353*, meaning that our hypothesis can not be rejected. In our case we chose the limit of the critical region such that the bilateral tail error assumes the value *0.05* (in other words: we used a level of significance *0.05*).

When an experiment is repeated once or more with the same animals, e.g. in our balance experiments which were continued during several weeks, it is possible to test the results in combination. For each experiment a rank is assigned to every observation by arranging the observations according to their magnitude. The ranks are added for each animal separately and the resulting series of totals of ranks are subjected to the test of WILCOXON.

The following example from our experiments about the endogenous fecal phosphorus excretion may make this method clear. The results of both experiments for 5 rats of dietary group PA and 5 rats of group PD are represented below:

| PA | | | | | PD | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 19.2 | 19.2 | 13.6 | 24.3 | 10.2 | 36.4 | 51.5 | 52.8 | 13.2 | 12.5 | 1 st exp. } obser- |
| 23.6 | 18.6 | 22.1 | 43.8 | 19.1 | 18.4 | 26.3 | 23.7 | 17.7 | 36.7 | 2nd exp. } vations |
| 5½ | 5½ | 4 | 7 | 1 | 8 | 9 | 10 | 3 | 2 | 1st exp. } ranks |
| 6 | 3 | 5 | 10 | 4 | 2 | 8 | 7 | 1 | 9 | 2nd exp. } ranks |
| 11½ | 8½ | 9 | 17 | 5 | 10 | 7 | 17 | 4 | 11 | totals of ranks |



0264

For the totals of ranks we find u = 13½, while m = n = 5. From the afore mentioned tables of u and k we find k = *0.921*. Therefore the PA and PD group do not differ significantly.

We encounter a different case when an experiment, giving two series of observations of a certain quantity for each animal, is carried out with several animals. Then the two series of observations may be compared by means of WILCOXON's test for each animal separately. For each of the animals WILCOXON's $u$ is approximately normally distributed with mean $\frac{mn}{2}$ and standard deviation $\sigma = \sqrt{\frac{mn\,(m+n+1)}{12}}$, if the hypothesis tested holds true. In that case the sum of $u$ for all animals, $u_{tot}$, also has an approximately normal distribution, with mean $\Sigma \frac{mn}{2}$ and standard deviation $\sigma_{tot} = \sqrt{\Sigma \sigma^2}$ The corresponding bilateral tail error is found from these data in a way, which is analogous to the way described above for the test of WILCOXON itself.

This method was used in the statistical treatment of the data obtained from our metabolism experiments. The animals received the control diet PA during 6 weeks and were then kept on the acid diet PD during 8 weeks. The phosphorus balances were determined for each of these weeks. As an example we give the treatment of the results from 3 of the animals:

| | *Diet PA* | | | | | | *Diet PD* | | |
|---|---|---|---|---|---|---|---|---|---|
| WEEK: | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| rat 1 . | 0.44 | 0.59 | 0.83 | 0.93 | 0.47 | 0.45 | 0.84 | 0.81 | 0.58 |
| rat 2 . | 0.57 | 0.43 | 0.04 | 0.28 | 0.60 | 0.92 | 0.97 | 0.91 | — |
| rat 3 . | 1.57 | 0.38 | 0.32 | 0.40 | 0.26 | 0.76 | 1.14 | 0.79 | 0.58 |

| | *Diet PD* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| WEEK: | 10 | 11 | 12 | 13 | 14 | u | $\sigma^2$ | $\frac{mn}{2}$ |
| rat 1 . | 0.71 | 1.58 | 0.87 | 0.92 | 0.75 | 12 | 60 | 24 |
| rat 2 . | 1.01 | 0.64 | 0.93 | 0.92 | 0.55 | 5½ | 49 | 21 |
| rat 3 . | 0.59 | 0.83 | 0.87 | 1.29 | 1.48 | 10 | 60 | 24 |
| | | | | | | 27½ | 169 | 69 |

So $u_{tot} = 27.5$; $\frac{mn}{2}_{tot} = 69$ and $\sigma_{tot} = 13.0$ and therefore $\frac{\left|u - \frac{mn}{2}\right| - \frac{1}{2}}{\sigma_{tot}} = 3.15$. From a table of the normal distribution we find: k ≦ 0.002. In this case the phosphorus balance was increased markedly by the acid diet.

### *3.4.3 Rank correlation.*

To test, whether the random variables $x$ and $y$, possessing a simultaneous distribution, are independently distributed or not, a rank correlation method



given by KENDALL (1948) was used. In this method no suppositions need to be made about the form of the simultaneous distribution of $x$ and $y$. The hypothesis tested is that $x$ and $y$ are independent and the test is based on a number of pairs (x, y) observations.

The observations of $x$ are arranged according to increasing magnitude and below each value of $x$ the corresponding value of $y$ is placed. Then the quantity P, defined as the number of pairs of values of y of which the left one is smaller than the right one, is determined. To test the hypothesis we calculate: $S = 2P - \frac{1}{2}n(n-1)$, where n is the number of pairs of observations (x, y). It can be shown that $S$ possesses an approximately normal distribution with mean 0 and standard deviation

$$\sigma = \sqrt{\frac{n\,(n-1)\,(2n+5)}{18}}$$

For $\frac{S}{\sigma}$ we find the corresponding bilateral tail error k in a table of the normal distribution. A table of S and k for $n \leq 10$ is given by KENDALL (1948) and for $n \leq 40$ by KAARSEMAKER and VAN WIJNGAARDEN (1952). Corrections may be applied if there are equal values of $x$ or $y$. But as these corrections are only important when there is a considerable number of equal values, we did not use them and shall not describe them here.

As an example we give the application of this test to the results of the experiment in which the phosphorus retention of the tibia was determined. We wanted to test the independence of the weight loss of the animals ($x$) and the retention ($y$) during the experimental period of 6 weeks. In one group of rats the following results were obtained:

| x | −7 | −1 | +2 | +7 | +11 | +14 | +15 | +22 | +39 | +48 | +54 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| y | 64 | 45 | 46 | 58 | 43 | 70 | 60 | 127 | 50 | 61 | 71 |

$P = 3 + 8 + 7 + 5 + 6 + 2 + 3 + 0 + 2 + 1 + 2 + 0 = 37$

$S = 2P - \frac{1}{2}n(n-1) = 74 - 55 = 19.$ $\sigma = \sqrt{\frac{11.10.27}{18}} = 12.85$

$\frac{S}{\sigma} = 1.48 \rightarrow k = 0.139$

The hypothesis of independence is not rejected.

This test can also be applied to several independent series of observations by adding the values of S and calculating $\sigma_{tot} = \sqrt{\Sigma \sigma^2}$, as described under [3.4.2] for the test of WILCOXON. In this case the theoretical mean of Σ S is equal to zero.

### *3.4.4 A non-parametric test against trend.*

The rank correlation method of KENDALL can be applied to test against trend in a series of n observations of a random variable $y$ (MANN, 1945). To do this, we use the row of natural numbers 1, 2, . . . . . , n as a second series and apply KENDALL's rank correlation test to these two series. Under the hypothesis



0264

tested, that no trend is present, all possible permutations of the arrangement of the y-values according to magnitude are equally probable. The same S and $\sigma$, as described under [3.4.3], are calculated. A positive value of S belonging to the critical region indicates a positive trend (i.e. increasing values of y), a negative value a negative trend (values of y decreasing). This test can also be applied to several independent series of observations, even if they are of different length. In that case for each of the series S and $\sigma$ are calculated and from these

$$S_{tot} = \Sigma\ S \text{ and } \sigma_{tot} = \sqrt{\Sigma\ \sigma^2}.$$

and from a table of the normal distribution the corresponding bilateral tail error k is found (ELFVING and WHITLOCK, 1950).

As an example we give the application of this test to the results of the bone retention experiment. For each of the three dietary groups PA, PD and PG a series of observations was obtained:

| x | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | P | S | $\sigma^2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| y | 127 | 61 | 71 | 50 | 58 | 70 | 60 | 45 | 46 | 43 | 64 | 15 | —25 | 165 |
| y' | 99 | 98 | 104 | 43 | 52 | 54 | 80 | 38 | 40 | 41 | 43 | 14½ | —26 | 165 |
| y'' | 58 | 96 | 93 | 64 | 47 | 85 | 84 | 31 | 60 | 77 | 64 | 21½ | —12 | 165 |
| | | | | | | | | | | | | | —63 | 495 |

So here we find:

$$S_{tot} = -63,\ \sigma_{tot} = 22.2 \text{ and } k = 0.0046.$$

We conclude that a negative trend is present.

### *3.4.5 Combination of bilateral tail errors.*

When a certain effect is investigated in some independent experiments, which cannot be pooled, it is possible to combine the bilateral tail errors of each ($k_1$, $k_2$..... and $k_n$ resp.). In order to do this we calculate

$$\chi^2 = -2 \ln \frac{k_1 \cdot k_2 \ldots\ldots k_n}{2^n}$$

and find the combined bilateral tail error, $k_{tot}$, from a table of $\chi^2$ (FISHER, 1948, p. 112-113), assuming 2n degrees of freedom (where n represents the number of tail errors combined) and multiplying by 2 (the $\chi^2$-table giving unilateral tail errors). This method is a modification of the method given by FISHER (1948, p. 99), which was communicated to us by Dr. J. HEMELRIJK.

As an example we apply this method to the results for the calcium balances obtained in our metabolism experiments. In both experiments a slight tendency to an increased Ca-balance for dietary group PD was found, but in neither case significant:

$$k_1 = 0.074 \text{ and } k_2 = 0.177.$$

$$\text{Here } \chi^2 = -2 \ln \frac{0.074 \times 0.177}{4} = -4.606 \log 0.00325 = 11.46$$

From the table of $\chi^2$ we find after multiplying by 2:

$$k_{tot} = 0.044$$

So the combined evidence from both experiments shows a result that is just significant.



# 4. BIOLOGICAL PART

## 4.1 GROWTH.

In this chapter and the next one we shall describe the results of our investigations, carried out with more than 700 rats during a period of two years. As all techniques have been treated in detail in the foregoing chapter, we can confine ourselves to the presentation of our results.

For 203 animals we followed the growth during the 20 weeks immediately following the nursing period. There were 101 males and 102 females of the first, second and third generation, receiving the diets PA, PD, PG and PBB (see table 2 under [3.1.4]). The members of each litter of the first generation were distributed as equitably as possible among the dietary groups. Hereafter the term "first generation" will be used to designate the original animals, with whom the investigation started.

In table 3 the results are shown. For every group the average weight for each week is given. For all groups PD and PG there is always a control group PA of the same generation, born at the same time and grown up under similar circumstances. For the groups PBB however there were no corresponding control groups PA. If we therefore compare the 2nd and 3rd generations of the PBB group with the 2nd generation of the PA group, we must be careful with our conclusions.

The statistical treatment of the results given in table 3 was carried out in this way that the weight of the rats of each dietary group were compared at 0, 10 and 20 weeks with those of the corresponding control group by means of the test of WILCOXON. The bilateral tail errors obtained in this way are given in table 4.

A significant difference was only found in the case of the PG group of the second generation after 20 weeks of diet. When however in this case the weight increases over 20 weeks were compared no difference was found: k = *0.358*. At 10 weeks the average weight of the males of the PBB group, 2nd generation was significantly higher than that of the control group; with the weight increase over 10 weeks the difference was even more outspoken (k = **0.00070**). In the next 10 weeks the difference disappeared. It will probably have been due to the fact that the PA group used for comparison was not the real control group in this case. This is probably also the case for the corresponding females, where the average starting weight for the PBB group was significantly lower than that of the PA group. This difference also disappeared in the following weeks. From the average weights for the third generation of group PBB it will be noticed that there was a sudden and transient retardation of growth between the 14th and 15th week. The weights increased again during the following weeks and this



0264

## TABLE 3
*Growth*

| GROUP | GENERAT. | SEX | NR. OF ANIMALS | WEEKS OF DIET | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| PA | 1st gen. | ♂ | 16 | 34 | 54 | 80 | 102 | 124 | 154 | 173 | 189 | 209 | 227 | 242 | 258 | 277 | 285 | 291 | 297 | 304 | 309 | 313 | 317 | 319 |
| PD | | | 16 | 34 | 54 | 88 | 106 | 129 | 151 | 168 | 184 | 201 | 214 | 229 | 245 | 264 | 274 | 278 | 287 | 292 | 299 | 304 | 307 | 313 |
| PG | | | 16 | 34 | 51 | 79 | 104 | 127 | 149 | 170 | 186 | 205 | 217 | 233 | 248 | 269 | 278 | 285 | 292 | 297 | 305 | 311 | 314 | 317 |
| PA | 2nd gen. | | 9 | 34 | 49 | 69 | 90 | 115 | 140 | 160 | 175 | 194 | 206 | 221 | 229 | 237 | 245 | 254 | 264 | 267 | 272 | 278 | 284 | 290 |
| PD | | | 11 | 33 | 54 | 69 | 86 | 104 | 128 | 152 | 173 | 190 | 205 | 210 | 232 | 244 | 254 | 261 | 277 | 282 | 290 | 295 | 298 | 303 |
| PG | | | 11 | 33 | 49 | 69 | 89 | 108 | 125 | 147 | 169 | 189 | 205 | 213 | 223 | 237 | 245 | 262 | 263 | 269 | 278 | 283 | 288 | 295 |
| PBB | | | 7 | 31 | 53 | 81 | 110 | 137 | 174 | 197 | 219 | 236 | 249 | 256 | 270 | 278 | 281 | 287 | 293 | 302 | 308 | 309 | 309 | 308 |
| PBB | 3rd gen. | | 15 | 38 | 55 | 72 | 96 | 114 | 141 | 163 | 184 | 201 | 218 | 229 | 242 | 246 | 253 | 274 | 252 | 256 | 259 | 264 | 269 | 274 |
| PA | 1st gen. | ♀ | 16 | 35 | 53 | 75 | 89 | 103 | 120 | 130 | 141 | 150 | 156 | 163 | 171 | 179 | 184 | 186 | 188 | 189 | 191 | 192 | 194 | 197 |
| PD | | | 16 | 34 | 51 | 73 | 92 | 102 | 123 | 134 | 144 | 153 | 159 | 167 | 173 | 181 | 185 | 186 | 189 | 190 | 193 | 195 | 197 | 199 |
| PG | | | 15 | 31 | 47 | 71 | 89 | 105 | 117 | 130 | 139 | 150 | 158 | 163 | 170 | 176 | 181 | 184 | 187 | 191 | 193 | 194 | 197 | 199 |
| PA | 2nd gen. | | 11 | 39 | 53 | 69 | 84 | 99 | 111 | 127 | 138 | 146 | 154 | 164 | 167 | 173 | 177 | 181 | 184 | 187 | 190 | 195 | 196 | 200 |
| PD | | | 11 | 36 | 48 | 65 | 83 | 97 | 111 | 127 | 141 | 149 | 158 | 166 | 172 | 178 | 181 | 189 | 192 | 197 | 201 | 203 | 205 | 206 |
| PG | | | 11 | 35 | 49 | 65 | 82 | 99 | 110 | 125 | 132 | 141 | 151 | 159 | 162 | 169 | 171 | 174 | 175 | 180 | 182 | 184 | 188 | 189 |
| PBB | | | 7 | 27 | 48 | 69 | 91 | 106 | 129 | 140 | 147 | 155 | 161 | 162 | 170 | 177 | 178 | 180 | 182 | 189 | 191 | 192 | 193 | 193 |
| PBB | 3rd gen. | | 15 | 44 | 61 | 74 | 94 | 114 | 130 | 143 | 154 | 163 | 164 | 175 | 181 | 185 | 188 | 188 | 188 | 193 | 195 | 195 | 196 | 196 |

TABLE 4

*Growth - Statistical comparison*

| DIETARY GROUPS | GENE-RATION | MALES | | | FEMALES | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 10 | 20 | 0 | 10 | 20 |
| PA – PD | 1st gener. | *0.984* | *0.308* | *0.290* | *0.940* | *0.355* | *0.777* |
| PA – PG | ,, ,, | *0.880* | *0.440* | *0.376* | *0.119* | *0.859* | *0.813* |
| PA – PD | 2nd gener. | *0.542* | *0.968* | *0.523* | *0.294* | *1.000* | *1.000* |
| PA – PG | ,, ,, | *0.968* | *1.000* | *0.653* | *0.263* | *0.555* | **0.0094** |
| PA – PBB | ,, ,, | *0.681* | **0.023** | *0.299* | **0.0084** | *1.000* | *0.112* |
| PA – PBB | 2nd-3rd gen. | *0.234* | *0.497* | *0.440* | *0.148* | *0.082* | *0.682* |

continued also after the 20th week (av. weight after 29 weeks, when it was observed for the last time, for the males 292 g, for the females 214 g). Therefore it is highly improbable that this phenomenon should have to be ascribed to a harmful effect of the diet. More probably it is due to an intercurrent infection of the animals (the cages of the animals of this group were placed together and somewhat apart from the other animals).

Summarizing we believe that there was no evidence of a harmful effect on the growth of three generations of rats receiving either a diet with 0.40 or 0.75% of phosphoric acid or a diet with 1.20% of citric acid during the 20 weeks following the weaning of the litters.

### 4.2 Reproduction.

In special experiments the reproduction of the rats in the dietary groups PA, PD, PG, and PBB was investigated. Of each of the dietary groups PA, PD and PG 8 females and 3 males of the first generation were taken. They were all 32 weeks old and had received the diets during 29 weeks. Eleven weeks later the same animals were mated again. The results of both experiments are given in table 5. One of the females of the PA group died in the period between the experiments and was not replaced, as we wanted to know if any effects of the diet on the reproduction would be noticeable on repeating the experiment with the same animals. We also include the data concerning the reproduction of 7 females of the PBB group (second generation, 40 weeks old), although strictly speaking they are not quite comparable to the other data. To facilitate comparison the means are given with their standard errors (written in parentheses below the mean).

From the result given in table 5 we may conclude that there was no detrimental effect of the acid diets on the reproduction, neither in the first, nor in the second experiment.

## TABLE 5

*Reproduction*

| GROUP | WEEKS OF DIET | NR. OF FEMALES | NR. OF LITTERS | AV. WEIGHT | | TOTAL NR. OF YOUNG | | AV. NR. OF YOUNG PER DELIVERY | | AV. PERCENTAGE STILLBORN PER DELIVERY | AV. WEIGHT PER LIVING YOUNG | NR. OF YOUNG MISSING AFTER 3 WEEKS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | BEFORE PREGNANCY | AFTER DELIVERY | LIVING | DEAD | LIVING | DEAD | | | |
| | | | | | | *1st experiment* | | | | | | |
| PA | 29 w | 8 | 6 | 205 (6.1) | 213 (4.3) | 37 | 5 | 6.2 (1.22) | 0.83 (0.48) | 11.9% | 5.4 (0.31) | 7 |
| PD | ,, | 8 | 5 | 207 (8.0) | 217 (8.4) | 43 | 2 | 8.6 (0.51) | 0.40 (0.25) | 4.4% | 5.6 (0.12) | 1 |
| PG | ,, | 8 | 6 | 193 (5.4) | 206 (2.1) | 47 | 1 | 7.8 (1.30) | 0.17 (0.17) | 2.1% | 5.7 (0.39) | 1 |
| PBB (2nd gen.) | 40 w. | 7 | 7 | 203 (3.2) | 210 (5.9) | 48 | 0 | 6.9 (0.63) | 0 (—) | 0.0% | 5.9 (0.31) | 7 |
| | | | | | | *2nd experiment* | | | | | | |
| PA | 40 w | 7 | 6 | 220 (5.8) | 232 (3.3) | 37 | 5 | 6.2 (1.38) | 0.83 (0.24) | 12.8% | 6.4 (0.26) | 2 |
| PD | ,, | 8 | 8 | 225 (6.5) | 238 (7.0) | 59 | 2 | 7.4 (0.98) | 0.25 (0.25) | 2.1% | 6.3 (0.26) | 0 |
| PG | ,, | 8 | 7 | 222 (3.6) | 233 (8.0) | 46 | 2 | 6.6 (1.65) | 0.29 (0.29) | 5.7% | 6.2 (0.12) | 4 |



0264

The results for the animals on the acid diets, including those of group PBB (0.75% of phosphoric acid), even tend to be slightly better, although these effects are not significant, judging from the standard errors.

The results for the second experiment are also quite comparable to those of the first experiment.

### 4.3 Death rate.

As a considerable number of the animals in our investigation had to be killed at a relatively young age, only part of them could be used for estimating the death rate in the different dietary groups. And of these animals it was possible only to record the death rate in the first half year of life, as after that period a progressive number of them were killed for experimental purposes.

Table 6 gives the death rate among 399 animals of three generations divided over different dietary groups during the first half year of life after the weaning of the litters. As the first two months constitute the most critical period, these are subdivided into weeks.

Most of the animals of group PA, PD and PG of the second generation had to be killed already during the 6th month for the tissue analyses. The rather high death rate of these three groups, which cannot be ascribed to a harmful effect of the acid diets judging from the figures, is most probably due to an intercurrent infection. Postmortems of some of the dead animals revealed no other indications as to the cause of death but pneumonia.

On the whole the results given in table 6 do not give the impression that the mortality is increased by any one of the acid diets PD, PBB or PG.

### 4.4 Blood picture.

The blood picture was determined for 20 animals of the first generation (age: 8 months, diet during 7 months) and 20 animals of the second generation (age: 8 months, diet during 7 months), in each case 10 animals of the PA group and an equal number of the PD group were investigated. Both male and female animals were used.

The results are given in table 7a for the first generation and in table 7b for the second generation. The erythrocyte count is expressed in extinction units (see under [3.2.2]), the leucocyte count in hundreds per cubic millimeter and the results of the differential count in cells per hundred leucocytes counted.
A statistical treatment of the results obtained for the erythrocytes, total leucocytes, lymphocytes and neutrophiles by means of the test of Wilcoxon was carried out. The results are tabulated in table 8 in the form of the bilateral tail errors k. The comparison was always made between the corresponding observations for the PA group and the PD group.

## TABLE 6

*Death rate*

| | GROUP | TOTAL NR. OF ANIMALS AT WEANING | TOTAL NR. OF DEATHS | | 1ST W. | 2ND W. | 3RD W. | 4TH W. | 5TH W. | 6TH W. | 7TH W. | 8TH W. | 3RD M. | 4TH M. | 5TH M. | 6TH M. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1st generation | PA | 92 | 5 | 5.4% | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 |
| | PD | 40 | 1 | 2.5% | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | PG | 40 | 2 | 5.0% | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| | PBB | 4 | 0 | 0.0% | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2nd generation | PA | 43 | 16 | 37% | 4 | 2 | 3 | 1 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | – |
| | PD | 71 | 23 | 32% | 6 | 10 | 1 | 5 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | – |
| | PG | 56 | 17 | 30% | 6 | 9 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | – |
| | PBB | 18 | 1 | 8.5% | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| 3RD generat. | PBB | 35 | 7 | 20% | 0 | 0 | 4 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| | | 399 | | | | | | | | | | | | | | |

## TABLE 7a

*Blood picture first generation*

| GROUP | NR. OF RAT | SEX | ERYTHR. | LEUCOC. | LYMPHOC. | NEUTROPH. | EOSINOPH. | BASOPH. | YOUNG LEUC. | MONOC. |
|---|---|---|---|---|---|---|---|---|---|---|
| PA | 5207 | ♂ | 0.234 | 98 | 71 | 29 | – | – | – | – |
| ,, | 5208 | ,, | 0.306 | 120 | 64 | 35 | – | – | 1 | – |
| ,, | 5211 | ,, | 0.150 | – | 66 | 29 | – | – | 5 | – |
| ,, | 5213 | ,, | 0.241 | 77 | 81 | 16 | 1 | – | 2 | – |
| ,, | 5216 | ,, | 0.228 | 78 | 69 | 19 | 1 | 1 | 10 | – |
| | | av. ♂♂ | 0.232 | 93 | 70% | 26% | <1% | <1% | 3.6% | <1% |
| PA | 5206 | ♀ | 0.183 | 120 | 81 | 17 | 1 | – | – | 1 |
| ,, | 5209 | ,, | 0.346 | 107 | 74 | 21 | 1 | 1 | 1 | 2 |
| ,, | 5210 | ,, | 0.243 | 108 | 62 | 35 | – | – | 3 | – |
| ,, | 5214 | ,, | 0.212 | 85 | 82 | 15 | 2 | 1 | – | – |
| ,, | 5215 | ,, | 0.248 | 93 | 75 | 23 | 1 | – | – | 1 |
| | | av. ♀♀ | 0.246 | 103 | 75% | 22% | 1% | <1% | <1% | <1% |
| | | aver. ♂+♀ | 0.239 | 98 | 73% | 24% | <1% | <1% | 2.2% | <1% |
| PD | 5256 | ♂ | 0.230 | 98 | 68 | 32 | – | – | – | – |
| ,, | 5260 | ,, | 0.201 | 72 | 73 | 22 | 2 | 2 | 1 | – |
| ,, | 5261 | ,, | 0.181 | 108 | 51 | 36 | 6 | – | 5 | 2 |
| ,, | 5264 | ,, | 0.223 | 115 | 80 | 15 | 1 | – | 4 | – |
| ,, | 5266 | ,, | 0.235 | 93 | 73 | 24 | 1 | 1 | 1 | – |
| | | av. ♂♂ | 0.214 | 97 | 69% | 26% | 2,0% | <1% | 2.2% | <1% |
| PD | 5258 | ♀ | 0.217 | 107 | 79 | 17 | – | – | 4 | – |
| ,, | 5259 | ,, | 0.191 | 85 | 68 | 25 | 2 | 2 | 1 | 2 |
| ,, | 5262 | ,, | 0.214 | 88 | 79 | 19 | 1 | – | 1 | – |
| ,, | 5263 | ,, | 0.208 | 122 | 85 | 10 | 3 | – | – | 2 |
| ,, | 5267 | ,, | 0.237 | 79 | 74 | 24 | 1 | – | – | 1 |
| | | av. ♀♀ | 0.213 | 96 | 77% | 19% | 1.4% | <1% | 1.2% | 1.0% |

TABLE 8

*Blood picture — statistical comparison*

| GENERATION | ERYTHROCYTES | LEUCOCYTES | LYMPHOCYTES | NEUTROPHILES |
|---|---|---|---|---|
| 1st gener. | | | | |
| ♂♂ | *0.421* | *1.000* | *0.841* | *1.000* |
| ♀♀ | *0.421* | *0.548* | *0.921* | *0.921* |
| ♂+♀ | *0.143* | *0.968* | *0.882* | *0.882* |
| 2nd gener. | | | | |
| ♂♂ | *0.902* | *0.535* | *0.757* | *0.710* |
| ♀♀ | *1.000* | *0.700* | *0.400* | *1.000* |
| ♂+♀ | *0.353* | *0.353* | *0.133* | *0.505* |

From this table it will be clear at once that there was not a single significant difference. Moreover our results are in good agreement with those of GRIFFITH and FARRIS (1942, p.356) for the normal albino rat. They found:

leucocytes — 9000 per cu.mm. (range 6000-18000)
lymphocytes — 78% (range 55-96%)
neutrophiles — 20% (range 8 -39%)
eosinophiles — 2% (range 0 - 4%)
basophiles — <1% (range 0 - 0%)
monocytes — <1% (range 0 - 3%)

These results were obtained — like ours — without avoidance of emotional stimulation and therefore marked deviations from the actual values may exist. We may conclude that the diet containing 0.40% of phosphoric acid had no influence on the blood picture of both male and female rats of the first and second generation who had received the diet during 7 months.

4.5 AUTOPSY[1]).

A large number of animals that were killed in our investigations were subjected to autopsy. They belonged to all three generations and to the dietary groups PA, PD, PG and PBB and were from 1 to 500 days of age.

No gross anatomical changes were found in any of the animals, but for more or less marked pulmonic lesions due to pneumonia. These lesions were of the type described by GRIFFITH and FARRIS (1942, p.460-2) and occurred in a considerable number of adult animals of all groups, although not visible from

[1]) The autopsies and the subsequent histological examinations were carried out by Miss M. P. J. VAN DER RIJST, to whom our sincere thanks are due. We also wish to thank Dr J. F. HAMPE of the Laboratory of Pathological Anatomy (director: Prof. Dr H. F. DEELMAN), Wilhelmina University Hospital, Amsterdam, for his assistance in the histological examinations.

their outward appearance. We can confirm the well-known observation that pneumonia is probably the most common of all morbid conditions of adult rats. In the new-born animals in our reproduction experiments no gross anatomical changes were found either.

Histological examination of the liver, spleen, adrenals, testicles, skeletal muscle and femur revealed no pathological changes or any peculiarity. As in some cases abnormalities of the kidney were found, the investigation of this organ was extended to more animals in order to see if there might be any relation with the diet.

The histological changes found in the kidneys may be classified as follows:

1. an indication of degenerative changes of the tubular epithelium.
2. dilatation of the tubules with epithelial changes, often combined with fibrosis and infiltration of lymphocytes.
3. medullary fibrosis in limited areas.
4. tubular necrosis in the medulla, generally connected with the fibrosis mentioned under 3. and accompanied by calcification.

The changes described under 3. and 4. never occurred in a serious degree.

These changes were distributed more or less equally among all dietary groups according to a preliminary investigation. In a more extensive examination the kidneys of 24 animals of the PA group (first generation, diet during 12 months) and 20 animals of the PBB group (second generation, diet during 12 months) were compared. The frequency of the phenomena described above was found to be as follows:

| | PA | PBB |
|---|---|---|
| 1. | 8 out of 24 (33%) | 6 out of 20 (30%) |
| 2. | 4 „ „ 24 (17%) | 5 „ „ 20 (25%) |
| 3. | 10 „ „ 24 (42%) | 9 „ „ 20 (45%) |
| 4. | 4 „ „ 24 (17%) | 5 „ „ 20 (25%) |

Similar lesions of the kidneys with about the same frequency were described by GRIFFITH and FARRIS (1942, p.469) under the heading: spontaneous diseases. We therefore think to be justified in assuming that there is no evidence that the acid diets (PBB, PD, PG) caused renal damage, even in the second generation after one year. A possible explanation of the changes found may be degeneration due to chronic infections, e.g. pneumonia.

Summarizing we may say that autopsy and histological examination of many organs revealed no changes that could be attributed to the effects of the acid diets.



### 4.6 INVESTIGATION OF THE TEETH[1]).

The teeth of a number of rats of the dietary groups PA, PD, PG and PBB were investigated. The animals had received the diets during 3 to 16 months. No extensive lesions were found; only some fissural caries. The caries percentage (giving an estimate of the amount of dental tissue lost, due to a carious process) was exceptionally low (about 0.1%) and about the same for all dietary groups. This caries percentage was one of the lowest ever recorded for any group of animals at our laboratory (compare the results given by JANSEN et al. 1951).

In all groups dental attrition of the molars was found to occur with increasing age. At the age of two months practically no attrition was found in any of the groups (diet during 5 weeks). At a higher age all groups showed dental attrition, especially of the lower molars. The highest degree of attrition was found in the animals receiving phosphoric acid. The animals receiving citric acid also showed a higher degree of attrition than the animals of group PA. No caries was observed in the abraded cuspal parts. Apparently there was no connection between attrition and caries, the latter being always fissural and independent of the diet. A remarkable feature of the molars of the animals receiving phosphoric acid was the white colour, caused partly by the proper colour of the molars, partly by the reduced plaque.

The histological examination of the teeth revealed a deposition of a calcium containing precipitate in the dentine of the animals receiving the acid diets, being an indication of a reaction of the dentine to the effect of the acid diets. This process had not progressed to a further degree in the animals receiving the diets PD and PG for 10 months than in those receiving these diets during 3 months (6 specimen). Neither after 10 nor after 3 months any indication of a reaction of the pulp was visible; only normal odontoblasts were found. No secondary dentine formation had occurred. In the histological examination no traces of caries were apparent, which is in accordance with the very low caries percentage found.

### 4.7 DISCUSSION.

In the biological part of our investigation comprising growth, reproduction, death rate, blood picture, autopsy and histological examination of three successive generations of rats, receiving diets containing either phosphoric acid (up to 0.75%) or citric acid (up to 1.20%) during periods up to 12 months, we found no evidence of any harmful effect of the acid diets.

Our results for the growth are in accordance with those of MITCHELL and MILLER (1931) and LAMB and EVVARD (1931). ZILZER (1930) found a slightly

1) We are much indebted to Miss L. DALDERUP and Mrs M. NEDERVEEN-FENENGA, D.D.S., M.D. for the examination of the teeth. The sections for the histological examination were prepared by Mrs L. BULT-VREUGDE.



0297

slower growth for acid-fed rats, but his results are difficult to compare with ours, because the abstract fails to mention the dosage. The absence of an effect on the reproduction in our experiments is also in agreement with the findings of LAMB and EVVARD (1931).

The changes in the blood picture on administration of acid, found by HOUSEHOLDER (1921), HALDANE et al (1928), SAKAI (1932), KUGA (1934) and TERZIOGLU (1944), cannot be compared to our results very well. These authors investigated the effect of an acute acidosis, in some cases obtained by injection of acid. But no acidotic condition existed in our animals, as will be demonstrated later (see under [5.2.2]). And moreover we searched for an effect on the blood picture of a prolonged administration of an acid diet. In that case no effect was found however.

With regard to degenerative changes of the kidney the same remark can be made. Positive effects were found by MAC NIDER (1923) after two injections of HCl in dogs resulting in an acute acidosis. But the continuous feeding of a diet containing 2% $CaCl_2$ to rats during one year caused no kidney damage (ADDIS et al, 1927), similar to the results of our experiments. SEGAL (1927) found reversible changes in the kidneys of rabbits under these circumstances. Rabbits however are specially susceptible to acid ingestion because of their inability to produce ammonia in the kidneys. Moreover HCl and HCl-producing substances were given instead of phosphoric acid or citric acid. Urinary calculi as found in rats by HAMMARSTEN and LUNDGREN (1940) after an acid diet were not present in our rats. Liver damage was found by HESS and GOLDSTEIN (1925), TATSUZAWA (1933) and GOVAN and PARKES (1946; 1949), in all cases in the more susceptible rabbits. In our rats no signs of liver necrosis were apparent.

The investigation of the teeth yielded results consistent with the literature on this subject. Absence of a relation between dental caries and the dietary acid-base balance was also found by PRICE (1935), FORSHUFVUD (1938), THOMAS and BODECKER (1942). The increased dental attrition for the rats receiving the acid diets in our experiments can probably be compared to the effect of phosphoric acid containing beverages found by McCAY and WILL (1949) and MÜLLER and GORTNER (1949) and also to the effect of fruit juices described by Wynn and Haldi (1948), Jarvis et al (1949) and Miller (1950). Probably the effect in our case, which seems to have been weaker than that observed by these authors, was due to the free acid present in the diets. From the low caries percentage and the histological examination we concluded that the increased attrition is not to be regarded as a harmful action of the acid diets.



# 5. CHEMICAL PART

## 5.1 METABOLISM EXPERIMENTS.

### *5.1.1 Introduction.*

In this part the results of the metabolism experiments, tissue analyses, and the experiments with radioactive phosphorus will be described. For obtaining the data represented about 12.000 chemical analyses were carried out. Obviously it will not be possible to give all experimental data in detail; in many cases it will be necessary for the sake of brevity to tabulate the averages for the groups only.

We carried out two metabolism experiments in the course of our investigation. In preliminary experiments we found female rats adapting themselves better to the metabolism cages than male rats.

Most of the male rats, we put in metabolism cages, refused to take food during the first two to four days resulting in a considerable loss of weight (up to 20%). In some cases it took up to four weeks before the weight was normal again. This happened with all dietary groups.

We therefore used adult female rats in both experiments.

In the first experiment we used 24 female rats of group PA (age $8^1/_2$ months). During the first 6 weeks they were kept on the diet PA; during the next 8 weeks 8 animals were continued on this diet, while 8 others received the diet PD and the remaining 8 rats received the diet PG. The experiment was completed with another control period of 6 weeks during which all animals received the diet PA. We determined calcium and phosphorus balances, the urinary excretion of ammonia, urea, total base and citric acid and the volume, pH and titratable acidity of the urine.

The first experiment could yield only knowledge concerning a relatively short period of acid administration, while we wanted to know the effect of a prolonged intake. Therefore a second metabolism experiment was carried out without a control period at the beginning and at the end. In this experiment 8 animals of dietary group PA were compared with 8 animals of dietary group PD. All animals had received the diets during 8 months previously, enabling us to compare the metabolism of animals who had received the diets already during a considerable part of their life time. We determined the calcium, phosphorus and nitrogen balances as well as the urinary excretion of ammonia and urea and the volume, pH and titratable acidity of the urine. Contrary to the first experiment there was no check on possible differences between the groups, due to causes other than dietary ones. Parallel interpretation of both experiments should make it possible however to draw conclusions that are not influenced by extraneous effects.



0264

In order to obtain values that could be compared irrespectively of differences in body size of the animals, we divided the results by the $^2/_3$ power of the body weights. The $^2/_3$ power of the body weight should approximately be proportional to the body surface. RUBNER (1882) found already the metabolic rate to be proportional to the body surface.

The following calculations may show that division by the power of the body weight yields results that are better comparable if differences in body weight exist. The average intake of calories, nitrogen, calcium and phosphorus for man and for the rats (av. weight 236 g) in our first metabolism experiment are given below. The ratio of the body weights and of the $^2/_3$ powers of the body weights are resp. 297 : 1 and 44 : 1.

| | CAL/D | G N/D | G CA/D | G P/D |
|---|---|---|---|---|
| man . . . . | 2500–3000 | 11.2 | 0.80 | 1.3 |
| rat . . . . . | 61 | 0.36 | 0.10 | 0.14 |
| ratio . . . . | 41–49 | 31 | 8.0 | 9.3 |

The results of the metabolism experiments may be transformed into values per average rat by multiplication with 38.2 for the first experiment and with 36.0 for the second one.

### *5.1.2 Phosphorus balance.*

In both metabolism experiments we determined the phosphorus balance, being the difference between the total phosphorus intake and the sum of urinary and fecal phosphorus excretion in mg/d of phosphorus. Strictly spoken we determined only inorganic urinary phosphorus, but as this comprises generally 95 to 99% of the total urinary phosphorus the difference is negligible.

In the first experiment we obtained from the 24 animals during 20 weeks 480 balance figures, which cannot be represented here in their entirety. In table 9 the averages for each group and for each period are given. The following abbreviations are used to designate the periods: EP — experimental period,

TABLE 9

*phosphorus balance (first experiment)*

| GROUP | CP 1 (6 WEEKS) (ALL RATS PA DIET) | EP (8 WEEKS) | CP 2 (6 WEEKS) (ALL RATS PA DIET) |
|---|---|---|---|
| PA | 0.53 | 0.67 | 0.70 |
| PD | 0.50 | 0.76 | 0.63 |
| PG | 0.55 | 0.76 | 0.63 |

All values in mg/d/weight $^{2/3}$.



CP 1 and 2 — first and second control period. From table 9 it is apparent that the phosphorus balance tended to rise during the experimental period for the groups PD and PG. This was not a gradual rise during the 8 weeks of the experimental period, as the application of a test against trend (see under [3.4.4]) for these groups revealed no trend (k resp. *0.431* and *0.541*). To establish whether the increase of the phosphorus balance was significant, the test of WILCOXON (see under [3.4.2] )was used in two directions: vertically and horizontally. In the first case ranks were assigned to the results for one week of the two groups compared. This was done for all weeks of the same period and the resulting ranks were added for each rat. On the sums the test of WILCOXON was carried out. In the second case for each animal the value of $u$ for the two periods compared was calculated. The results were added as well as the variances ($\sigma^2$) for each animal. From these the corresponding k-value was found by means of a table of the normal distribution. The results are given in table 10.

TABLE 10

*phosphorus balance — statistical comparison (first experiment)*

| GROUPS | CP 1 | EP | CP 2 |
|---|---|---|---|
| PA – PD | *0.798* | *0.065* | *1.000* |
| PA – PG | *0.878* | *0.412* | *0.956* |
| **GROUP** | **CP 1 – EP** | **EP – CP 2** | **EP – CP 1+2** |
| PA | **0.011** | *1.000* | *0.140* |
| PD | **<0.0001** | **0.0070** | **<0.0001** |
| PG | **0.0006** | *0.578* | **0.034** |

The upper part represents the results of the vertical comparison; the lower part those of the horizontal comparison. In the lower part CP 1 — EP means comparison of experimental period with the first control period; EP — CP 1 + 2 comparison of experimental period with both control periods. It will be clear from this table that there was a significant increase of the phosphorus balance for the PD group on receiving the diet PD. Less certain is the effect of the diet PG on the PG group. From the horizontal comparison, which forms the sharpest test (comparison of the same animals), a significant increase follows. From the vertical comparison no affirmation can be obtained. We shall return to this presently.

Table 11 gives the data for the phosphorus balances obtained in the second metabolism experiment. In this case we found again an increased phosphorus retention. To test if the increase was significant, the "vertical" comparison described above was carried out. The corresponding value of k was found to be: **0.044**.



0267

TABLE 11

*Phosphorus balance (second experiment)*

| | 1st week | 2nd w. | 3rd w. | 4th w. | 5th w. | aver. |
|---|---|---|---|---|---|---|
| PA | +0.59 | +0.98 | +0.46 | +0.63 | —0.63 | +0.41 |
| | +0.54 | +0.96 | +1.21 | +0.44 | +0.20 | +0.67 |
| | +1.20 | +1.82 | +0.58 | +0.13 | +0.02 | +0.75 |
| | +1.75 | +1.16 | +0.45 | +0.46 | +0.56 | +0.88 |
| | +0.22 | +1.54 | +0.34 | +0.33 | +0.39 | +0.56 |
| | +0.50 | +0.70 | +0.26 | +0.35 | +0.35 | +0.43 |
| | +0.41 | +0.78 | +0.51 | +0.13 | +0.28 | +0.42 |
| | +0.56 | +1.63 | +0.69 | +0.45 | +0.57 | +0.78 |
| | | | | | | +0.61 |
| PD | —0.20 | +0.40 | +0.87 | +0.59 | +0.63 | +0.46 |
| | +0.63 | +0.63 | +0.98 | +0.52 | +0.65 | +0.68 |
| | +0.77 | +1.38 | +1.24 | +0.99 | +1.00 | +1.08 |
| | +0.68 | +0.64 | +0.84 | +0.81 | +1.00 | +0.79 |
| | +0.96 | +0.44 | +1.00 | +0.36 | +0.03 | +0.56 |
| | +0.67 | +0.88 | +1.23 | +0.27 | +1.70 | +0.95 |
| | +1.15 | +0.38 | +0.69 | +0.37 | +0.66 | +0.65 |
| | +1.01 | +1.28 | +1.16 | +0.57 | +0.33 | +0.87 |
| | | | | | | +0.76 |

(All values in mg/d/weight $^{2/3}$)

So in both metabolism experiments a significantly increased phosphorus retention was found on administration of phosphoric acid. In the first case this retention amounted to about 0.17 mg/d/weight $^{2/3}$; in the second case it was about 0.15 mg/d/weight $^{2/3}$.

It is important to see how the urinary and fecal excretions of phosphorus were affected by the acid diets. The averages of the results obtained in the first experiment are given in table 12 (upper part). The results of the horizontal comparison are given in the lower part.

As in some cases also significant differences for the control group were found, it was necessary to test if the changes between the periods differed for the different groups of rats. We therefore calculated for each rat Wilcoxon's $u$ for the two periods compared. For each of the groups of rats a number of values of $u$ were obtained. If the hypothesis tested (i.e. the diet has no influence on the difference between the two periods) holds true, these groups of values of $u$ are samples of the same universe. This can be tested by applying Wilcoxon's test to these values. The resulting k-values are given in the two bottom rows of table 12. This method was repeatedly used in the course of the metabolism experiments.

There was a very marked rise in urinary phosphorus excretion for the PD diet, but no change for the PG diet. The fecal phosphorus excretion was only

## TABLE 12

*Phosphorus excretion (first experiment)*

| GROUP | URINE | | | FECES | | | RATIO URIN./FECAL P | | |
|---|---|---|---|---|---|---|---|---|---|
| | CP 1 | EP | CP 2 | CP 1 | EP | CP 2 | CP 1 | EP | CP 2 |
| PA | 0.96 | 0.85 | 0.89 | 1.98 | 2.13 | 2.27 | 49.3 | 41.9 | 39.9 |
| PD | 0.85 | 1.18 | 0.86 | 2.12 | 2.24 | 2.24 | 41.2 | 54.4 | 38.9 |
| PG | 0.86 | 0.84 | 0.87 | 1.96 | 2.08 | 2.20 | 44.6 | 42.5 | 40.9 |
| | | | | (All values in mg/d/weight $^{2/3}$) | | | | | |
| GROUP | CP 1 – EP | EP – CP 2 | EP – CP 1+2 | CP 1 – EP | EP – CP 2 | EP – CP 1+2 | CP 1 – EP | EP – CP 2 | EP – CP 1+2 |
| PA | **0.020** | *0.072* | **0.013** | **0.026** | **0.0035** | *0.466* | **0.002** | *0.855* | *0.086* |
| PD | **<0.0001** | **<0.0001** | **<0.0001** | **0.0026** | *0.820* | **0.049** | **<0.0001** | **<0.0001** | **<0.0001** |
| PG | *0.796* | *0.616* | *0.630* | *0.678* | **0.0022** | *0.066* | *0.438* | *0.178* | *0.683* |
| PA – PD | **0.0002** | **0.0006** | — | *0.574* | *0.161* | — | **0.0006** | *0.065* | — |
| PA – PG | *0.178* | *0.610* | — | *0.412* | *1.000* | — | *0.094* | *0.760* | — |



0264

doubtfully increased by the PD diet. The evidence for the PG diet is not very clear: in table 10 a tendency to an increased balance is indicated, but in table 12 no significant lowering of the urinary or fecal phosphorus excretion is apparent. Comparing the changes in the balance for group PA and PG we found no significant difference : CP 1 — EP k = *0.195*

EP — CP 2 k = *0.539*

We are therefore inclined to believe that the diet PG did not change either the balance or the excretion of phosphorus.

TABLE 13

*Phosphorus excretion (second experiment)*

| GROUP | URINE | FECES | RATIO URIN./FECAL P |
|---|---|---|---|
| PA | 0.99 | 1.90 | 0.56 |
| PD | 1.20 | 1.89 | 0.65 |
| k | *0.130* | *0.646* | *0.105* |

In the second metabolism experiment the same increase of the urinary phosphorus excretion was found the for PD diet (table 13), although it was not significant this time. There was no change in the fecal excretion in this case. The ratio of urinary to fecal phosphorus excretion was increased in both experiments, but significantly only in the first experiment.

Summarizing we can say the phosphoric acid increased the phosphorus

TABLE 14

*Calcium balance (first experiment)*

| GROUP | CP 1 | EP | CP 2 |
|---|---|---|---|
| PA | 0.59 | 0.46 | 0.51 |
| PD | 0.45 | 0.44 | 0.43 |
| PG | 0.56 | 0.57 | 0.58 |
| | (All values in mg/d/weight$^{2/3}$) | | |
| **GROUPS** | **CP 1** | **EP** | **CP 2** |
| PA - PD | *0.130* | *0.646* | *0.256* |
| PA - PG | *0.760* | *0.214* | *0.612* |
| **GROUP** | **CP 1 - EP** | **EP - CP 1** | **EP - CP 1+2** |
| PA | 0.026 | *0.110* | 0.015 |
| PD | *0.469* | *0.649* | *0.475* |
| PG | *0.756* | *1.000* | *0.889* |
| PA - PD | *0.074* | *0.442* | — |
| PA - PG | 0.010 | *0.955* | — |



balance and the urinary phosphorus excretion markedly, while the fecal phosphorus excretion was not increased or only very little. The citric acid caused no change of the balance and the excretion of phosphorus.

*5.1.3 Calcium metabolism.*

The calcium balance was determined in both experiments. The averages for each group and each period in the first experiment are given in table 14 (upper

TABLE 15

*Calcium balance (second experiment)*

| | 1ST WEEK | 2ND W. | 3RD W. | 4TH W. | 5TH W. | AVER. |
|---|---|---|---|---|---|---|
| PA | +0.34 | +0.53 | +0.62 | +0.69 | +0.83 | +0.60 |
| | +0.16 | +0.36 | +0.67 | +0.70 | +0.45 | +0.47 |
| | +0.10 | +0.61 | +0.74 | +0.61 | —0.17 | +0.38 |
| | +1.64 | +0.71 | +0.69 | +0.68 | +0.53 | +0.85 |
| | +0.38 | +0.57 | +0.57 | +0.40 | +0.47 | +0.48 |
| | +0.46 | +0.71 | +1.07 | +0.51 | +0.31 | +0.61 |
| | +0.15 | +0.45 | +0.64 | +0.27 | +0.28 | +0.36 |
| | +0.37 | +0.39 | +0.37 | +0.63 | +0.31 | +0.41 |
| | | | | | | +0.52 |
| PD | +0.09 | +0.34 | +0.91 | +0.62 | +0.47 | +0.49 |
| | +0.42 | +0.58 | +1.01 | +0.39 | +0.80 | +0.64 |
| | +0.51 | +0.45 | +0.90 | +0.75 | +0.82 | +0.69 |
| | +0.44 | +0.52 | +0.85 | +0.78 | +1.13 | +0.74 |
| | +0.80 | +0.36 | +0.99 | +0.28 | +0.40 | +0.57 |
| | +0.16 | +0.67 | +1.24 | +0.44 | +0.49 | +0.60 |
| | +0.99 | +0.53 | +0.91 | +0.52 | +0.45 | +0.68 |
| | +0.24 | +0.59 | +0.83 | +0.54 | +0.47 | +0.53 |
| | | | | | | +0.62 |

(All values in mg/d/weight $^{2/3}$)

part). In the lower parts the results of the statistical comparison are represented (k – values). It is clear that the acid diets did not lower the calcium balance. A significant decrease occurred however in the balance of the PA group during the experimental period. Consequently a significant difference is found on comparison of the changes between the first control period and the experimental period for the PA and PG groups (bottom row in table 14). For the PD group the corresponding difference is almost significant. It is not certain if these differences are due to an abnormality in the calcium balances of the control group PA or to an increase of the calcium balance for the groups PD and PG during the experimental period, superimposed on a general decrease of the



0264

calcium balance in all three groups during this period. In this respect it is interesting to notice that in the second experiment also a slight tendency towards an increased calcium balance for the PD group — though not significant (k = *0.177*) — was found (table 15). Combination of the bilateral tail errors for both experiments (*0.074* for the first and *0.177* for the second experiment) according to the method described under [3.4.5] yielded a result that is just significant: **0.046.**

In table 16 we give the urinary and fecal excretion of calcium found in the first

TABLE 16

*Calcium excretion (first experiment)*

| GROUP | URINE | | | FECES | | |
|---|---|---|---|---|---|---|
| | CP 1 | EP | CP 2 | CP 1 | EP | CP 2 |
| PA | 3.5 | 4.1 | 5.0 | 1.93 | 2.21 | 2.23 |
| PD | 2.3 | 3.2 | 5.0 | 2.10 | 2.25 | 2.26 |
| PG | 2.3 | 3.6 | 4.7 | 1.91 | 2.14 | 2.30 |
| | (In $10^{-2}$ mg/d/weight $^{2/3}$) | | | (In mg/d/weight $^{2/3}$) | | |
| GROUP | CP 1 – EP | EP – CP 2 | EP – CP 1+2 | CP 1 – EP | EP – CP 2 | EP – CP 1+2 |
| PA | *0.079* | **0.0057** | *0.662* | <0.0001 | *0.963* | **0.016** |
| PD | **0.024** | <0.0001 | *0.165* | <0.0001 | *0.622* | **0.0087** |
| PG | <0.0001 | **0.0054** | *0.590* | **0.0041** | **0.029** | *0.838* |
| PA – PD | *0.610* | *0.161* | — | *0.919* | *0.760* | — |
| PA – PG | *0.234* | *0.959* | — | *0.382* | *0.161* | — |

experiment. In all groups both the urinary and the fecal excretion increased regularly from the beginning to the end of the experiment. In no case a significant difference in the increase between two groups existed (bottom rows of table 16). Similar results were obtained in the second experiment (table 17). The fecal excretion for the PD group was lower — though not significantly — than for the PA group. This decrease (0.17 mg/d/weight $^{2/3}$) is of the same order of magnitude as the increase of the calcium balance found in this experiment (0.10 mg/d/weight $^{2/3}$). In table 17 the ratio of calcium to phosphorus in the feces is also given. This ratio tends to decrease, though not significantly. This would point to an increased fecal excretion of acid calcium phosphates instead of $Ca_3(PO_4)_2$.



Summarizing we found a probable but not quite certain increase of the calcium balance for both acids and no appreciable changes in the excretion that could be attributed to the acid diets. The increase of the calcium balance, if real,

TABLE 17

*Calcium excretion (second experiment)*

| GROUP | URINE | FECES | CA/P IN FECES |
|---|---|---|---|
| PA | 3.0 | 2.03 | 1.13 |
| PD | 2.7 | 1.86 | 1.00 |
| | ($10^{-2}$ mg/d/weight $^{2/3}$ | (mg/d/weight $^{2/3}$ | |
| k | *0.195* | *0.146* | *0.083* |

could be ascribed to a slightly increased food intake during the experimental period without a correspondingly increased excretion (first experiment) as well as to a slightly decreased fecal calcium excretion (second experiment).

TABLE 18

*Nitrogen balance*

| | 1ST WEEK | 2ND W. | 3RD W. | 4TH W. | 5TH W. | AVER. |
|---|---|---|---|---|---|---|
| PA | +2.27 | +1.33 | +0.91 | +1.40 | +2.11 | +1.60 |
| | +0.92 | +1.31 | —1.72 | +3.95 | +2.38 | +1.37 |
| | +2.10 | +2.76 | +2.75 | +1.81 | +1.31 | +2.15 |
| | +2.88 | +2.13 | +1.39 | +1.92 | +2.03 | +2.07 |
| | +1.29 | +4.04 | +1.00 | +1.67 | +2.39 | +2.08 |
| | +0.69 | +1.32 | +0.87 | +0.97 | +1.37 | +1.04 |
| | +0.83 | +1.79 | +1.07 | +1.12 | +0.87 | +1.14 |
| | +1.12 | +1.49 | +3.26 | +2.93 | +1.73 | +2.11 |
| | | | | | | +1.70 |
| PD | —0.92 | +1.79 | +1.69 | +1.96 | +1.35 | +1.17 |
| | +1.28 | +1.82 | +1.64 | +1.68 | +1.65 | +1.61 |
| | —1.29 | +3.52 | +2.84 | +3.06 | +2.23 | +2.97 |
| | +1.11 | +1.72 | +1.58 | +0.22 | +2.52 | +1.43 |
| | +0.53 | +0.95 | +1.44 | +0.88 | +0.66 | +0.89 |
| | +1.82 | +2.72 | +2.20 | +1.91 | +2.12 | +2.15 |
| | +1.67 | +0.87 | +1.91 | +0.97 | +1.89 | +1.46 |
| | +2.35 | +3.54 | —0.72 | +2.69 | +0.31 | +1.63 |
| | | (All values in mg/d/weight $^{2/3}$) | | | | +1.55 |



0264

### 5.1.4 *Nitrogen metabolism.*

The nitrogen balance was determined in the second experiment only. The results are given in table 18. It should be kept in mind that these are somewhat high due to loss of fecal ammonia on drying of the feces as described under [3.3.6.4]. The diet PD had no effect on the nitrogen balance, as the statistical

TABLE 19

*Ammonia and urea in urine (first experiment)*

| GROUP | AMMONIA – N | | | AMMONIA + UREA – N | | |
|---|---|---|---|---|---|---|
| | CP 1 | EP | CP 2 | CP 1 | EP | CP 2 |
| PA | 0.190 | 0.205 | 0.232 | 4.21 | 4.05 | 4.81 |
| PD | 0.219 | 0.342 | 0.266 | 3.85 | 4.08 | 4.60 |
| PG | 0.192 | 0.220 | 0.222 | 3.98 | 4.08 | 5.04 |
| | (mg/d/weight $^{2/3}$) | | | (mg/d/weight $^{2/3}$) | | |
| GROUP | CP 1 – EP | EP – CP 2 | EP – CP 1+2 | CP 1 – EP | EP – CP 2 | EP – CP 1+2 |
| PA | *0.267* | 0.0080 | *0.340* | *0.139* | <0.0001 | 0.00014 |
| PD | <0.0001 | <0.0001 | <0.0001 | *0.714* | 0.0025 | *0.089* |
| PG | *0.119* | *0.749* | *0.259* | 0.027 | <0.0001 | *0.216* |

comparison taught us (k = *0.959*). It is very improbable that this result would have been different if no loss of fecal ammonia would have occurred, as the nitrogen metabolism is chiefly regulated by the kidney in man (PETERS and VAN SLYKE, 1932, p.269-272) as well as in the rat (GRIFFITH and FARRIS, 1942, p.183).

In both experiments the urinary excretion of ammonia and urea was determined. The results for the first experiment are given in table 19. There was a very marked rise in the ammonia excretion during the experimental period for group PD, but not for group PG. It is more difficult to draw a conclusion concerning the effect of the diet on the sum of the ammonia and urea nitrogen excretion. Comparison of the changes between the periods for the different groups of rats (see under [5.1.2.]) revealed no significant differences however, as will be apparent from the corresponding bilateral tail errors given below:

| GROUPS | CP 1 – EP | EP – CP 2 |
|---|---|---|
| PA – PD | *0.442* | *0.412* |
| PA – PG | *0.063* | *0.867* |

This is in good agreement with the fact that in the other experiment no change in the nitrogen balance was found, as the variations in the total nitrogen ex-



cretion are mainly due to changes in the urinary ammonia and urea excretion (see e.g. SMITH, 1926).

In table 20 the average ammonia and urea excretion in the second experiment are given. Here the excretion of urea instead of the total ammonia and urea

TABLE 20

*Ammonia and urea in urine (second experiment)*

| GROUP | AMMONIA – N | UREA – N |
|---|---|---|
| PA | 0.207 | 4.00 |
| PD | 0.294 | 3.58 |
| | (All values in mg/d/weight $^{2/3}$) | |
| k | 0.0087 | *0.130* |

excretion was compared in order to see more clearly if the rise in the ammonia excretion was accompanied by a corresponding decrease in the urea excretion. While the change for ammonia was significant, the decrease of the urea excretion was not significant. There is however a tendency to a decrease, which is sufficient to compensate the increased ammonia excretion.

Our results can be summarized as follows. The phosphoric acid had no effect on the nitrogen balance and the total ammonia and urea excretion, but a very marked increase of the ammonia excretion occurred. There was an indication for a parallel decrease of the urea excretion. The citric acid had no effect at all on the nitrogen metabolism.

### 5.1.5. *Total base excretion.*

In the first experiment the urinary total base content was determined in order to see whether the acid diet caused an increased excretion of fixed base. With the ion exchange method used in our investigation (see under [3.3.5.6.]) we also determined ammonia beside Na, K, Ca and Mg. Therefore we subtracted from the total base content found the ammonia content to obtain the fixed base concentration of the urine. The averages for each group and period, expressed in milliequivalents per day and weight $^{2/3}$, are given in table 21.

From the results of the statistical comparison, given in the lower part of the table, it is clear that no increase in the urinary fixed base excretion occurred, for either of the groups PD and PG during the experimental period.

### 5.1.6. *Citric acid excretion.*

In the first experiment we determined the urinary citric acid excretion for the PA and PG group during all periods. The averages of the results, expressed in mg/d of citric acid and in % of the intake, are given in table 22. The bilateral



0264

tail errors given in the lower part of the table belong to the excretion in mg/d. These values show that the addition of 1.2% of citric acid to the diet of group PG during the experimental period did not increase the urinary citric acid excretion significantly if compared to the control group PA.

TABLE 21

*Fixed base excretion*

| GROUP | CP 1 | EP | CP 2 |
|---|---|---|---|
| PA | 9.6 | 9.5 | 9.6 |
| PD | 9.2 | 9.5 | 9.5 |
| PG | 8.8 | 9.2 | 9.7 |
| | (All values in $10^{-2}$ meq/d/weight $^{2/3}$) | | |
| **GROUP** | **CP 1 – EP** | **EP – CP 2** | **EP – CP 1+2** |
| PA | *0.350* | *0.101* | *0.122* |
| PD | *0.276* | *0.523* | *0.924* |
| PG | *0.150* | *0.216* | *0.662* |
| PA – PD | *0.959* | *1.000* | — |
| PA – PG | *0.161* | *0.955* | — |

The basal diet PA contained 0.051% of citric acid in the form of sodium citrate. This was included in the total intake of citric acid in calculating the relative excretion of citric acid. The very large decrease of the relative citric acid excretion for group PG during the experimental period confirms that the oxidative degradation of citric acid by the body can be almost complete.

Interesting results were obtained in other experiments, when the citric acid excretion was determined for groups of young animals, receiving the diets PA,

TABLE 22

*Citric acid excretion*

| GROUP | CP 1 | | EP | | CP 2 | |
|---|---|---|---|---|---|---|
| | mg/d | % | mg/d | % | mg/d | % |
| PA | 6.1 | 76 | 8.4 | 99 | 11.6 | 126 |
| PG | 5.7 | 67 | 11.0 | 5.3 | 12.2 | 137 |
| **GROUP** | **CP 1 – EP** | | **EP – CP 2** | | **EP – CP 1+2** | |
| PA | <0.0001 | | 0.039 | | *0.219* | |
| PG | <0.0001 | | 0.016 | | *0.230* | |
| PA – PG | *0.758* | | *0.805* | | — | |



PE, PF and PG (citric acid content 0.051, 0.20, 0.50 and 1.25% resp.) during periods of from 6 to 23 weeks. The results are given in table 23, being averages for 6 animals each in the case of the 6 and 12 weeks groups and for 3 animals each of the 23 weeks groups. Statistical examination proved the excretion in mg/d for the groups PF and PG to be significantly higher than that for the

TABLE 23

*Citric acid excretion*

| GROUP | 6 WEEKS | | 12 WEEKS | | 23 WEEKS | | AVERAGE | |
|---|---|---|---|---|---|---|---|---|
| | mg/d | % | mg/d | % | mg/d | % | mg/d | % |
| PA | 5.03 | 73 | 4.91 | 58 | 5.97 | 113 | 5.30 | 81 |
| PE | 4.27 | 14.4 | 2.52 | 9.6 | 4.45 | 13.0 | 3.75 | 12.2 |
| PF | 7.67 | 9.7 | 8.47 | 11.9 | 7.07 | 7.8 | 7.72 | 9.8 |
| PG | 10.36 | 5.8 | 10.12 | 5.5 | 9.47 | 5.4 | 9.98 | 5.6 |

control group PA (k = **0.011** and **0.0016** resp.), while the excretion for the PE group did not differ significantly (k = *0.131*). So in the case of young rats the degradation of the citric acid ingested was not large enough to prevent the citric acid excretion from rising, when the diet contained 0.50% or more of the acid. The excess excreted in the urine was however only about 3% of the amount of citric acid added to the diet.

5.1.7. *Titratable acidity, pH, volume and chloride content of the urine.*

In both metabolism experiments the titratable acidity and pH of the urine were determined. The averages for each group and each period for the first experiment are given in table 24. In the lower part of table 24 the bilateral tail errors are represented as usual.

TABLE 24

*Urinary titratable acidity and pH (first experiment)*

| GROUP | TITRATABLE ACIDITY | | | pH | | |
|---|---|---|---|---|---|---|
| | CP 1 | EP | CP 2 | CP 1 | EP | CP 2 |
| PA | 1.01 | 1.17 | 1.75 | 5.88 | 5.90 | 5.80 |
| PD | 1.11 | 1.75 | 1.76 | 5.91 | 5.69 | 5.75 |
| PG | 0.93 | 1.33 | 1.60 | 5.92 | 5.78 | 5.71 |
| | ($10^{-2}$ meq/d/weight $^{2/3}$) | | | | | |
| **GROUP** | **CP 1 – EP** | **EP – CP 2** | **EP – CP 1+2** | **CP 1 – EP** | **EP – CP 2** | **EP – CP 1+2** |
| PA | *0.317* | <0.0001 | 0.012 | *0.617* | 0.044 | *0.124* |
| PD | <0.0001 | *0.775* | 0.0026 | <0.0001 | 0.046 | 0.00027 |
| PG | <0.0018 | 0.0037 | *1.000* | 0.0017 | *0.247* | *0.223* |



0264

The pH was decreased markedly during the experimental period for group PD only. For all groups there was a gradual decrease in the pH from the beginning to the end of the experiment. To this general decrease the difference in pH for group PG between the first control period and the experimental period

TABLE 25

*Urinary titratable acidity, pH and volume (second experiment)*

| GROUP | TITR. ACID. | pH | VOLUME | |
|---|---|---|---|---|
| | | | ml/d | ml/d/weight $^{2/3}$ |
| PA | 1.12 | 5.89 | 47.3 | 1.31 |
| PD | 1.46 | 5.66 | 37.9 | 1.05 |
| | ($10^{-2}$ meq/d/weight $^{2/3}$) | | | |
| k | 0.013 | 0.0019 | 0.028 | *0.161* |

apparently has to be ascribed. Similar changes occurred in the titratable acidity. Here a gradual rise from the beginning to the end was observed. While the titratable acidity for the control group PA was significantly decreased during the experimental period compared to both control periods (1.17 against 1.38; k = 0.012), it was increased significantly for group PD (1.75 against 1.43;

TABLE 26

*Daily urinary volume (first experiment)*

| GROUP | CP 1 | EP | CP 2 |
|---|---|---|---|
| PA | 49.8 ml/d | 52.1 ml/d | 58.6 ml/d |
| PD | 37.3 | 42.5 | 49.9 |
| PG | 34.1 | 41.5 | 50.0 |
| **GROUP** | **CP 1 – EP** | **EP – CP 2** | **EP – CP 1+2** |
| PA | *0.079* | 0.0019 | *0.362* |
| PD | 0.0048 | 0.0005 | *0.624* |
| PG | 0.012 | 0.0016 | *0.624* |
| PA – PD | *0.721* | *0.959* | — |
| PA – PG | *0.721* | *0.798* | — |

k = 0.0026). For group PG no change that can be attributed to the acid diet was observed (1.33 against 1.27; k = *1.000*).

Only the diet PD containing phosphoric acid increased the titratable acidity and decreased the pH of the urine. The diet PG containing citric acid had no effect on either of these, which is in accordance with the fact that this diet did not increase the urinary citric acid excretion (see under [5.1.6.]).



The average values of the titratable acidity and pH found in the second metabolism experiment are given in table 25.
They are in good accordance with the corresponding results from the first experiment. The titratable acidity was increased and the pH was lowered, both significantly.

TABLE 27

*Chloride excretion*

| GROUP | DIET DURING | | | |
|---|---|---|---|---|
| | 6 weeks | 12 weeks | 23 weeks | average |
| PA | 36.7 | 64.6 | 35.3 | 45.5 |
| PD | 31.9 | 70.7 | 76.7 | 59.8 |
| PG | 31.6 | 61.8 | 67.0 | 53.8 |

(All values in mg/d Cl)

In table 25 the average daily volume of the urine in the second metabolism experiment, expressed in ml/d and in ml/d/weight $^{2/3}$ is represented. There is no sign of a diuretic effect of the acid diet PD. On the contrary the daily urinary volume, expressed in ml/d, was significantly lower for the group PD. When expressed in ml/d/weight $^{2/3}$, which affords a better comparison, the difference was not significant. The corresponding results from the first metabolism experiment are given in table 26.
The statistical comparison shows the absence of a dietary effect on the daily urine production very clearly.

The chloride excretion was not affected either by the acids diets. In a special experiment we determined the daily chloride excretion for groups of young rats receiving the diets PA, PD, or PG during 6, 12 or 23 weeks. Each group consisted of three rats. The averages for each group are given in table 27. The statistical comparison of the three series together yielded the following bilateral errors:

PA—PD k = *0.208*
PA—PG k = *0.313*

*5.1.8. Fate of the ingested acids.*

In our metabolism experiments a fairly complete picture of the effects of the acid diets on the mineral and nitrogen metabolism was obtained. It is possible to conclude from the results described under [5.1.2.] to [5.1.7.] which mechanisms have been active in the neutralization and excretion of the phosphoric acid ingested.

In both experiments we found an increased urinary titratable acidity and ammonia excretion. We also found a phosphorus retention and probably a



0267

calcium retention. From the average results found it was possible to make a quantitative estimate of the amount of acid accounted for by these mechanisms in both experiments. The phosphoric acid was considered to be equivalent to 1.8 meq/mM. The results obtained are given below, expressed in $\mu$eq/d/weight$^{2/3}$:

| | FIRST EXPERIMENT | | SECOND EXPERIMENT | |
|---|---|---|---|---|
| Titratable acidity . . . . . | — | 5.2 $\mu$eq/d | — | 3.1 $\mu$eq/d |
| Ammonia excretion . . . . | 0.105 mg/d | 7.5 ,, | 0.087 mg/d | 6.2 ,, |
| Phosphorus retention . . . . | 0.15 ,, | 8.7 ,, | 0.15 ,, | 8.7 ,, |
| Calcium retention . . . . . | 0.09 ,, | 4.5 ,, | 0.10 ,, | 5.0 ,, |
| | | 25.9 $\mu$eq/d | | 23.0 $\mu$eq/d |
| Phosphoric acid intake . . . | | 30.7 $\mu$eq/d | | 30.0 $\mu$eq/d |
| Accounted for . . . . . | | 84% | | 77% |

The values for the first experiment were obtained taking into account the simultaneous changes for the control group PA. Considering the limited accuracy of the values used in this calculation we believe the conclusion that the mechanisms mentioned account for all of the phosphoric acid ingested to be allowed. In the second experiment the ratio of fecal Ca to P decreased from 1.13 to 1.00. This must have caused a saving of about 12.5 $\mu$eq/d of base. Part of these have been expressed in the calcium retention (5.0 $\mu$eq/d). The excess (7.5 $\mu$eq/d) would just about close the gap between the intake of acid and the quantity of acid accounted for.

An interesting result of our metabolism experiment is the phosphorus retention that occurred after ingestion of the diet PD containing phosphoric acid. The calcium retention which probably also occurred might account for about 33% of the phosphoric acid retained of $Ca_3(PO_4)_2$ and for about 98% if $Ca(H_2PO_4)_2$ would be formed. Another possibility would be that part of the retained phosphoric acid is used for the synthesis of organic phosphorus compounds. We shall return to this point when relating the results of the tissue analyses.

The only effect of the citric acid containing diet PG we found in our metabolism experiments was a doubtful calcium retention. If this was real, it might be caused by a deposition of calcium citrate in the bones (see also under [5.2.4.]). As the calcium retention amounted to about 3.4 mg/d this could account for 10.7 mg/d of citric acid only. Of the average daily intake of 200 mg of citric acid less than 3% or 6 mg/d were excreted in the urine. So probably at least 183 mg/d or 92% of the intake were oxidized in our experiments.

We should like to stress once more the approximative character of the calculations given above. They can only indicate the order of magnitude of the effects found.



## 5.2. Tissue analyses.

### 5.2.1. *Introduction.*

Several series of tissue analyses were carried out comprising the blood serum, liver, muscle, kidneys and tibiae of rats of different dietary groups after receiving the diets during increasing lengths of time. Table 28 gives a survey of the series of analyses. The first 5 series were done, when the

TABLE 28

*Tissue analyses*

| NR. OF SERIES | NR. OF RATS total | NR. OF RATS p. group | SEX | AGE | DIETARY GROUPS | DIET DURING | GENERATION |
|---|---|---|---|---|---|---|---|
| | | | | | PA, | 6 weeks | 1 st |
| 1 | 21 | 3 | ♂ | 9 weeks | PB, | | |
| 2 | 21 | 3 | ♀ | 9 ,, | PC, | 6 ,, | 1 ,, |
| 3 | 21 | 3 | ♀ | 15 ,, | PD, | 12 ,, | 1 ,, |
| 4 | 21 | 3 | ♂ | 15 ,, | PE, | | |
| 5 | 21 | 3 | ♂ | 26 ,, | PF, | 12 ,, | 1 ,, |
| | | | | | PG. | 23 ,, | 1 ,, |
| 6 | 36 | 12 | ♀ | 15 months | PA, PD, | 14 months | 1 ,, |
| 7 | 36 | 14, 15, 7 | ♂ | 6 ,, | PG. | 5 ,, | 2 nd |

results of the biological and metabolism experiments were not yet available. As we did not know if and if so, in what degree the acid diets would affect the animals we took animals from all dietary groups. When no harmful effects of the acid diets were found in the biological and metabolism experiments, we decided to use only the dietary groups PD and PG with the highest content of acid for the series 6 and 7. This enabled us to have larger groups.

The statistical analysis was executed at the Mathematical Centre, Statistical Department (director: Prof. Dr. D. van Dantzig), Amsterdam, under the supervision of Dr. J. Hemelrijk by Mr. J. van Klinken. The test applied was Wilcoxon's test (see under [3.4.2.]). In view of the small number of rats per group in the first 5 series the results for the groups PB, PC and PD were treated as one group and the same was done for the groups PE, PF and PG. Then these 5 series were tested in combination by adding Wilcoxon's $u$ and the variances $\sigma^2$ for all series as described under [3.4.2.]. The 6th and 7th series were tested separately. When the non-parametric test against trend, described under [3.4.4.], was applied to the results of the determinations within each of the dietary groups, a trend was found in a number of cases. As generally the determinations were carried out in the order: PA, (PB, PC,) PD, (PE, PF,) PG, this



0264

trend might cause a faulty result of the application of WILCOXON's test in these cases. At present a method is being worked out to apply a correction in these cases. Meanwhile our conclusions must have a preliminary character. Only those results are mentioned which could not have been caused by trend within the groups.

It should be kept in mind that the absence of evidence of a difference does not prove that there is really no difference. This reserve which should always be made with biological experiments is somewhat stronger in our case because the application of the correction mentioned above might reveal differences which have not yet been found. The conclusions drawn by the Mathematical Centre from the statistical analysis are printed in italics.

In view of the large number of analyses carried out it will be necessary to give only the average values for each group.

*5.2.2. Blood.*

All analyses were carried out in the blood serum which was obtained and handled as described under [3.3.1.1.]. The results from the determination of inorganic phosphorus and calcium are given in table 29. The numbers above the columns refer to the series of experiments outlined in table 28.

TABLE 29

*Inorganic phosphorus and calcium in blood serum*

| GROUP | MG % PHOSPHORUS | | | | | | MG % CALCIUM | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 6 | 7 | 1 | 2 | 4 | 5 | 6 | 7 |
| PA | 9.1 | 7.0 | 8.7 | 7.9 | 7.0 | 8.1 | 10.5 | 11.4 | 11.7 | 11.4 | 10.4 | 9.4 |
| PB | 9.2 | 8.4 | 8.1 | 8.5 | — | — | 10.7 | 11.8 | 11.9 | 11.4 | — | — |
| PC | 10.6 | 9.1 | 8.5 | 8.6 | — | — | 11.3 | 12.1 | 12.7 | 11.3 | — | — |
| PD | 9.0 | 9.6 | 8.5 | 8.9 | 6.3 | 7.7 | 10.0 | 11.4 | 12.1 | 10.8 | 10.7 | 8.8 |
| PE | — | 8.9 | — | 8.1 | — | — | 10.8 | 11.6 | 12.4 | — | — | — |
| PF | — | 9.1 | — | 8.2 | — | — | 9.1 | 9.7 | 11.8 | 10.1 | — | — |
| PG | 8.9 | 8.7 | 7.2 | 8.5 | 5.9 | 8.1 | 11.8 | 11.0 | 10.5 | 9.7 | 11.7 | 9.2 |

*From the statistical analysis it was concluded that the inorganic phosphorus content of the serum was increased by both acids in the series 1—4.*

The increase of the inorganic phosphorus by an ingestion of acid is in accordance with the findings of KAY (1924). With increasing age we found a decreasing phosphorus content for all groups:



9 weeks — 9.1 mg%
15 „ — 8.3 „
6 months — 8.0 „
15 „ — 6.4 „

Decreases of similar magnitude were found for man (DE TONI, 1933) and for the horse (PEARSON, 1934).

*The calcium content of the serum in the series 1–5 was lower in the animals receiving citric acid than in those receiving phosphoric acid.*

This finding might be similar to the hypocalcemia found on injection of sodium citrate in rats by LEADINGHAM and SANDERS (1938).

In table 30 the total base and chloride content of the serum are given.

The total base content, expressed in meq/ml, does not include the base bound to the serum bicarbonate, as was explained under [3.3.1.4.]. In a separate experiment the alkaline reserve of the blood serum was determined for 10 male rats of the PA group (12 months old, 1st generation), and 10 male rats of the PBB group (13 months old, 2nd generation). The group averages found were:

PA 70.7 vol. % (standard error: ± 2.2 vol. %)
PBB 71.4 vol. % ( „ „ : ± 2.0 vol. %)

There was no difference between the groups.

The values were higher than those found by HAWKINS (1924): 44-60 vol. %, but this will probably be caused by the fact that we used serum saturated with air containing 5% of $CO_2$, while Hawkins used whole blood kept under a layer of paraffin.

This result is very important, as it shows that even the extreme diet PBB (0.75% of phosphoric acid) did not cause acidosis in the second generation after one year.

TABLE 30

*Total base and chloride in blood serum*

| GROUP | MEQ/ML TOTAL BASE | | | | | | MG % CHLORIDE | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 | 3 | 5 | 6 | 7 |
| PA | 0.112 | 0.133 | 0.115 | 0.116 | 0.123 | 0.121 | 321 | 342 | 336 | 331 |
| PB | 0.115 | 0.134 | 0.118 | 0.116 | — | — | — | — | — | — |
| PC | 0.116 | 0.134 | 0.121 | 0.115 | — | — | — | — | — | — |
| PD | 0.118 | 0.150 | 0.116 | 0.116 | 0.120 | 0.116 | 331 | 346 | 333 | 333 |
| PE | 0.121 | — | 0.120 | 0.115 | — | — | — | — | — | — |
| PF | 0.118 | — | 0.123 | 0.117 | — | — | — | — | — | — |
| PG | 0.118 | 0.147 | 0.111 | 0.118 | 0.121 | 0.117 | 329 | 345 | 340 | 337 |



0264

*A slight indication for an increase of the total base content of the serum by both acids in the series 2–5 was found.*

*The chloride content of the serum was increased by the citric acid in the series 3–5.*

The alkaline phosphatase activity of the blood serum is represented in table 31. *There were no significant changes in any of the series attributable to either of the acids ingested.*

TABLE 31

*Serum alkaline phosphatase*

| GROUP | MILLIMOLE UNITS PER ML | | | | | |
|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 |
| PA | 12.7 | 12.2 | 10.9 | 2.6 | 6.0 | 18.7 |
| PD | 14.6 | 10.6 | 9.2 | 4.5 | 5.3 | 17.9 |
| PG | 11.6 | 8.8 | 12.5 | 7.7 | 5.1 | 15.8 |

Our findings for the blood serum may be summarized as follows. No important changes were found in total base content, alkaline reserve and in alkaline phosphatase activity for either of the acids. An increase of the inorganic phosphorus content by both acids was found for the younger animals. The serum chloride was very slightly increased for the younger animals by the citric acid.

TABLE 32

*Total weight of liver*

| GROUP | GRAMS PER 100 G BODYWEIGHT | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| PA | 4.75 | 4.60 | 3.94 | 4.13 | 3.30 | 3.41 | 3.79 |
| PB | 5.85 | 4.41 | 3.53 | 3.63 | 3.10 | — | — |
| PC | 6.16 | 5.04 | 3.44 | 3.87 | 3.45 | — | — |
| PD | 4.77 | 4.60 | 3.84 | 4.29 | 3.66 | 3.49 | 3.80 |
| PE | 4.86 | 4.28 | 3.62 | 4.02 | 3.64 | — | — |
| PF | 6.71 | 4.31 | 3.31 | 3.75 | 3.50 | — | — |
| PG | 5.15 | 4.68 | 3.43 | 3.74 | 3.99 | 3.46 | 3.66 |



*5.2.3. Liver and muscle.*

The total fresh weight of the liver was determined (table 32) and expressed in g per 100 g body weight. *No changes in any of the series were found for either of the acids.*

The total phosphorus content of liver and muscle were determined in all series. The results are given in table 33, expressed in mg of phosphorus per g of fresh

TABLE 33

*Total phosphorus in liver and muscle*

| GROUP | LIVER | | | | | | | MUSCLE | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| PA | 3.66 | 3.11 | 3.61 | 3.57 | 3.69 | 3.53 | 3.69 | 2.65 | 2.73 | 2.43 | 2.57 | 2.32 | 2.63 | 2.63 |
| PB | 3.71 | 2.99 | 3.86 | 3.57 | 3.67 | — | — | 2.78 | 2.50 | 2.43 | 2.60 | 2.32 | — | — |
| PC | 3.42 | 3.06 | 3.61 | 3.58 | 3.27 | — | — | 2.85 | 2.41 | 2.71 | 2.93 | 2.53 | — | — |
| PD | 3.66 | 3.12 | 3.66 | 3.53 | 3.48 | 3.46 | 3.60 | 2.85 | 2.55 | 2.59 | 2.63 | 2.58 | 2.57 | 2.63 |
| PE | 3.91 | 3.06 | 3.70 | 3.40 | 3.24 | — | — | 2.81 | 2.44 | 2.53 | 2.51 | 2.60 | — | — |
| PF | — | 3.08 | 3.91 | 3.46 | 3.29 | — | — | 3.02 | 2.42 | 2.31 | 2.51 | 2.44 | — | — |
| PG | 3.52 | 3.26 | 3.84 | 3.40 | 3.34 | 3.38 | 3.66 | 3.08 | 2.37 | 2.55 | 2.51 | 2.66 | 2.43 | 2.36 |

tissue. *No changes in the phosphorus content of the liver for either of the acids were found in any of the series. The phosphorus content of the muscle however was influenced by the diets. In the younger animals (series 1–5) it was increased by the phosphoric acid containing diets, while a decrease was found for the animals receiving citric acid in series 7. In series 7 the phosphorus content of the muscle for group PG was lower than for group PD.*

TABLE 34

*Total base, K and Na in liver and muscle*

| GROUP | LIVER | | | | | | | | MUSCLE | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | total base | | | | K | | Na | | total base | | | | K | | Na | |
| | 2 | 3 | 4 | 5 | 6 | 7 | 6 | 7 | 2 | 3 | 4 | 5 | 6 | 7 | 6 | 7 |
| PA | 178 | 168 | 144 | 129 | 78 | 81 | 40 | 29 | 181 | 172 | 164 | 155 | 94 | 98 | 23 | 23 |
| PD | 163 | 168 | 139 | 162 | 75 | 80 | 40 | 33 | 172 | 185 | 153 | 169 | 95 | 96 | 23 | 25 |
| PG | 183 | 160 | 134 | 130 | 73 | 81 | 37 | 31 | 178 | 174 | 164 | 141 | 91 | 85 | 23 | 28 |



0267

In the series 2–5 the total base content of both liver and muscle was determined, while in the series 6 and 7 the potassium and sodium content were determined. The results are compiled in table 34; they are expressed in $\mu$eq/g of fresh tissue.

*In the series 2–5 the total base content of the liver was higher for the phosphoric acid receiving groups than for those receiving citric acid. In the series 6 and 7 the potassium content of the liver was not influenced by the acids ingested. A slight*

TABLE 35

*Chloride in liver*

| GROUP | 1 | 3 | 5 |
|---|---|---|---|
| PA | 61 | 56 | 97 |
| PD | 72 | 49 | 93 |
| PG | 60 | 52 | 105 |

*indication for a decrease of the sodium content of the liver by citric acid was found.*

*The potassium content of the muscle was decreased by the citric acid in series 7; it was also lower in the group PG than in the group PD. In this series the sodium content of the muscle was increased by both acids. This increase was larger in the case of the group PG than for the group PD.*

For the series 6 and 7 the ratio $PO_4/(K+Na)$ in liver and muscle was calculated, in meq/meq:

| GROUP | LIVER | | MUSCLE | |
|---|---|---|---|---|
| | 6 | 7 | 6 | 7 |
| PA | 0.95 | 1.08 | 0.73 | 0.70 |
| PD | 0.97 | 1.03 | 0.71 | 0.70 |
| PG | 0.99 | 1.05 | 0.68 | 0.67 |

A decrease of this ratio was found in muscle for the PG group. This proves that the retained phosphorus in the case of the PD group (see under [5.1.2.]) was not stored in organic phosphorus compounds in the soft tissues. The decrease of this ratio for the PG group might be explained by a partial exchange of phosphate ions of the muscle for citrate ions. Another possible explanation would be that the phosphate of the muscle is mobilized for the oxidation of the citric acid ingested.

The chloride content of the liver was determined in the series 1, 3 and 5; the



results, expressed in mg %, are given in table 35. *No significant differences between the dietary groups were found.*

Summarizing we can say that no important effects of the acid diets on the total weight, as well as on the total phosphorus, potassium, sodium and chloride contents of the liver were found. The total phosphorus content of the muscle was increased by phosphoric acid in the younger animals and decreased by citric acid in the older animals. Finally the sodium content of the muscle was probably raised by both acids in the animals of the second generation. The fact that generally no decrease of the total base content or of the sum of potassium and sodium of liver and muscle was found is in accordance with the absence of an increased total fixed base excretion in the urine (see under [5.1.5.]).

*5.2.4. Kidney.*

In all series the fresh weight, the water content and the ash content of one of the kidneys were determined, while the other one was used for the determination of the alkaline phosphatase activity. The weights, expressed in mg per 100 g

TABLE 36

*Weight of kidney*

| GROUP | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| PA | 437 | 456 | 383 | 437 | 362 | 384 | 373 |
| PB | 533 | 429 | 394 | 349 | 337 | — | — |
| PC | 534 | 457 | 369 | 383 | 354 | — | — |
| PD | 538 | 433 | 392 | 397 | 348 | 377 | 389 |
| PE | 485 | 406 | 383 | 383 | 353 | — | — |
| PF | 600 | 413 | 347 | 356 | 373 | — | — |
| PG | 536 | 435 | 408 | 344 | 372 | 393 | 391 |

of body weight, are given in table 36. In table 37 the water content and the ash content of the kidney are given.

*No differences were found in the weight of the kidneys that could be attributed to the acid diets. The ash content was neither changed in any of the series. The water content was decreased in the series 2–5 by both acids.*

The decrease of the renal water content in series 2–5 is difficult to interpret, as there was no parallel increase of the ash content. This would have been the case if the decrease of the water content was due to calcification of the kidney. Moreover the histological examination revealed no renal abnormalities due



0264

to the acid diets that might have any relation to this finding (see under [4.5]).

The results of the determination of the renal alkaline phosphatase activity, expressed in millimole units per mg of fresh tissue, are given in table 38. *No significant differences were found in the series 2–5 and 7.*

TABLE 37

*Water content and ash content of kidney*

| GROUP | % WATER | | | | | | % ASH | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 | 1 | 2 | 4 | 5 | 6 | 7 |
| PA | 78.8 | 78.0 | 77.5 | 77.5 | 76.8 | 76.6 | 1.67 | 1.18 | 1.15 | 1.25 | 1.46 | 1.16 |
| PB | 77.2 | 77.7 | 76.8 | 76.9 | — | — | 1.35 | 1.33 | 1.25 | 1.20 | — | — |
| PC | 78.0 | 77.0 | 76.8 | 75.3 | — | — | 1.42 | 1.38 | 1.38 | 1.21 | — | — |
| PD | 78.8 | 75.5 | 77.6 | 75.8 | 77.1 | 76.6 | 1.29 | 1.05 | 1.19 | 1.22 | 1.54 | 1.12 |
| PE | 77.1 | 76.7 | 77.3 | 75.2 | — | — | 1.30 | 1.10 | 1.18 | 1.30 | — | — |
| PF | 77.5 | 75.7 | 76.5 | 77.0 | — | — | 1.43 | 1.14 | 1.31 | 1.07 | — | — |
| PG | 77.7 | 78.0 | 76.1 | 76.3 | 77.9 | 77.0 | 1.37 | 1.10 | 1.34 | 1.31 | 1.34 | 1.14 |

Summarizing our findings for the kidney we are able to say that no effects of the acid diets were found on the weight and the ash content. The water content, while not affected in the older animals (series 6 and 7), was decreased by both acids in the younger animals (series 2–5). No effect on the renal alkaline phosphatase activity was found.

TABLE 38

*Renal alkaline phosphatase activity*

| GROUP | MILLIMOLE UNITS PER MG | | | | | |
|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 |
| PA | 2.99 | 1.88 | 2.71 | 2.31 | 1.09 | 2.75 |
| PD | 3.07 | 1.75 | 2.42 | 1.28 | 0.82 | 2.31 |
| PG | 2.99 | 1.73 | 2.66 | 1.74 | 0.78 | 2.46 |

*5.2.5 Bone.*

Of the tibia the ash content, as well as the calcium and the phosphorus content of the ash were determined. Table 39 gives the ash contents, expressed in % of the dry, alcohol-ether extracted bone. *In series 6 the ash content was*



*decreased significantly by both acids. There was a slight indication that the decrease was larger for group PD than for group PG.*

The decrease of the ash content in the case of series 6 is rather remarkable, as we found no evidence of an increased calcium and total base excretion in the metabolism experiments and even an indication of a slight calcium retention for the group PD (see under [5.1.3]). Also in the literature no cases of a demineralizing effect of an acid diet on the skeleton were found. To obtain additional

TABLE 39

*Ash content of the tibia*

| GROUP | % OF DRY WEIGHT | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| PA | 59.8 | 59.8 | 59.6 | 64.2 | 60.8 | 60.8 | 64.3 |
| PB | 58.7 | 59.1 | 58.0 | 63.7 | 60.5 | — | — |
| PC | 55.9 | 62.7 | 65.7 | 64.5 | 62.4 | — | — |
| PD | 56.0 | 60.3 | 62.7 | 67.0 | 59.8 | 58.0 | 62.7 |
| PE | 59.3 | 61.5 | 62.5 | 64.4 | 56.3 | — | — |
| PF | 56.3 | 62.3 | 60.5 | 64.8 | 61.6 | — | — |
| PG | 57.6 | 60.8 | 65.1 | 64.0 | 59.8 | 58.2 | 63.3 |

evidence we determined the ash content of the tibia of 30 rats of the PBB group (15 males and 15 females of the 3rd generation, age 9 months, diet during 8 months), taking special precautions against faulty results. The crucibles with bones were distributed at random in the electrical furnace; they were first heated for one night at 600° C and weighed and then heated for another night at 600° C and weighed again. There were no differences larger than a few tenths of a milligram after the second night, so ashing was complete within one night. The average ash content found was:

PBB, 3rd gener., ♂♂ – 63.4%
♀♀ – 63.9%
♂+♀ – 63.7%

When these results were compared with those for the PA group of series 7 (2nd generation, males, age 6 months, diet during 5 months) no difference was found.

The results of the calcium and phosphorus determinations in the bone ash, expressed in % of the ash, are given in table 40. No changes in the phosphorus



0264

content were found for either of the acids in any series. There was a slight indication that the calcium content was increased by citric acid in series 1-5 and 6. In series 7 it was increased for the group PD.

TABLE 40

*Calcium and phosphorus in bone ash*

| GROUP | CALCIUM | | | | | | PHOSPHORUS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 4 | 5 | 6 | 7 | 1 | 2 | 4 | 5 | 6 | 7 |
| PA | 29.6 | 35.9 | 32.6 | 36.6 | 34.4 | 34.2 | 18.9 | 19.1 | 19.1 | 19.2 | 19.3 | 19.2 |
| PB | 32.8 | 38.7 | 34.3 | 39.0 | — | — | 17.5 | 20.6 | 19.3 | 19.1 | — | — |
| PC | 35.1 | 35.1 | 32.5 | 38.8 | — | — | 19.3 | 18.9 | 19.0 | 19.8 | — | — |
| PD | 29.5 | 37.7 | 31.9 | 36.5 | 33.4 | 35.6 | 19.2 | 19.5 | 19.4 | 19.6 | 19.2 | 19.0 |
| PE | 34.0 | 37.5 | 33.1 | 38.4 | — | — | 19.2 | 19.6 | 18.9 | 19.4 | — | — |
| PF | 32.6 | 35.9 | 33.3 | 38.6 | — | — | 18.6 | 19.1 | 19.7 | 19.2 | — | — |
| PG | 28.0 | 36.1 | 32.9 | 39.9 | 36.9 | 34.7 | 19.4 | 19.0 | 19.6 | 20.0 | 19.2 | 19.3 |

For series 6 and 7 we calculated the ratio Ca/P in the bone ash and found:

| GROUP | 6 | 7 |
|---|---|---|
| PA | 1.78 | 1.79 |
| PD | 1.74 | 1.87 |
| PG | 1.94 | 1.75 |

We found an increase for the PG group in series 6. From this fact and from the decreased ash content in series 6, it seems to be possible that part of the phosphate of the bone was replaced by citrate in the rats fed citric acid.

Citrate was found to be a regular component of the bone by DICKENS (1941), who demonstrated the presence of as much as 70% of the body content of citrate in the hard substance of bone.

More work on this subject seems to be needed before more definite conclusions concerning the possible effects of the acid diets on the composition of the bone may be drawn. If existent, these effects are rather small, judging from the results we obtained.

## 5.3 EXPERIMENTS WITH RADIOACTIVE PHOSPHORUS.

### *5.3.1 Endogenous fecal phosphorus excretion.*

In this experiment the percentage of endogenous phosphorus in the total fecal phosphorus excretion was determined. Of each of the groups PA, PD and PG 10



female rats (age 7 months, diet during 6 months) were used. A week after intraperitoneal injection of 20 $\mu$C of radioactive phosphate we started to collect the urine and feces for two consecutive periods of seven days. Of the urine and feces collected the specific activities were determined, their ratio giving the percentage of endogenous phosphorus. In this way we obtained two values for each animal.

The results obtained are given in table 41, expressed in % of total fecal phosphorus. Also represented are the ratio of the urinary to fecal activity ex-

TABLE 41

*Endogenous fecal phosphorus excretion*

| GROUP | %ENDOG. FECAL P | | URIN./FECAL ACTIV. | | URINE/FECAL P | | TOTAL ACTIV./DAY | |
|---|---|---|---|---|---|---|---|---|
| | 1st w. | 2nd w. | 1st w. | 2nd w. | 1st w. | 2nd w. | 1st w. | 2nd w. |
| PA | 20.1 | 22.1 | 2.09 | 1.85 | 0.40 | 0.38 | 0.30 | 0.15 |
| PD | 25.9 | 20.8 | 2.65 | 2.73 | 0.50 | 0.50 | 0.31 | 0.15 |
| PG | 32.6 | 19.2 | 2.34 | 1.88 | 0.48 | 0.35 | 0.37 | 0.16 |

creted per day, the ratio of urinary to fecal phosphorus excretion per day as well as the total activity excreted per day (in $\mu$C/d). The values given are averages for each group. No significant differences were found for either of the acid diets in the percentage of endogenous fecal phosphorus, in the ratio of urinary to fecal activity excreted per day and in the total activity excreted per day. Only the ratio urinary to fecal phosphorus excretion per day for the PD group was increased significantly, a fact found already in our metabolism experiments (see under [5.1.2]). There is however a tendency towards a parallel increase in the ratio of the activities excreted daily in urine and feces.

### *5.3.2 Phosphorus retention of the bone.*

The phosphorus retention of the tibia was determined for 33 male rats of the groups PA, PD, and PG. Of each group there were 11 animals, aged 13 months, who received the diets during 12 months previously. The animals were injected intraperitoneally with 50 $\mu$C of radioactive phosphate and after 24 hrs. urine samples of all animals were collected by means of light ether anesthesia. Of the urine samples the specific activity was determined in order to see if no faulty injections (into gut or bladder) had taken place. As all specific activities were found to ly between 0.10 and 0.27 $\mu$C/mg of phosphorus, the injections were carried out rightly.

One hindleg was amputated under ether anesthesia and with ordinary sur-



0297

gical precautions[1]) on the day after the injection. Apart from two animals of the PA group who died from an overdosage of ether and who were replaced by two others from this group, no animals died during and after the operation. Wound healing and recovery from surgical shock were complete within 10 days after the operation. The animals were kept for 6 weeks on the ordinary diets and were then killed. At the site of operation no abnormalities were found but

TABLE 42

*Phosphorus retention of the bone*

| PA | PD | PG |
|---|---|---|
| 127% | 99% | 58% |
| 61 | 98 | 96 |
| 71 | 104 | 93 |
| 50 | 43 | 64 |
| 58 | 52 | 47 |
| 70 | 54 | 85 |
| 60 | 80 | 84 |
| 45 | 38 | 31 |
| 46 | 40 | 60 |
| 43 | 41 | 77 |
| 64 | 43 | 64 |
| av. 63% | av. 63% | av. 69% |

a small amount of pus in a few cases (two animals from the group PA and two from PD). The remaining tibia was taken out and, like the first one, the specific activity was determined. From the ratio of the two specific activities the phosphorus retention was found. The period was about the longest one that could be chosen in view of the radioactive decay; HEVESY et al (1940) had a period of 50 days in their experiments.

In view of the limited number of animals that could be handled at one time the experiment was carried out in three parts. In each part an equal number of rats from all three groups were treated together (3 or 4 of each group). The results obtained are given in table 42, expressed in % of radioactive phosphorus retained after six weeks.

The dotted lines indicate the three groups of rats treated at the same day. As a significant downward trend existed for all three dietary groups, we applied WILCOXON's test to each of the three parts and combined the results. The differences between the dietary groups were not significant.

[1]) The operations were carried out by Dr R. LUYKEN, M.D. We are very much indebted to him for his unceasing interest and helpfulness throughout our investigation.



As most of the animals lost some weight during the six weeks after the operation, it occurred to us that this might have an effect on the retention. We therefore tested for the presence of a correlation between weight loss and retention by means of KENDALL's rank correlation method (see under [3.4.3]). But no correlation was found. There were no important differences in dry weight between both tibiae in each animal.

In table 42 two values of the retention are higher than 100%. As there was no reason to ascribe these to faulty technique, they were retained. A possible explanation would be that in these cases after amputation a considerable transfer of radioactive phosphorus from plasma to bone substance has taken place.

Our conclusion must be that in this experiment no appreciable effect of the acid diets on the phosphorus metabolism of the bone in our animals was found.

## 5.4 DISCUSSION.

The most outstanding results of the chemical experiments are:

1. the absence of an acidotic condition in our animals, following from the alkaline reserve of the blood.
2. the absence of a decrease in the calcium balance.
3. the absence of an increase in the fixed base excretion.
4. the absence of a decrease in the nitrogen balance as well as of an increase in the urinary ammonia plus urea nitrogen excretion.
5. the complete similarity between the two metabolism experiments, proving that the effects of the phosphoric acid on the mineral metabolism was the same after a prolonged intake as after a relatively short one.

These results prove that the animals could ingest both acids in the quantities given during a considerable period of life without disturbance of the fixed base and nitrogen metabolism. A noteworthy feature of our results for the nitrogen metabolism is the decrease of the urea output accompanying the increased ammonia excretion, the sum of uriary ammonia and urea nitrogen remaining constant. Early investigators considered this a proof for the hypothesis that the ammonia was formed from urea (PETERS and VAN SLYKE, 1932, p. 373). But there is no contradiction between this decreased urea excretion and the fact that glutamine is the main source of urinary ammonia. The glutamine amide group, in so far as it is not used in the synthesis of other amino acids, will be converted partly into urea and partly into ammonia. The more ammonia is formed, the less nitrogen will be available for the synthesis of urea and this will result in a parallel decrease in the urea excretion.

According to the results of the metabolism experiments the phosphoric acid activated the following neutralization mechanisms.

1. the excretion of ammonia by the kidney.



0264

2. the phosphate shift, resulting in an increased titratable acidity and a decreased pH of the urine.
3. the excretion of acid calcium phosphates in the feces instead of $Ca_3(PO_4)_2$.

Neutralization by means of the blood bicarbonate was not found to have taken place. As we did not find a decrease in serum chloride content (only a very small increase in the younger animals by citric acid) or an increased chloride content of the liver, neutralization was apparently neither attained by means of a chloride shift from plasma to blood cells and tissues.

The results of the tissue analyse for the phosphoric acid groups were in good accordance with the aforementioned conclusions. No important changes were found in the fixed base and chloride content of the blood serum and soft tissues nor in the ash content of the kidneys. The increase found in the inorganic phosphorus content of the serum is in accordance with the findings of KAY (1924) and POTOP (1935).

In our experiments no sign of renal edema, as was found after injection of hydrochloric acid by MACNIDER (1923), was apparent. There was no increase of the renal water content in our case. Neither did we find renal enlargement, as was found by MACKAY and OLIVER (1935) when rats were fed a diet containing excessive amounts of phosphates. No diuretic effect of the acid diets was found.

A remarkable effect of the phosphoric acid intake on the phosphorus metabolism was the increased retention of this element. A similar retention of the anion of an ingested acid was found by SHOHL and SATO (1923). After administration of 25 meq of hydrochloric acid with the diet to infants they found a retention of 12 to 15% of the acid ingested. SALTER et al (1932) found a similar retention on feed acid phosphates to subjects with a constant neutral diet. About 25% of the ingested phosphate was stored. According to the estimates given under [5.1.8] in our case the retention was about 29% of the phosphoric acid added. It should be stressed, that this amount retained represented only about 4% of the total phosphorus intake. And as our diets represented an intake of phosphoric acid corresponding to the daily consumption of enough soft drink to satisfy the daily caloric need, there can be no possibility of overmineralisation of the bones or soft tissues. Moreover no signs of this were detected in our tissue analyses. Most probably the increased retention of phosphoric as well as of calcium (if real) will have resulted from an increased resorption due to the acid regime, as was also found for calves by WELLMANN (1943).

This would not result in an increased percentage of endogenous fecal phosphorus excretion, as in this case the absolute endogenous phosphorus excretion increases correspondingly (KJERULF-JENSEN, 1941). The constancy of alkaline phosphatase activity of the serum and the kidney also make it improbable



that important changes in the phosphorus metabolism occurred. This conclusion is supported by the fact that no change in the phosphorus retention of the bone occurred and also by the fact that no increase of the total activity excreted per day in the determination of the endogenous fecal phosphorus excretion was found.

In accordance with these facts the ash content of the tibia was not changed in the series 1–5 and 7 for the diet PD and in the third generation of animals receiving the PBB diet. The composition of the ash was not changed either, except for a slight increase of the calcium content in the second generation. The lowered ash content found in series 6 of the tissue analyses is completely incomprehensible in the light of this evidence. The possibility of rejecting a true hypothesis is always present in a statistical analysis when a large number of tests (in the case of the tissue analyses about 120) are applied, one should except a small number of the conclusions drawn from the analysis to be false. The statistical analysis does not, of course, indicate which of the conclusions may be false. This may, however, sometimes be guessed on the basis of further evidence. In this case we are inclined to consider the possibility, that the lowered ash content found in series 6 is such a false conclusion. Further experiments should give more definite results.

The phosphorus content of the soft tissues was only changed in the case of the younger animals, where an increase of the muscular phosphorus content was found. No evidence of a deposition of the retained phosphorus in organic compounds was found.

About the effect of the citric acid we can be short. As less than 3% of the amount ingested was excreted, no difficu'ties with the neutralization would arise. The only effect on the mineral metabolism found was a possible, slight calcium retention. If real, this will probably have been due to a promotion of the calcium absorption by the citric acid. A similar calcium retention, due to lactic acid in a dose of 12 meq/d/kg, was demonstrated by FLORIS (1923) in dogs. Some slight effects of the citric acid were found in the tissue analyses: a slight rise of the inorganic phosphorus and possibly also of the chloride of the serum, esp. in the younger animals. The muscular phosphorus content was decreased in the older animals. Of the tibia the ash content was decreased, the calcium content and the Ca/P ratio increased in the old animals (age 15 months), which might be explained by a partial exchange of phosphate by citrate. An effect which was not found by LEONARDS and FREE (1944) on prolonged feeding of sodium citrate to rats.



0264

# 6. CONCLUSIONS

The object of our investigation was to establish whether any harmful effect could result from the prolonged intake of phosphoric acid or citric acid incorporated in soft drinks or lemonades as acidulants. As we had to use rats in our investigations, the results obtained are strictly spoken valid only for the rat. We know of no facts however that could make us expect different results for man.

Our conclusions are valid for diets containing up to 0.40% of phosphoric or up to 1.20% of citric acid. The daily intake of acid with these diets corresponded to a quantity of beverage containing an amount of sugar equivalent to the daily caloric need of the animals. The amounts of acid daily ingested may therefore be considered as the absolute maximum. When some of the experiments were carried out with a diet containing 0.75% of phosphoric acid, the same results were obtained.

Two successive generations of rats, receiving the diets during periods up to 12 months were used in our investigation. In some cases even a third generation of rats, receiving the diet with 0.75% of phosphoric acid was investigated. Together more than 700 rats were used in this investigation.

No evidence of any deleterious effect of the acid diets on the growth could be found. This includes also rats of the second and third generation of the group receiving the diet with 0.75% of phosphoric acid.

The diets, including the one with 0.75% of phosphoric acid, had no effect on the reproduction and on the resulting young. The growth of the young was also normal.

The death rate was not affected by the acid diets, including the one with 0.75% of phosphoric acid, in either of the generations.

The blood picture was not affected by the acid diets in the first and second generation.

No evidence of any harmful effect in any of the generations, including those receiving the diet with 0.75% of phosphoric acid, was found in the autopsy and histological examination of many organs. In the examination of the teeth an increased dental attrition was found for both acids, but no increase of the caries percentage. It should be remarked that only the teeth of the amimals receiving the diets highest in acid content were investigated.

In the metabolism experiments the same effects on the mineral metabolism were found with animals receiving the phosphoric acid during 8 to 9 months as with those receiving it during 2 months only.

The evidence obtained from the metabolism experiments and the tissue analyses proved that the prolonged intake of the acid diets did not produce an acidotic condition.



No evidence of a demineralizing effect of the acid diets was found in the metabolism experiments as well as in the tissue analyses.

No decrease of the nitrogen retention was found, nor an increase of the sum of the urinary ammonia and urea excretion.

The phosphoric acid was neutralized and excreted partly by means of an increased ammonia excretion and an increased titratable acidity of the urine as well as by the excretion of acid calcium phosphates instead of $Ca_3(PO_4)_2$ in the feces. Part of the phosphorus was retained. From experiments with radioactive phosphorus no evidence of changes in the endogenous fecal phosphorus excretion and in the phosphorus retention of the bone was found. Apart from an increased retention of phosphorus and an increased urinary phosphorus excretion no evidence of any fundamental changes in the phosphorus metabolism was found. Some further experiments concerning the effect on the mineral composition of the bone are needed.

Of the citric acid ingested less than 3% was excreted in the urine. Probably at least 92% was oxidized. It caused some changes in the ash content and calcium content of the tibia that might be explained by a partial replacement of phosphate by citrate. A similar effect was noticed in the muscle in some cases.

As in man the same mechanisms are known to function in the neutralization and excretion of ingested acids as found in the rat, we believe to be justified in expecting from the results of our experiments that no harm can result from a prolonged consumption of beverages acidulated with either phosphoric acid or citric acid.



0264

# 7. SAMENVATTING

In de INLEIDING wordt het doel van het verrichte onderzoek beschreven. In verband met het wijdverspreide gebruik van phosphorzuur als een middel om een zure smaak te geven aan verfrissende dranken werd een grondig onderzoek naar de werking op het organisme nodig geoordeeld. Uit een studie van de litteratuur maakten wij de gevolgtrekking dat ook meer bekend diende te worden betreffende de werking van de organische zuren, die in dit verband gebruikt worden. Daarom werd besloten gelijktijdig experimenten met phosphorzuur en met citroenzuur uit te voeren.

In het LITTERATUUROVERZICHT worden de verschillende mechanismen, die werkzaam zijn bij de regulering van het zuur-base evenwicht, beschreven in de eerste drie paragrafen. De volgende vijf paragrafen zijn gewijd aan een bespreking van de effecten van de opname van zuur resp. op de algemene toestand van het organisme, de minerale samenstelling van het weefsel, de stofwisseling in het algemeen, de calcium- en phosphorstofwisseling en de stofwisseling van stikstof. Uit dit overzicht van de litteratuur wordt de gevolgtrekking gemaakt, dat in het bijzonder gewenst zijn proeven betreffende het effect van een langdurige opname, die zich uitstrekken over een breed terrein, omvattende stofwisseling, samenstelling van de weefsels, groei, voortplanting, bloedbeeld en sectie.

Het volgende hoofdstuk, dat de TECHNIEKEN beschrijft, vangt aan met een paragraaf over de opzet der proeven. We bespreken de keuze van de rat als proefdier in onze experimenten. De samenstelling van het basisdieet wordt vermeld. De keuze van citroenzuur als voorbeeld van een organisch zuur, dat gebruikt wordt om een zure smaak aan dranken te verschaffen, wordt besproken. Eveneens wordt besproken de toediening der zuren aan de dieren (toevoeging aan het dieet) en de hoeveelheden, die gegeven werden. In de volgende paragraaf worden de biologische technieken (groei, voortplanting, bloedbeeld, sectie en onderzoek der tanden) beschreven. De derde paragraaf is gewijd aan een uitvoerige beschrijving van de chemische technieken, die in ons onderzoek gebruikt werden. Dit moge ertoe dienen om andere onderzoekers in de gelegenheid te stellen onze proeven te herhalen. Wij zouden hier willen noemen de bepaling van het totaal basegehalte van bloed, weefselas en urine door middel van ionenuitwisseling. De methode voor bloedserum, die in de litteratuur was beschreven, werd verbeterd en uitvoerig beproefd op zijn betrouwbaarheid, totdat een geschikte uitvoering werd gevonden. Deze methode werd ook toegepast op weefselas en urine; goede resultaten werden verkregen als de oplossing geneutraliseerd werd vóór de percolatie door de kolom van de ionenuitwisselaar. Voor uitvoering van de natte verassing met salpeterzuur en perchloorzuur werden omstandigheden gevonden, waarbij het gevaar van explosies vermeden wordt. Deze methode was zeer geschikt voor seriewerk, daar ongeveer dertig verassingen tegelijkertijd konden worden uitgevoerd. Voor de bepaling van chloride in weefsel werkten wij een methode uit, waarbij het chloride met trichloorazijnzuur geextraheerd werd uit het fijngemalen weefsel. Destructie van het organische materiaal door droge verassing of door koken met loog gaf geen betrouwbare resultaten. Tenslotte moge de verzameling en de behandeling van urine en faeces in the stofwisselingsexperimenten genoemd worden. In verband met de beperkte hoeveelheden materiaal – vooral in de bloedanalysen – werden de meeste bepalingen uitgevoerd op de semimicro schaal. Eenvoudige technieken, geschikt voor seriewerk, werden gebruikt in verband met het grote aantal analysen (ong. 12000), dat moest worden uitgevoerd. Een uitvoerige beschrijving van de methoden, die bij de statistische analyse van onze resultaten werden gebruikt, besluit dit hoofdstuk. Parametervrije methoden (de toets van WILCOXON, de rangcorrelatiemethode volgens KENDALL en een trendtoets) werden gebruikt om de aanname van een normale verdeling van de waargenomen veranderlijken te vermijden. Het combineren van overschrijdingskansen wordt eveneens beschreven. Daar deze methoden verdienen wijder bekend te zijn en veelvuldiger te worden toegepast bij biochemische experimenten, geven wij voorbeelden van hun toepassing ontleend aan de bewerking van onze eigen resultaten.

In het vierde hoofdstuk, het BIOLOGISCHE GEDEELTE, worden de resultaten van de biologische proeven vermeld. Ten aanzien van groei, voortplanting, sterfte, bloedbeeld en bij de sectie en het onderzoek der tanden konden geen schadelijke invloeden van de beide toegediende zuren worden waargenomen bij drie opeenvolgende geslachten. In de discussie, die dit hoofdstuk besluit, worden onze resultaten vergeleken met die uit de litteratuur.

Het CHEMISCHE GEDEELTE beschrijft de resultaten van de stofwisselingsproeven, de weefselanalysen en de proeven met radioactief phosphor. Twee uitvoerige stofwisselingsexperimenten werden uitgevoerd. In het eerste kregen 24 volwassen ratten eerst gedurende zes weken het basis dieet (controleperiode); gedurende de volgende acht weken ontvingen acht dezer dieren phosphorzuur en acht anderen citroenzuur (proefperiode). Het experiment werd besloten met een tweede controleperiode van zes weken. In het tweede stofwisselingsexperiment werden acht volwassen ratten van de controle groep gedurende vijf weken vergeleken met acht dieren, die het phosphorzuur bevattende dieet tijdens de proefperiode alsmede tijdens de acht voorafgaande maanden ontvingen. In beide experimenten onderzochten wij de stofwisseling van phosphor, calcium en stikstof; titreerbare aciditeit, zuurgraad, volumen en chloridegehalte van de urine werden eveneens bepaald. In het eerste experiment werden bovendien de totale baseuitscheiding en de citroenzuurexcretie vervolgd. De resultaten van beide experimenten waren in goede overeenstemming met elkaar, hetgeen

bewijst dat de invloed van het phosphorzuur op de mineraalstofwisseling dezelfde was na een langdurige toediening als na een betrekkelijk kort gebruik. Er werden geen aanwijzingen voor een verlaagde calciumretentie, een verhoogde totale alkaliuitscheiding of een verlaagde stikstofretentie gevonden. Het phosphorzuur werd gedeeltelijk geneutraliseerd en uitgescheiden door middel van een verhoogde ammoniakuitscheiding en een toegenomen titreerbare aciditeit van de urine alsmede doordat een deel van het in de faeces uitgescheiden neutrale calciumphosphaat in de zure zouten overging. Tevens werd de phosphorretentie verhoogd. Het citroenzuur werd blijkens de zeer geringe uitscheiding vrijwel geheel geoxydeerd in het lichaam. In de weefselanalysen werd de minerale samensteiling van het bloedserum, de zachte weefsels en het bot bepaald bij dieren van verschillende leeftijd alsmede van de tweede generatie. De gegevens, die werden verkregen, stemden in het algemeen overeen met de resultaten van de stofwisselingsproeven. Wij vonden geen enkele aanwijzing voor het bestaan van een acidotische toestand bij onze dieren. In het eerste experiment met radioactieve phosphor werd de endogene phosphoruitscheiding in de faeces bepaald door vergelijking der specifieke activiteiten van de faeces en de urine. Geen invloed van de zure dieëten hierop werd geconstateerd. In het tweede experiment met radioactieve phosphor werd de phosphorretentie van de tibia bepaald. Bij het begin van het experiment werd een achterpoot geamputeerd en de specifieke activiteit van de tibia bepaald. Na zes weken werden de dieren gedood en de specifieke activiteit van de andere tibia bepaald. Vergelijking van de beide specifieke activiteiten leverde de retentie. Geen effect van de zure dieëten hierop kon worden vastgesteld. Het hoofdstuk wordt besloten met een bespreking van de resultaten.

In het laatste hoofdstuk wordt een opsomming van onze conclusies gegeven. Onze slotconclusie luidt, dat wij geen schadelijke gevolgen verwachten voor de mens van een langdurig gebruik van dranken, die aangezuurd zijn met phosphorzuur of citroenzuur, daar bij de mens dezelfde mechanismen voor de neutralisatie en uitscheiding van opgenomen zuren werkzaam zijn als bij de rat.



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0264

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0264

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0264

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# Method for the Enzymic Determination of Citric and Pyruvic Acids in Sugar Factory Products

L. Buesching

This study was submitted to the Committee for Research and Examination of prize papers of the Association of German Sugar Technicians and was awarded a prize.

Citric acid plays an important role not only in the metabolism of plants and animals, but a knowledge of the citric acid concentration during sugar juice purification is also important for an estimate of various sugar juice purification methods, since an increased residual citrate content in thin juice may cause technical difficulties, such as an incrustation of the evaporation unit and a precipitation of calcium citrate during the pan boiling of white sugar. If data on the possible citrate content in intermediate products of sugar manufacturing are only sparsely found in the literature (1) this is due primarily to the lack of a rapid and reproducible determination method.

For example, the determination of citric acid in solutions not containing saccharoses could so far be carried out by a colorimetric method (2). This method is based on the fact that citric acid is first converted to pentabromoacetone, whereby interfering substances such as acetone, acetaldehyde and beta-keto acids must first be removed. Thus, a prerequisite of this method is the isolation of acids from the juices by means of anion exchangers. Similarly, Wallenstein and Bohn (3), after isolation of acids from the juices, have separated individual acid components by displacement chromatography on ion exchangers. These methods are very laborious and therefore unsuited for serial analyses.

Recently, Gruber and Moellering (4) have worked out an enzymic method for the determination of citric acid, which makes it possible to obtain reproducible results in a relatively short period of time. The method is based on the fact that the enzyme citrate lyase, obtained from bacteria of the Aerobacter aerogenes strain, catalyzes the splitting of citrate into oxalacetate and acetate in the presence of zinc ions. Since citrate lyase also contains a certain amount of oxalacetate decarboxylase, pyruvate is formed along with oxalacetate. In order that the reaction may lead to a complete splitting of the citrate, definite amounts of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) are added

to the reaction solution prior to addition of citrate lyase. Thus, every pyruvate which has been formed from oxalacetate by a non-enzymic reaction is converted by LDH and unchanged oxalacetate is converted by MDH. In this reaction, in which the above enzymes act as catalysts, pyruvate and oxalacetate are hydrogenated by reduced nicotinamide-adenine-dinucleotide (NADH). These reactions can be quantitatively measured by the decrease in extinction, caused by the NADH oxidation. By separate addition of LDH and MDH it is possible to determine separately any pyruvate or oxalacetate present in the sample.

The course of the reactions can be summarized as follows:

$$\text{Citrate} \xrightarrow{\text{Citrate-Lyase}} \text{Oxalacetate} + \text{Acetate} \qquad [1]$$

$$(\text{Oxalacetate} \xrightarrow{\text{Decarboxylase?}} \text{Pyruvate} + CO_2) \qquad [2]$$

$$\text{Oxalacetate} + \text{NADH} + \text{H+} \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD} + \qquad [3]$$

$$\text{Pyruvate} + \text{NADH} + \text{H+} \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD+} \qquad [4]$$

Method of Investigation

1. Reagents

   a) Triethanolamine buffer

   b) NADH, nicotinamide-adenine-dinucleotide reduced, Na-salt (enzymically reduced)

      Empirical formula: $C_{21}\ H_{27}\ O_{14}\ N_7\ P_2\ Na_2$

   c) Zinc chloride, for analysis

   d) LDH, lactate dehydrogenase from skeleton muscle (rabbit). Crystall suspension in 2.2 M (molar) ammonium sulfate solution, pH ca. 7

   e) MDH, malate dehydrogenase from swine heart. Suspension in 2.8 M ammonium sulfate solution, pH ca. 6

   f) CL, citrate lyase (lyophilised dry powder)

All enzymes and coenzymes were supplied by the firm C.F. Boehringer α Sohne, Mannheim.

2. Preparation of Solutions

   a) Triethanolamine buffer (0.1 M; pH 7.6). 4.65 g triethanolamine hydrochloride dissolved in ca. 200 ml distilled water, adjusted with ca. 3 ml 2N-caustic soda to pH 7.6, and filled to 250 ml with distilled water.

b) NADH (ca.0.01 M). Dissolve 10 mg NADH in 1 ml of distilled water.

c) Zinc chloride (ca. 0. 03M). Dissolve 400 mg. $ZnCl_2$ (dry) in 100 ml distilled water and 1 ml 2N HCl.

d) LDH (2 mg protein/ml) Mix 0.4 ml substance (25 mg protein/5.0 ml) with 0.6 distilled water.

e) MDH (2 mg protein/ml). Mix 0.2 ml substance (25 mg protein/2.5 ml) with 0.8 ml distilled water.

f) CL. The dry preparation, containing 15 mg citrate lyase in one tablet, is taken up, prior to use, in 3 ml of an ice-cold buffer solution of the following composition: 6 g ammonium sulfate and 4 mg zinc chloride in 100 ml of a 0.01 M triethanolamine buffer, pH 7.6.

The stability of the solutions is different. According to data supplied by the Boehringer firm, traces of heavy metals and higher temperatures in strongly diluted solutions can result in activity losses. The enzyme preparations should be stored in a refrigerator at ca. +4° C. and should be kept in an ice bath during the tests. Water distilled in glass equipment should best be used to dissolve the reagents. For example, the stability of a NADH solution is reported to be equal to 3 weeks, while the citrate lyase solution can be used for only about 8 days when it is stored at +4°C.

3. Carrying out the determination.

The above authors (4) state that citric acid can be determined in wine or fruit juices and in samples with a very low protein content without any pretreatment, that the reaction is specific for citrate, and that the following compounds do not react: isocitrate, cis-aconitate, oxalate, succinate, fumarate, alpha-ketoglutarate, L-glutamate, tartrate, lactate, malate, acetate, ascorbate, glucose, fructose and ethanol.

Measuring instrument: Elko II, Hg 365 filter, glass cell with 0.5 cm thickness of the layer, measuring volume 3.00 ml, ambient temperature, measurement against a reference cell filled with distilled water.

Preparation (pretreatment) of samples.

a) Beet pressed juice

5 g of pressed juice are filled up to 100 ml with distilled water and then filtered through a membrane filter (Sartorius membrane filter No. 1100b.)

b) Crude juice

Same treatment as for pressed juice.

c) Predefecation, first saturation, and main defecation juices.
These juices are first clear filtered, then a 20 to 40 g batch is weighed, depending upon the citrate content, the pH is adjusted to 7.3-7.5, and the volume is filled up to 100 ml with distilled water.

d) Thin juice
This juice can be used directly for measurement

e) Syrup (thick juice).
30 g syrup are filled up to 100 ml with distilled water.

f) Sugar mill discharges (effluents)
According to the turbidity and color of the discharged products, a simple filtration is sufficient, or a pre-treatment with active carbon followed by filtration must be carried out. Naturally, in this case also the samples must be neutralized prior to measurement. The weighed samples are different depending upon the level of the citrate content. Thus, in the case of molasses, a sample of 3 g is sufficient in most cases, which is filled up to 100 ml with distilled water.

g) Sugar
250 g sugar (more or less depending upon the citric acid content) are dissolved with distilled water to a clear liquid of 10 to 15° Bx (Brix), 20 ml of a cation exchanger in the $H^+$ form (e.g. Amberlite 200) is added, and the mixture is stirred for 1 hour. The mixture is then allowed to flow through a column filled with the same cation exchanger, and then through a column filled with an anion exchanger in the $OH^-$ form (e.g. IRA-68); the mixture is then finally washed (rinsed) until it is free of saccharose. The anion exchanger is eluted with a 5% ammonia solution, the ammonia distilled off in vacuo, and the solution is concentrated to a certain amount (5 or 10 ml). This concentrate is then used for the determination.

4. Measurement

The following amounts are successively introduced into the cell with a pipet:

2.39 ml buffer solution
0.06 ml buffer solution
0.01 ml $ZnCl_2$ solution
0.50 ml sample solution

The contents of the cell are mixed (stirred) with a plastic rod flattened downwards, and the extinction $E_1$ is measured. Then, 0.01 ml LDH solution and 0.01 ml MDH solution are added. Pyruvate and oxalacetate present in the sample react within 3 minutes. After the reaction is terminated, the extinction $E_2$ is recorded.
Then, 0.02 ml CL-solution is mixed (stirred) in, and the extinction $E_3$ is measured after 5 minutes.

5. Calculation

The difference $E_2-E_3$, converted to a layer thickness of 1 cm, gives $\Delta E_{citr.}$

In general, the following formula is applicable:

$$\frac{\Delta E.\ V}{\Sigma.d.v.} = \text{micromoles/ml sample, where}$$

$\Sigma$= extinction coefficient of NADH $=3.3.10^3\ Cm^2$ per micromole for 366 nm.

d = layer thickness = 1 cm

V = test volume = 3 ml

v = volume of sample used = 0.5 ml.

For the previously described citric acid determination, the following formula is applicable:

$$\frac{\Delta E_{citr} \cdot 3.00.\ 192.1}{3,3\ .\ 10^3.\ \ 0.5} = \text{mg citric acid/ml}$$

192.1 = molecular weight of citric acid; thus, by conversion we get:

$$\frac{\Delta E_{citr.} \cdot 1\ \ 0.1746}{0.5}$$

From the difference $E_1 - E_2$ we can calculate the total content of pyruvate and oxalacetate. The concentration of each single component can be determined by adding successively LDH and MDH. From a calculation standpoint, this yields the following equations:

1) $\frac{\Delta \text{ pyruvate} \cdot 0.0793}{v}$ = mg pyruvic acid/ml.

2) $\frac{\Delta \text{ oxalacetate} \cdot 0.1193}{v}$ = mg oxalacetic acid/ ml

Figure 2 shows a typical extinction course (curves) during citrate determinations in different sugar juices and discharge products. On the basis of the decrease in extinction after addition of LDH, one can conclude that pyruvic acid is present, and after addition of MPH, that oxalacetic acid is present.

EXTINCTION CURVES IN CITRIC ACID DETERMINATIONS WITH SEPARATE ADDITION OF THE ENZYMES LDH, MDH and CL

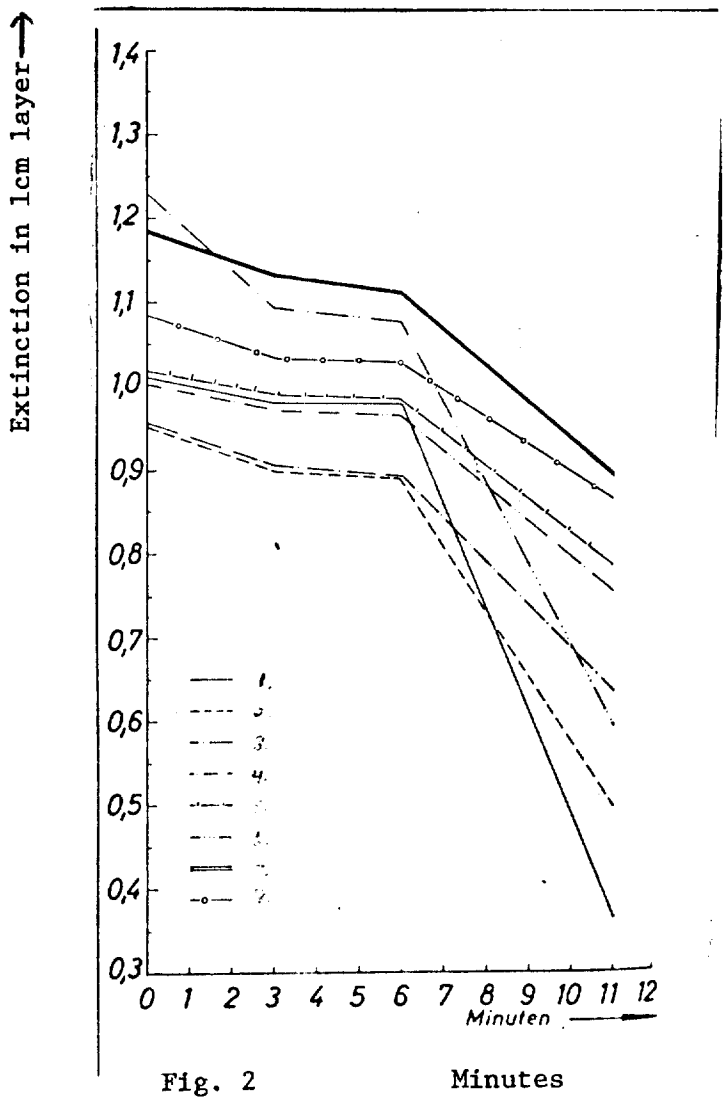


Fig. 2 Minutes

1. Predefecation juice
2. IA - saturation juice
3. IB - saturation juice
4. II - saturation juice
5. Thick juice (syrup)
6. White sugar discharge product
7. Middle product discharge
8. Molasses

6. Check of the accuracy of the determination method.

Prior to carrying out tests to determine the citrate content of factory juices, it was necessary, at first, to check the accuracy of the method. The following series of tests were performed for this purpose.

a) Determination of citric acid in a pure aqueous solution:

| ..tric acid added, mg/ml | Citric acid found mg/ml | Deviation (discrepancy) mg | Percent citric acid found (detected again) |
|---|---|---|---|
| 0.0238 | 0.0246 | + 0.0008 | 103 |
| 0.0357 | 0.0380 | + 0.0023 | 106 |
| 0.0476 | 0.0490 | + 0.0014 | 103 |
| 0.0595 | 0.0602 | + 0.0007 | 101 |
| 0.0714 | 0.0745 | + 0.0031 | 104 |
| 0.0833 | 0.0829 | - 0.0004 | 100 |
| 0.0952 | 0.0975 | + 0.0023 | 102 |
| 0.1071 | 0.1084 | + 0.0013 | 101 |
| 0.1190 | 0.1195 | + 0.0005 | 100 |
| 0.1309 | 0.1310 | + 0.0001 | 100 |
| 0.1428 | 0.1438 | + 0.0010 | 101 |
| 0.1547 | 0.1566 | + 0.0019 | 101 |
| 0.1666 | 0.1681 | + 0.0015 | 101 |

In these tests, an average deviation of +1.8% was found.

b) Determination of citric acid in 20% refined sugar solution.

In these tests, increasing amounts of citric acid were replenished to a constant volume with a 20% refined sugar solution and analyzed. The following figures were obtained:

| Citric acid added, mg/ml | Citric acid found mg/ml | Deviation (discrepancy) mg | Percent citric acid found (detected again) |
|---|---|---|---|
| 0.0696 | 0.0682 | - 0.0014 | 98 |
| 0.0812 | 0.0827 | + 0.0015 | 102 |
| 0.0928 | 0.0916 | - 0.0012 | 99 |
| 0.1044 | 0.1020 | - 0.0024 | 98 |
| 0.1160 | 0.1142 | - 0.0018 | 98 |
| 0.1276 | 0.1312 | + 0.0036 | 103 |
| 0.1392 | 0.1435 | + 0.0043 | 103 |
| 0.1508 | 0.1525 | + 0.0017 | 101 |
| 0.1624 | 0.1685 | + 0.0061 | 104 |

Average deviation: +0.7%

c) Determination of citric acid in syrup with added citric acid.

his case, a syrup was analyzed, containing 0.0480 mg citric acid.

In this case, a syrup was analyzed, containing 0.0480 mg citric acid per ml in a prepared dilution. Increasing amounts of citric acid were filled up to a constant volume with this diluted syrup, and the citric acid content in this mixture was then determined.

| mg from syrup | mg from citric acid | Total mg/ml | Citric acid found, mg/ml | Deviation, mg | Percent citric acid found (detected again) |
|---|---|---|---|---|---|
| 0.0336 | 0.0696 | 0.1032 | 0.1055 | + 0.0023 | 102 |
| 0.0312 | 0.0812 | 0.1124 | 0.1206 | + 0.0082 | 107 |
| 0.0288 | 0.0928 | 0.1216 | 0.1292 | + 0.0076 | 106 |
| 0.0264 | 0.1044 | 0.1308 | 0.1352 | + 0.0044 | 103 |
| 0.0240 | 0.1160 | 0.1400 | 0.1391 | - 0.1391 | 99 |
| 0.0216 | 0.1276 | 0.1492 | 0.1531 | + 0.0039 | 103 |
| 0.0192 | 0.1392 | 0.1584 | 0.1634 | + 0.0050 | 103 |
| 0.0168 | 0.1508 | 0.1676 | 0.1709 | + 0.0033 | 102 |
| 44 | 0.1624 | 0.1768 | 0.1822 | + 0.0054 | 103 |

Average deviation: + 3.1%

In summary, it can be stated that the above series of tests gives very good results which lie within the limits (range) also found by Gruber and Moeffering, namely 96 to 107% recovered citric acid in a test in which the above authors added increasing amounts of citric acid to a deproteinated, homogenized rabbit liver.

In the third series of tests, the greatest deviations were found with higher syrup fractions. These deviations were attributed at first to the relatively high intense color of the syrup. A further series of tests was carried out to study the effect of different pretreatment methods on the preparation of an unobjectionable satisfactory sample solution.

Results: No. 1 : Syrup dilution 30g/100 mP water

No. 2 : Same dilution as under No. 1, but filtration through a membrane filter (MF 100).

No. 3: Same dilution mixed with active carbon, first filtered through a paper filter, and then filtered a second time through a MF 100 membrane filter.

Extinction values: layer thickness of 0.5 cm, measured in an Elko II instrument with an Hg 365 filter.

Table 4 - CITRIC ACID DETERMINATION IN A SYRUP

| Additions, ml | | Extinction of Sample Solution No. 1 | Extinction of Sample Solution No. 2 | Extinction of Sample Solution No. 3 |
|---|---|---|---|---|
| 5. | 0.50 sample immediately | 0.6498 | 0.6478 | 0.6126 |
| 6. | 0.01 LDH after 3 minutes | 0.6271 | 0.6253 | 0.5867 |
| 7. | 0.01 MDH after 3 minutes | 0.6253 | 0.6234 | 0.5850 |
| 8. | 0.02 CL after 1 minute | 0.5544 | 0.5528 | 0.5100 |
| 9. | 0.02 CL after 2 minutes | 0.5513 | 0.5513 | 0.5085 |
| 10. | 0.02 CL after 3 minutes | 0.5498 | 0.5498 | 0.5058 |
| 11. | 0.02 CL after 4 minutes | 0.5482 | 0.5482 | 0.5045 |
| 12 | 0.02 CL after 5 minutes | 0.5482 | 0.5482 | 0.5045 |
| 13. | mg citric acid/ml | 0.0539 | 0.0526 | 0.0563 |
| 14. | mg citric acid/100 parts separating juice | 20.85 | 20.36 | 21.78 |

The deviations of the three values are very small, since -0.49 mg/100 parts separating juice and + 0.93 mg/100 parts separating juice, relative to the original solution, lie within the possible limits of error. These tests were repeated with additional juices and discharge products, and similar figures were obtained. However, when a turbidity is present in addition to the color, a careful pretreatment as described above is necessary.

The reason for the deviations observed in the single values of test series 6c is attributed to the very difficult exact dosage caused by the viscosity of the syrup.

Results of citric acid determinations in sugar-containing products.

During and after the 1967/68 working season, juices, discharge products, beet slices and sugars from factories of the "Sudzucker-AG" were tested for their citric acid content, and the following figures were found:

| | |
|---|---|
| Beet pressed juice | : 896-425 mg/100 parts separation juice |
| Dried pressed slices | : 244-36 mg/100 g slices |
| Sugar slices | : 499-343 mg/100 slices |
| Crudejuice | : 843-540 mg/100 parts separation juice |
| Predefecation juice | : 558-534 mg/100 parts separation juice |

| | |
|---|---|
| Saturation juice IA | : 354-194 mg/100 parts separation juice |
| Saturation juice IB | : 132-89 mg/100 parts separation juice |
| Thin juice | : 93-22 mg/100 parts separation juice |
| Syrup | : 123-28 mg/100 parts separation juice |
| White Sugar Discharge Product | : 102-54 mg/100 parts separation juice |
| Middle product discharge | : 210-189 mg/100 parts separation juice |
| Refined clarified liquid sugar | : 1.2 mg/100 parts separation juice |
| Molasses | : 272-200 mg/100 parts separation juice |
| Raw Sugar | : 21 mg/100 pol (100 parts polarized sugar?) |
| White Sugar | : 2.20-0-0.30 mg/100 Pol. |
| Second time sediment | 2.71 - 0.58% |
| Dried syrup filter residue: | 0.55% |

Results of pyruvic acid determinations in juices and discharge products.

| | Pyruvic acid/100 parts separation juice |
|---|---|
| Beet Pressed juice | 4.7 mg |
| Raw juice | 17.5 mg |
| Predefecation juice | 9.0- 17.4 mg |
| Saturation juice IA | 7.8- 12.1 mg |
| Saturation juice IB | 4.5- 7.7 mg |
| Thin juice | 6.6- 8.5 mg |
| Syrup | 10.0- 14.9 mg |
| White sugar discharge product | 8.2- 13.5 mg |
| Middle product discharge | 22.0- 23.9 mg |
| Molasses | 40.2- 64.4 mg |

The samples tested for pyruvic acid include juices and discharge products of the Rain sugar mill from the 1967/68 working season.

Along with pyruvic acid, oxalacetic acid was also determined, whereby values of about 10 mg/100 parts separation juice were found in raw juice, 1mg/100 parts separation juice in thin juice, and 20mg/100 parts separation juice in molasses.

In conclusion, I would like to thank especially Dr. H. Schiweck of the central laboratory of the "Suddeutschen Zucker-AG", Neuoffstein, for his valuable encouragement and support of this work.

SUMMARY

In the present paper a method has been described permitting determination of citric and pyruvic acid in all products which occur during sugar production. This method gives reproducible values even in a relatively short time and therefore is suitable for factory control. The results obtained are most valuable for judging technological processes, e.g. juice purification.

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# Methode zur enzymatischen Bestimmung von Citronensäure und Brenztraubensäure in Zuckerfabriksprodukten

## Method of Enzymatic Determination of Citric and Pyruvic Acid in Sugar Factory Products / Méthode de la détermination enzymatique des acides citrique et pyruvique dans des produits de la sucrerie

Von L. Büsching

Diese Arbeit wurde dem Ausschuß für Forschung und Prüfung von Preisarbeiten des Vereins Deutscher Zuckertechniker eingereicht und mit einem Preis bedacht.

Die Citronensäure spielt nicht nur im Stoffwechsel von Pflanzen und Tieren eine bedeutende Rolle, sondern die Kenntnis der Citronensäurekonzentration während der Saftreinigung ist auch für die Beurteilung verschiedener Saftreinigungsmethoden von Bedeutung, da erhöhte verbleibende Citratgehalte im Dünnsaft zu technologischen Schwierigkeiten führen können (Inkrustation der Verdampfstation, Ausfällen von Calciumcitrat beim Verkochen von Weißzuckern). Wenn bisher Angaben über den möglichen Citratgehalt in Zwischenprodukten der Zuckerfabrikation in der Literatur nur spärlich vorhanden sind (1), so liegt das in erster Linie an dem Fehlen einer schnellen und reproduzierbaren Bestimmungsmethode.

Die Bestimmung der Citronensäure konnte z. B. bisher in nichtsaccharosehaltigen Lösungen auf kolorimetrischem Wege erfolgen (2). Die Methode beruht darauf, daß zunächst die Citronensäure in Pentabromaceton übergeführt wird, wobei störende Substanzen wie Aceton, Acetaldehyd und $\beta$-Ketosäuren vorher entfernt werden müssen. Die Methode setzt also die Isolierung der Säuren aus den Säften durch Anionenaustauscher voraus. Ebenso haben Wallenstein und Bohn (3) nach Isolierung der Säuren aus den Säften eine Auftrennung in die Einzelkomponenten durch Verdrängungschromatographie an Ionenaustauschern durchgeführt. Diese Methoden sind mit einem großen Arbeitsaufwand verbunden und eignen sich daher nicht für Serienanalysen.

In neuerer Zeit haben nun Gruber und Moellering (4) ein enzymatisches Verfahren zur Bestimmung der Citronensäure entwickelt, das bei einem relativ geringen Zeitaufwand reproduzierbare Ergebnisse zu erzielen gestattet. Die Methode beruht darauf, daß das Enzym Citrat-Lyase, das aus Bakterien der Gattung Aerobacter aerogenes gewonnen wird, in Gegenwart von Zinkionen die Spaltung von Citrat in Oxalacetat und Acetat katalysiert. Da die Citrat-Lyase aber noch gewisse Mengen an Oxalacetat-Decarboxylase enthält, entsteht neben Oxalacetat auch Pyruvat. Damit die Reaktion zur völligen Spaltung des Citrats führt, setzt man der Reaktionslösung vor dem Zusatz von Citrat-Lyase bestimmte Mengen an Lactat-Dehydrogenase (LDH) und Malat-Dehydrogenase (MDH) zu. So wird jedes Pyruvat, das aus Oxalacetat durch eine nicht enzymatische Reaktion entstanden ist, durch LDH und unverändertes Oxalacetat durch MDH umgesetzt. Bei dieser Reaktion, bei der die genannten Enzyme als Katalysatoren dienen, werden Pyruvat bzw. Oxalacetat durch reduziertes Nicotinamid-adenin-dinucleotid (NADH) hydriert. Diese Reaktionen lassen sich durch die Extinktionsabnahme, hervorgerufen durch die NADH-Oxydation, quantitativ erfassen. Durch getrennte Zugabe von LDH und MDH hat man die Möglichkeit, in der Probe vorhandenes Pyruvat und Oxalacetat einzeln zu bestimmen.

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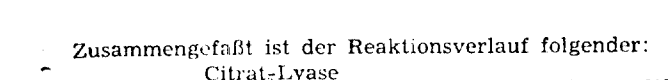
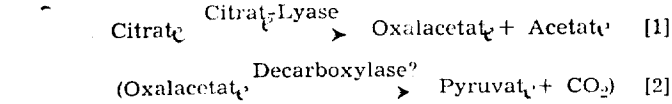
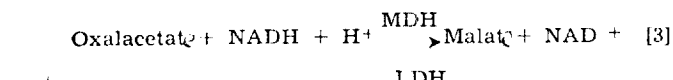
Zusammengefaßt ist der Reaktionsverlauf folgender:



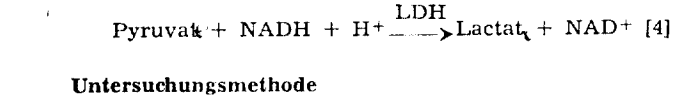
$$\text{Citrat}^{\ominus} \xrightarrow{\text{Citrat-Lyase}} \text{Oxalacetat}^{\ominus} + \text{Acetat}^{\ominus} \quad [1]$$



$$(\text{Oxalacetat}^{\ominus} \xrightarrow{\text{Decarboxylase}} \text{Pyruvat}^{\ominus} + CO_2) \quad [2]$$



$$\text{Oxalacetat}^{\ominus} + \text{NADH} + H^{+} \xrightarrow{\text{MDH}} \text{Malat}^{\ominus} + \text{NAD}^{+} \quad [3]$$



$$\text{Pyruvat}^{\ominus} + \text{NADH} + H^{+} \xrightarrow{\text{LDH}} \text{Lactat}^{\ominus} + \text{NAD}^{+} \quad [4]$$

**Untersuchungsmethode**

1. Reagenzien

a) Triäthanolamin-Puffer
b) NADH Nicotinamid-adenin-dinucleotid reduziert, Na-Salz (enzymatisch reduziert)
Summenformel: $C_{21}H_{27}O_{14}N_7P_2Na_2$
c) Zinkchlorid p. a.
d) LDH Lactatdehydrogenase aus Skelettmuskel (Kaninchen). Kristallsuspension in 2,2m-Ammoniumsulfatlösung, pH ca. 7.
e) MDH Malatdehydrogenase aus Schweineherz. Suspension in 2,8m-Ammoniumsulfatlösung, pH ca. 6.
f) CL Citrat-Lyase (lyophilisiertes Trockenpulver).

Alle Enzyme bzw. Coenzyme sind von der Firma C. F. Boehringer & Söhne, Mannheim, bezogen.

2. Herstellen der Lösungen

a) Triäthanolamin-Puffer (0,1 m; pH 7,6)
4,65 g Triäthanolamin-hydrochlorid in ca. 200 ml dest. Wasser lösen, mit ca. 3 ml 2n-Natronlauge auf pH 7,6 einstellen und mit dest. Wasser auf 250 ml auffüllen.
b) NADH (ca. 0,01 m)
10 mg NADH in 1 ml dest. Wasser lösen.
c) Zinkchlorid (ca. 0,03 m)
400 mg $ZnCl_2$ (trocken) mit 100 ml dest. Wasser und 1 ml 2n-Salzsäure lösen.
d) LDH (2 mg Protein/ml)
0,4 ml Substanz (25 mg Protein/5,0 ml) mit 0,6 ml dest. Wasser vermischen.
e) MDH (2 mg Protein/ml)
0,2 ml Substanz (25 mg Protein/2,5 ml) mit 0,8 ml dest. Wasser vermischen.
f) CL
Das Trockenpräparat, das 15 mg Citrat-Lyase in einer Tablette enthält, wird vor Gebrauch mit 3 ml eiskalter Pufferlösung folgender Zusammensetzung aufgenommen: 6 g Ammoniumsulfat und 4 mg Zinkchlorid in 100 ml 0,01m-Triäthanolamin-Puffer; pH 7,6.

Die Haltbarkeit der Lösungen ist unterschiedlich. Nach Angaben der Firma Boehringer können Schwermetallspuren und höhere Temperaturen in stark verdünnten Lösungen zu Aktivitätsverlusten führen. Die Enzympräparate sollen im Kühlschrank bei ca. + 4° C aufbewahrt werden und während der Versuche in einem Eisbad stehen. Zum Auflösen der Reagenzien verwendet man am besten mit einer Glasapparatur destilliertes Wasser. Die Haltbarkeit einer NADH-Lösung wird zum Beispiel mit 3 Wochen angegeben, während die Citrat-Lyase-Lösung nur etwa 8 Tage verwendbar ist, wenn sie bei + 4° C aufbewahrt wird.

3. Durchführung der Bestimmung

Die Verfasser (4) geben an, daß man Citronensäure in Wein oder Fruchtsäften und in Proben mit sehr niedrigem Proteingehalt ohne Vorbehandlung bestimmen kann, daß die Reaktion für Citrat spezifisch ist und daß folgende Stoffe nicht reagieren: Isocitrat, Cis-Aconitat, Oxalat, Succinat, Fumurat, α-Ketoglutarat, L-Glutamat, Tartrat, Lactat, Malat, Acetat, Ascorbat, Glucose, Fruktose und Äthanol.

Meßgerät: Elko II; Filter Hg 365, Glasküvette 0,5 cm Schichtdicke, Meßvolumen: 3,00 ml, Raumtemperatur. Messung gegen Vergleichsküvette mit dest. Wasser.

Vorbereitung der Proben:

a) Rübenpreßsaft
5 g Preßsaft werden mit dest. Wasser zu 100 aufgefüllt und anschließend über Membranfilter (Sartorius-Membranfilter Nr. 11006) filtriert.

b) Rohsaft
Behandlung wie Preßsaft.

c) Vorscheide-, I. Saturations- und Hauptscheidesaft
Diese Säfte werden zunächst klarfiltriert, dann wägt man je nach Citratgehalt 20 bis 40 g ein, stellt auf einen pH-Wert von 7,3 bis 7,5 ein und füllt auf dest. Wasser zu 100 ml auf.

d) Dünnsaft
Dünnsaft kann man direkt zur Messung verwenden.

e) Dicksaft
30 g Dicksaft werden zu 100 ml mit dest. Wasser aufgefüllt.

f) Zuckerhaus-Abläufe
Je nach Trübung und Farbe der Abläufe genügt eine einfache Filtration, oder es muß eine Vorbehandlung mit Aktivkohle und anschließender Filtration durchgeführt werden. Selbstverständlich muß man auch in diesem Fall vor dem Messen die Proben neutralisieren. Die Einwaagen sind je nach der Höhe des Citratgehaltes unterschiedlich. So genügt bei Melassen in den meisten Fällen eine Einwaage von 3 g, die mit dest. Wasser auf 100 ml aufgefüllt wird.

g) Zucker
250 g Zucker (je nach Citronensäuregehalt mehr oder weniger) löst man mit dest. Wasser zu einer Kläre von 10 bis 15° Bx auf, gibt dazu 20 ml Kationenaustauscher in der $H^{+}$-Form (z. B. Amberlite 200) und rührt diese Mischung eine Stunde. Man läßt sie dann über eine Säule mit dem gleichen Kationenaustauscher und nachfolgend über eine mit einem Anionenaustauscher in der $OH^{-}$-Form (z. B. IRA-68) laufen und spült zum Schluß saccharosefrei. Man eluiert den Anionenaustauscher mit 5%iger Ammoniaklösung. Im Vakuum wird Ammoniak abdestilliert und die Lösung auf eine bestimmte Menge (5 oder 10 ml) eingedampft. Dieses Konzentrat verwendet man dann zur Bestimmung.

4. Messung

Man pipettiert nacheinander in die Küvette

2,39 ml Pufferlösung
0,06 ml NADH-Lösung
0,01 ml $ZnCl_2$-Lösung
0,50 ml Probelösung

Mit einem nach unten abgeflachten Kunststoffstab wird der Inhalt der Küvette durchgemischt und die Extinktion $E_1$ gemessen.
Anschließend gibt man

0,01 ml LDH-Lösung
0,01 ml MDH-Lösung

hinzu.
Innerhalb von 3 Minuten reagiert in der Probe vorhandenes Pyruvat und Oxalacetat. Nach Beendigung der Reaktion liest man die Extinktion $E_2$ ab.
Danach mischt man weiter ein:

0,02 ml CL-Lösung

Nach 5 Minuten bestimmt man dann die Extinktion $E_3$.

5. Berechnung

Die Differenz $E_2 - E_3$, umgerechnet auf eine Schichtdicke von 1 cm, ergibt $\Delta E_{Citr.}$

Allgemein gilt:

$$\frac{\Delta E \cdot V}{\varepsilon \cdot d \cdot v} = \mu \text{ Mol/ml Probe}$$

$\varepsilon$ = Extinktionskoeffizient von NADH = $3{,}3 \cdot 10^3$ cm² pro $\mu$ Mol für 366 nm
d = Schichtdicke = 1 cm
V = Testvolumen = 3 ml
v = Volumen der eingesetzten Probe = 0,5 ml

Für die vorher beschriebene Citronensäurebestimmung gilt:

$$\frac{\Delta E_{Citr.} \cdot 3{,}00 \cdot 192{,}1}{3{,}3 \cdot 10^3 \cdot 0{,}5} = \text{mg Citronensäure/ml}$$

192,1 = Molgewicht Citronensäure

umgerechnet also:

$$\frac{\Delta E_{Citr.} \cdot 0{,}1746}{0{,}5}$$

Aus der Differenz $E_1 - E_2$ kann man den Gesamtgehalt an Pyruvat und Oxalacetat errechnen. Die Konzentration jeder einzelnen Komponente bestimmt man, indem LDH und MDH nacheinander zugegeben werden. Rechnerisch ergibt das folgende Gleichungen:

1) $$\frac{\Delta \text{ Pyruvat} \cdot 0{,}0793}{v} = \text{mg Brenztraubensäure/ml}$$

2) $$\frac{\Delta \text{ Oxalacetat} \cdot 0{,}1193}{v} = \text{mg Oxalessigsäure/ml}$$

Abb. 2 zeigt den typischen Extinktionsverlauf bei Citratbestimmungen in verschiedenen Zuckersäften und Abläufen. Man kann auf Grund der Extinktionsabnahme nach der Zugabe von LDH auf das Vorhandensein von Brenztraubensäure sowie auch nach der Zugabe von MDH auf Oxalessigsäure schließen.

## 6. Überprüfung der Genauigkeit der Bestimmungsmethode

Bevor Versuche durchgeführt wurden, um den Citratgehalt in Fabriksäften festzustellen, war es notwendig, zunächst einmal die Genauigkeit der Methode zu überprüfen. Dazu dienten folgende Versuchsreihen:

a) Bestimmung von Citronensäure in rein wäßriger Lösung:

Tabelle 1

| (1) Zugefügte Citronensäure mg/ml | (2) Gefundene Citronensäure mg/ml | (3) Abweichung mg | (4) % wiedergefundene Citronensäure |
|---|---|---|---|
| 0,0238 | 0,0246 | + 0,0008 | 103 |
| 0,0357 | 0,0380 | + 0,0023 | 106 |
| 0,0476 | 0,0490 | + 0,0014 | 103 |
| 0,0595 | 0,0602 | + 0,0007 | 101 |
| 0,0714 | 0,0745 | + 0,0031 | 104 |
| 0,0833 | 0,0829 | − 0,0004 | 100 |
| 0,0952 | 0,0975 | + 0,0023 | 102 |
| 0,1071 | 0,1084 | + 0,0013 | 101 |
| 0,1190 | 0,1195 | + 0,0005 | 100 |
| 0,1309 | 0,1310 | + 0,0001 | 100 |
| 0,1428 | 0,1438 | + 0,0010 | 101 |
| 0,1547 | 0,1566 | + 0,0019 | 101 |
| 0,1666 | 0,1681 | + 0,0015 | 101 |

Bei diesen Untersuchungen ergab sich eine durchschnittliche Abweichung von + 1,8 %.

b) Bestimmung von Citronensäure in 20 %iger Raffinadelösung

Bei diesen Versuchen wurden steigende Mengen Citronensäure mit einer 20 %igen Raffinadelösung zu einem konstanten Volumen ergänzt und analysiert. Dabei ergaben sich folgende Werte:

Tabelle 2

| (1) Zugeführte Citronensäure mg/ml | (2) Gefundene Citronensäure mg/ml | (3) Abweichung mg | (4) % wiedergefundene Citronensäure |
|---|---|---|---|
| 0,0696 | 0,0682 | − 0,0014 | 98 |
| 0,0812 | 0,0827 | + 0,0015 | 102 |
| 0,0928 | 0,0916 | − 0,0012 | 99 |
| 0,1044 | 0,1020 | − 0,0024 | 98 |
| 0,1160 | 0,1142 | − 0,0018 | 98 |
| 0,1276 | 0,1312 | + 0,0036 | 103 |
| 0,1392 | 0,1435 | + 0,0043 | 103 |
| 0,1508 | 0,1525 | + 0,0017 | 101 |
| 0,1624 | 0,1685 | + 0,0061 | 104 |

Durchschnittliche Abweichung = + 0,7 %.

c) Bestimmung von Citronensäure in Dicksaft mit zugesetzter Citronensäure

In diesem Falle wurde zunächst ein Dicksaft analysiert, der in einer hergestellten Verdünnung 0,0480 mg Citronensäure pro ml enthielt. Steigende Mengen Citronensäure wurden mit diesem verdünnten Dicksaft zu einem konstanten Volumen aufgefüllt. In dieser Mischung bestimmte man dann den Gehalt an Citronensäure.

Tabelle 3

| (1) mg aus Dicksaft | (2) mg aus Citronensäure | (3) Summe mg/ml | (4) gefundene Citronensäure mg/ml | (5) Abweichung mg | (6) % wiedergef. Citronensäure |
|---|---|---|---|---|---|
| 0,0336 | 0,0696 | 0,1032 | 0,1055 | + 0,0023 | 102 |
| 0,0312 | 0,0812 | 0,1124 | 0,1206 | + 0,0082 | 107 |
| 0,0288 | 0,0928 | 0,1216 | 0,1292 | + 0,0076 | 106 |
| 0,0264 | 0,1044 | 0,1308 | 0,1352 | + 0,0044 | 103 |
| 0,0240 | 0,1160 | 0,1400 | 0,1391 | − 0,0009 | 99 |
| 0,0216 | 0,1276 | 0,1492 | 0,1531 | + 0,0039 | 103 |
| 0,0192 | 0,1392 | 0,1584 | 0,1634 | + 0,0050 | 103 |
| 0,0168 | 0,1508 | 0,1676 | 0,1709 | + 0,0033 | 102 |
| 0,0144 | 0,1624 | 0,1768 | 0,1822 | + 0,0054 | 103 |

Durchschnittliche Abweichung = + 3,1 %.

Zusammenfassend kann man sagen, daß die oben angeführten Untersuchungsreihen sehr gute Ergebnisse zeigen. Sie liegen in den Grenzen, die auch Gruber und Moellering gefunden haben, nämlich 96 bis 107 % wiedergefundene Citronensäure in einem Versuch, bei dem die Autoren zu einer deproteinisierten, homogenisierten Kaninchenleber steigende Mengen

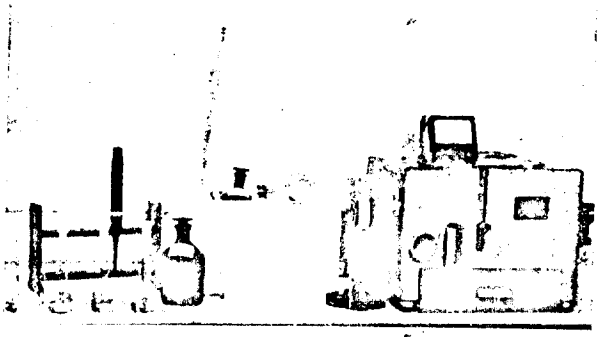


**Abbildung 1** Fig. 1
**Arbeitsgeräte zur Citratbestimmung**

Citronensäure zugesetzt haben. In der dritten Versuchsreihe wurden die größten Abweichungen bei höheren Dicksaftanteilen gefunden. Man führte diese Abweichungen zunächst auf die relativ hohe Dicksaftfarbe zurück. Um den Einfluß verschiedener Vorbereitungsmethoden zur Herstellung einer einwandfreien Probelösung zu studieren, wurde eine weitere Versuchsreihe durchgeführt.

Ergebnisse Nr. 1 Dicksaftverdünnung 30 g 100 ml Wasser.
2 Gleiche Verdünnung wie 1, aber über Membranfilter (MF 100) filtriert.
3 Gleiche Verdünnung mit Aktivkohle versetzt, vorfiltriert über Papierfilter, nachfiltriert über Membranfilter MF 100.

Extinktionswerte:
Schichtdicke 0.5 cm, gemessen im Elko II mit Filter Hg 365.

Citronensäurebestimmung in einem Dicksaft

| (1) Zusätze ml | (2) Probelösung 1 Extinktion | (3) Probelösung 2 Extinktion | (4) Probelösung 3 Extinktion |
|---|---|---|---|
| 2,39 Puffer | | | |
| 0,06 NADH | | | |
| 0,01 $ZnCl_2$ | | | |
| (5) 0,50 Probe sofort | 0,6498 | 0.6478 | 0,6126 |
| (6) 0,01 LDH nach 3 Minuten | 0.6271 | 0.6253 | 0,5867 |
| (7) 0,01 MDH nach 3 Minuten | 0,6253 | 0.6234 | 0,5850 |
| (8) 0,02 CL nach 1 Minute | 0.5544 | 0.5528 | 0,5100 |
| (9) nach 2 Minuten | 0.5513 | 0.5513 | 0.5085 |
| (10) nach 3 Minuten | 0,5498 | 0.5498 | 0,5058 |
| (11) nach 4 Minuten | 0,5482 | 0.5482 | 0,5045 |
| (12) nach 5 Minuten | 0,5482 | 0.5482 | 0.5045 |
| 1 Citronensäure | 0,0771 | 0,0752 | 0.0805 |
| Ergebnisse | | | |
| mg Citr./ml | 0,0539 | 0.0526 | 0.0563 |
| mg Citr./100 Tr. S. | 20.85 | 20.36 | 21.78 |

Die Abweichungen der drei Werte sind sehr gering, denn – 0,49 mg/100 Tr. S. bzw. + 0.93 mg/100 Tr. S., bezogen auf die Originallösung, liegen innerhalb der möglichen Fehlergrenzen. Diese Versuche wurden noch mit weiteren Säften und Abläufen wiederholt, wobei ähnliche Zahlen gefunden wurden. Kommt zu einer Färbung allerdings noch eine Trübung hinzu, so ist eine sorgfältige Vorbereitung, wie sie oben beschrieben wurde, unvermeidlich.

Die Ursachen der Abweichungen in den Einzelwerten der Versuchsreihe 6c werden nur auf die sehr schwierige genaue Dosierung, hervorgerufen durch die Viskosität des Dicksaftes, zurückgeführt.

Ergebnisse von Citronensäurebestimmungen in zuckerhaltigen Produkten

Während und nach der Kampagne 1967/68 wurden Säfte, Abläufe, Schnitzel und Zucker aus Werken der Südzucker-AG auf ihren Gehalt an Citronensäure untersucht. Folgende Werte wurden gefunden:

| | |
|---|---|
| Rübenpreßsaft | 896 — 425 mg/100 Tr.S. |
| getrocknete Preßschnitzel | 244 — 36 mg/100 g Schn. |
| Zuckerschnitzel | 499 — 343 mg/100 g Schn. |
| Rohsaft | 843 — 540 mg/100 Tr.S. |
| Vorscheidesaft | 558 — 534 mg/100 Tr.S. |
| Saturationssaft I A | 354 — 194 mg/100 Tr.S. |
| Saturationssaft I B | 132 — 89 mg/100 Tr.S. |
| Dünnsaft | 93 — 22 mg/100 Tr.S. |
| Dicksaft | 123 — 28 mg/100 Tr.S. |
| Weißzucker-Ablauf | 102 — 54 mg/100 Tr.S. |
| Mittelprodukt-Ablauf | 210 — 189 mg/100 Tr.S. |
| Raffinadekläre | 1.2 mg/100 Tr. |
| Melasse | 272 — 200 mg/100 Tr.S. |
| Rohzucker | 21 mg/100 Pol |
| Weißzucker | 2.20 — 0.30 mg/100 Pol |
| II. Saturationsschlamm getrocknet | 2.71 — 0.58 % |
| Dicksaft-Filter-Rückstand getrocknet | 0.55 % |

Ergebnisse von Brenztraubensäurebestimmungen Säften und Abläufen

| | Brenztraubensäure 100 Tr.S. |
|---|---|
| Rübenpreßsaft | 4.7 mg |
| Rohsaft | 17,5 mg |
| Vorscheidesaft | 9.0 — 17.4 mg |
| Saturationssaft I A | 7,8 — 12,1 mg |
| Saturationssaft I B | 4.5 — 7,7 mg |
| Dünnsaft | 6.6 — 8.5 mg |
| Dicksaft | 10,0 — 14,9 mg |
| Weißzucker-Ablauf | 8.2 — 13,5 mg |
| Mittelprodukt-Ablauf | 22.0 — 29.9 mg |
| Melasse | 40,2 — 64,4 mg |

Bei den auf Brenztraubensäure untersuchten Proben handelt es sich um Säfte und Abläufe des Werkes Rain aus der Kampagne 1967/68.

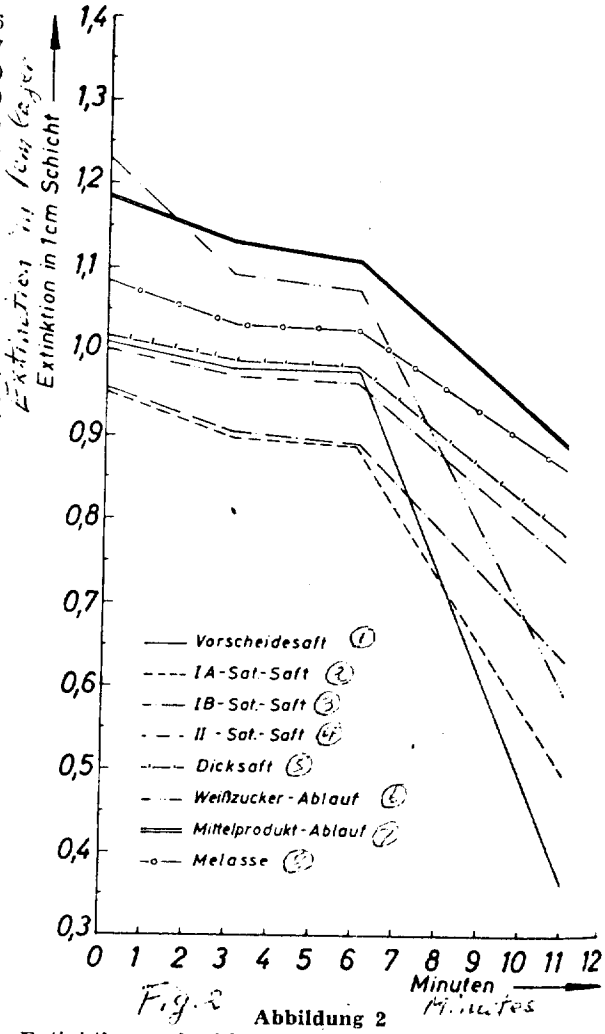



Abbildung 2
Extinktionsverlauf bei Citronensäurebestimmungen mit getrennter Zugabe der Enzyme LDH, MDH und CL

Neben der Brenztraubensäure wurde auch Oxalessigsäure bestimmt, wobei Werte um 10 mg/100 Tr. S. im Rohsaft, 1 mg/100 Tr. S. im Dünnsaft und 20 mg pro 100 Tr. S. in der Melassé gefunden wurden.

Abschließend möchte ich Herrn Dr. H. Schiweck, Zentral-Laboratorium der Süddeutschen Zucker-AG, Neuoffstein, für die wertvollen Anregungen und die Unterstützung dieser Arbeit besonders danken.

**Zusammenfassung**

In der vorliegenden Arbeit wird ein Verfahren beschrieben, das es ermöglicht, Citronensäure und Brenztraubensäure in sämtlichen Produkten zu bestimmen, die bei der Zuckerfabrikation anfallen. Die Methode zeichnet sich dadurch aus, daß sie reproduzierbare Werte ergibt und daß diese Werte auch in einer relativ kurzen Zeit erhalten werden, so daß man die Methode auch für die Betriebskontrolle einsetzen kann. Die Versuchsergebnisse haben wertvolle Erkenntnisse für die Beurteilung technologischer Prozesse, z. B. der Saftreinigung, geliefert.

**Summary**

In the present paper a method has been described permitting determination of citric and pyruvic acid in all products which occur during sugar production. This method gives reproducible values even in a relatively short time and therefore is suitable for factory control. The results obtained are most valuable for judging technological processes, e. g. juice purification.

**Résumé**

Dans l'article présent l'auteur a décrit une méthode qui permet la détermination des acides citrique et pyruvique dans tous les produits qui se présentent pendant la fabrication du sucre. Cette méthode est caractérisée par le fait qu'elle fournit des valeurs reproductibles, même après un temps relativement court, et par cela convient aussi au contrôle de la fabrication. Les résultats obtenus sont de grande valeur pour le jugement de procédés technologiques, par exemple de l'épuration des jus.

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# Organic Acids in Citrus Fruits. I. Varietal Differences[a]

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**SUMMARY**

**Organic acids were determined in juices and peels of mature fruits of Washington Navel and Valencia oranges, grapefruit, lemon, tangerine, and Palestine sweet lime. Juices and peels were freeze-dried, and organic acids were determined by silicic-acid chromatography. Cation-exchange resin in the hydrogen form was used for acidification of peel prior to extraction of acids with methanol. Citric acid predominated in all juices except that of the sweet lime (in which citrate level was so low that malate exceeded it). Malate occurred in all juices in low but measurable concentrations. Oxalate predominated in the peels, but in the lime, malonate appeared in equivalent amounts. Malonate appeared in all peels in measurable amounts, and in some peels was exceeded only by oxalate. Malic and citric acids were also determined in each peel. At least 15 peaks appeared with each peel, and paper chromatography of the effluents indicated the presence of more than one component in several peaks.**

The chemistry of citrus fruits and changes associated with maturation have been studied by many workers. This information has been summarized in recent reviews (Kefford, 1959; Sinclair, 1961). More recently, the author and co-workers have presented data regarding free amino acids in citrus fruits and the variations which accompany maturation (Clements and Leland, 1962a,b). This report is an extension of these studies to include the nonvolatile organic acids in peel and juice of different varieties, preliminary to investigations of maturation changes.

Knowledge regarding the citrus acids and the role they play in determining the acidity and buffering properties of the fruit is summarized in the aforementioned reviews (Kefford, 1959; Sinclair, 1961). In general, citric acid predominates in the flesh of the fruits, and oxalate predominates in the peels; several other acids have been reported in variable amounts in the different varieties. Citric acid has received much attention because of its obvious contribution to the properties of the juices of the commercial varieties, but quantitative data on the "minor" acids of the fruits are relatively limited. Such acids, as intermediates in the metabolic processes of the fruit, are directly involved in growth, maturation, and senescence. The present study was undertaken to determine differences among varieties, primarily to provide a basis for future studies correlating chemical composition with maturation behavior. Such information should also be of value in interpreting postharvest characteristics and taxonomic relationships.

The procedure of Bulen *et al.* (1952) was modified to serve as a method of analysis. For the juices, direct addition of the freeze-dried solids to the silicic-acid column was satisfactory. For peel studies, however, this technique resulted in poor resolution and low recoveries. Ether-extraction of the freeze-dried peel also presented several difficulties. Prolonged extraction (several days) was required for complete extraction, and the large amounts of sulfuric acid required to liberate oxalate from its calcium salt resulted in a moist product unsatisfactory for ether extraction without addition of adsorbent. Although such procedures have been applied in citrus peels (Sinclair and Eny, 1947), analysis of such extracts on silicic-acid columns gave erratic results and produced peaks which appeared to be artifacts.

Cation-exchange columns in the hydrogen form are used extensively for the acidification of liquids. Bradley (1960) used such

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a procedure for studies of tomato purée, and eliminated sulfuric acid from the analytical silicic-acid column. This technique made it possible to determine the slower-moving acids (including the mineral acids), the latter being eluted with methyl alcohol. It was concluded from this report that an analagous procedure might be used for acidification and extraction of acids from solids for consequent application to the conventional silicic-acid column. Treatment with cation-exchange resin in the hydrogen form is less drastic than addition of relatively concentrated sulfuric acid to the dry material. It also permits extraction with a more polar solvent (and therefore one in which most of the acids are more soluble), since the addition of extractable acid is avoided. Such an extract, free of extraneous acid, provides a measure of total-anion content. This technique was applied to peels in the following study, and should be applicable to other materials.

## EXPERIMENTAL

**Source of fruit.** Arizona and Texas grapefruits and one sample of Washington Navel orange (Sample III) were obtained from local markets. All other samples were picked from trees in plots of the Citrus Research Center at Riverside, California.

**Preparation of fruit.** Each sample (ten fruit) was washed and weighed. Pulps were pooled, weighed, sliced, and crushed in a mortar. Juice was expressed by squeezing through cheesecloth, and was finally vacuum-filtered through Celite (J-M Filter-Aid) to give a clear serum. Solids, pH, and total acid were determined, and the serum was freeze-dried and stored over calcium chloride. Peels were pooled and weighed, and ground in a homogenizer (Waring blender) without addition of water. The homogenate was frozen in a polyethylene bag, and several such samples were freeze-dried simultaneously in the apparatus previously described (Clements, 1962). The dry peel was stored over calcium chloride.

**Extraction of acids from peel.** Freeze-dried peel (1 g) was ground finely in a mortar with an equal weight of dry Amberlite IR-120 resin (Rohm and Haas Co.), converted to the hydrogen form as described by Moore *et al.* (1958), washed free of chloride, and dried at 60°C. The mixture was transferred to a wide-mouth screw-cap vial, and water was mixed in to give a smooth paste. The vial was capped and stored 4–5 days at 4°C. Silicic acid (washed and dried) was then mixed with the sample to give a free-flowing powder, and the mixture was slurried in acid-free methanol and transferred to a glass column (22 mm ID × 20 cm) equipped with a stopcock at the lower end, and with a glass-wool plug above the stopcock. A reservoir (200 ml), attached to the top of the column with a 19/38 ₮ joint and fitted with a squeeze bulb, facilitated the application of pressure. The liquid was forced out until level with the top of the solids, and additional methanol was forced through the column until the effluent was acid-free. The combined effluent (50–60 ml) was titrated with 0.2*N* sodium hydroxide, with phenol red as an indicator, and evaporated to dryness in an air stream. The residue was analyzed immediately or stored at −4°C.

**Determination of acids.** The following modification of Bulen *et al.* (1952) was used: Silicic acid (10 g), washed free of fines and dried, was ground with 0.5*N* sulfuric acid (7.2 ml). The mixture was slurried in washed chloroform and packed in a column (12 mm ID × 35 cm). The sample (50 mg juice solids or extract of 1 g peel) was acidified with 5*N* sulfuric acid (0.5 ml), taken up with a minimum amount of silicic acid, and added to the top of the column. The acids eluted with mixtures of *n*-butanol in chloroform equilibrated against 0.5*N* sulfuric acid according to the following schedule: 75 ml each of 2.5, 5, 10, 15, 20, and 25%, followed by 250 ml of 35% (butanol in chloroform, v/v). Fractions (3.3 ml) were collected, and titrated with 0.01*N* sodium hydroxide (aqueous), with phenol red as indicator. The flow rate was approximately 100 ml per hour.

**Paper chromatography.** Peaks were tentatively identified by comparison of elution thresholds with known compounds. For further identification, fractions representing individual peaks were combined, evaporated, and chromatographed on paper. Two dissimilar solvent systems were used: I) *i*-amyl alcohol equilibrated against 5*M* formic acid (Clements and Deatherage, 1957); and II) pyridine-ethyl acetate–acetic acid–water (5:5:1:3, v/v/v/v) (Fischer and Dorfel, 1955). Small-scale one-dimensional chromatograms were run as previously described (Clements and Deatherage, 1957) on S & S 589 White Ribbon acid-washed paper. $R_f$ values were measured, but in every case unknowns were checked against known compounds on the same chromatogram. Spots were detected by UV fluorescence and adsorption, bromophenol blue, and ammoniacal silver nitrate (Buch *et al.*, 1952). Color development with the last reagent was followed for several days, since it appears to be characteristic for individual compounds. Only those peaks which gave positive reactions with bromophenol blue on the chromatograms are reported quantitatively, however.

## RESULTS AND DISCUSSION

**Evaluation of methods.** Fig. 1 shows elution diagrams for a calibration column and for an analysis of a resin-treated lemon peel. Peak positions were determined for several other known acids, but the standard separation (which is illustrated) was primarily for the purpose of determining recoveries of those acids found to occur in citrus peels. These recoveries were determined by subjecting weighed amounts of acids to a resin treatment and extraction identical to the procedure used for peel analysis. Recoveries were comparable to those reported by Bulen *et al.* (1952) (i.e., 95–100% for most acids). Separate studies were performed on calcium oxalate, to determine the rate of solubilization. Results indicated that free oxalate was released from the relatively insoluble salt almost immediately. Recoveries exceeded those reported by Bulen *et al.* (85%). None of the acids studied appeared to be altered when subjected to contact with the resin (aqueous suspension) for several days at 25°C.

The period (4–5 days) used for peel treatment was determined by experiment, and appeared to be sufficient for equilibration (as evidenced by chromatograms from various treatments). The procedure produced sharp, reproducible separations, and appears to be especially advantageous for determinations of oxalate when it is present as the calcium salt. Total-anion values, determined by direct titration of the extract from duplicate samples, consistently agreed within 3–4%.

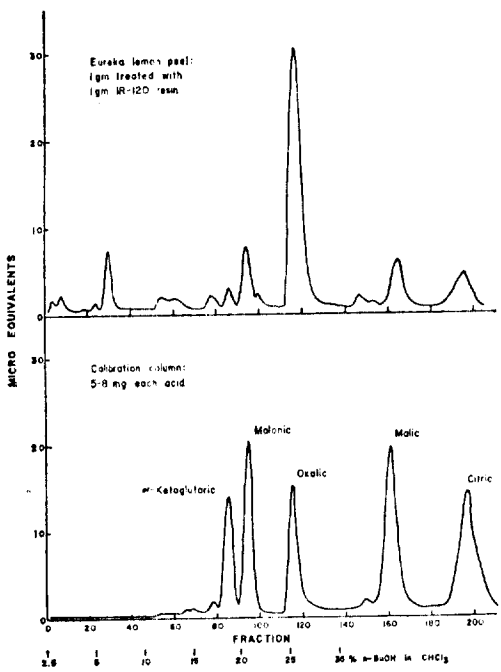


Fig. 1. Organic acids from lemon peel acidified with cation-exchange resin in the hydrogen form.

**Compositions of juices.** Data regarding the juices are presented in Table 1, and for those varieties which have been studied by other workers, the compositions are generally within the ranges reported (Sinclair, 1961; Wolf, 1958). Characteristically, citric acid predominated and malic acid appeared at low, but significant, levels. However, an exception was the Palestine sweet lime. The solids-acid ratio of the sample was 99 (compared to 12–13 for the oranges); the pH was 5.7, considerably higher than that of any other variety. The fruit which were analyzed displayed physical characteristics indicative of a physiological stage comparable to that of oranges during commercial maturity (i.e., with respect to solids content of juice, moisture content of peel, degree of turgidity, etc.). It is interesting to note that this was the only variety in which malic acid predominated in the juice (although malate concentration was comparable to that in the other varieties). The data suggest that the degree of acidity which characterizes a given variety is almost entirely a function of citrate content.

Silicic-acid separations of the various juices indicated that acids in addition to malate and citrate were present in the effluent, though at extremely low levels. Ascorbic acid, galacturonic acid, and other slow-moving acids would not appear in the effluent. Ting and Deszyck (1959) reported *l*-quinic acid in the peel and pulp of Pineapple oranges. Elution of this acid from a silicic-acid column requires the addition of 50% *n*-butanol in chloroform (following 35%). This sequence was followed on single samples of peels and juices of the varieties in preliminary stages of this study, but additional measurable peaks did not appear. However, sensitivity is reduced with this concentration of butanol (because of high

Table 1. Citric and malic acid contents of juices of some citrus varieties.

| Variety | Solids (%) | Acid (as citric) (%) | Solids acid | pH | Meq per 100 mg solids: Malic acid | Meq per 100 mg solids: Citric acid | Grams per 100 ml juice: Malic acid | Grams per 100 ml juice: Citric acid |
|---|---|---|---|---|---|---|---|---|
| Orange | | | | | | | | |
| Wash. Navel (I) | 6.8 | 0.55 | 12.4 | 3.6 | 13 | 129 | 0.06 | 0.56 |
| Wash. Navel (II) | 6.4 | .51 | 12.5 | 3.6 | 11 | 132 | .05 | .54 |
| Wash. Navel (III) | 12.7 | .95 | 13.4 | 3.6 | 23 | 115 | .20 | .93 |
| Valencia | 12.9 | 1.04 | 12.4 | 3.4 | 19 | 119 | .16 | .98 |
| Grapefruit | | | | | | | | |
| Calif. (Marsh) | 10.1 | 1.80 | 5.6 | 2.9 | 9 | 276 | .06 | 1.79 |
| Arizona | 11.6 | 1.98 | 5.9 | 3.1 | 5 | 284 | .04 | 2.10 |
| Texas (pink) | 9.3 | 1.14 | 8.2 | 3.3 | 9 | 201 | .06 | 1.19 |
| Lemon | | | | | | | | |
| Eureka (I) | 8.6 | 4.40 | 2.0 | 2.5 | 29 | 726 | .17 | 4.00 |
| Eureka (II) | 8.6 | 4.57 | 1.9 | 2.4 | 46 | 796 | .26 | 4.38 |
| Lime | | | | | | | | |
| Palestine Sweet | 9.9 | .10 | 99.0 | 5.7 | 30 | 1 | .20 | .08 |
| Tangerine | | | | | | | | |
| Dancy (I) | 11.7 | 1.00 | 11.7 | 3.5 | 23 | 164 | .18 | 1.22 |
| Dancy (II) | 11.3 | .84 | 13.5 | 3.6 | 28 | 119 | .21 | .86 |

background and broad peaks), and quinic acid must be present in substantial amounts to produce a distinct peak.

**Composition of peels.** Characteristics of the peels are presented in Table 2. In general, the varieties displayed elution patterns qualitatively similar to that of the lemon (Fig. 1). Paper chromatography of column effluents indicated that most of the peaks contained more than one compound detectable with ammoniacal silver nitrate. However, the predominant acids (oxalic, malic, malonic, and citric) produced peaks which appeared to be relatively pure. These acids also appeared at levels sufficient to give a strong positive reaction with bromophenol blue when the peaks were chromatographed on paper. The fractions containing malonic acid consistently contained another compound which exhibited $R_f$ values and color

Table 2. Organic acids in the peel of some citrus varieties.

| Variety | Weight (g) (av. per fruit): Fruit | Pulp | Peel: Wet | Peel: Dry | Meq. per g dry weight of peel: Total anion extd. | Citric acid | Malic acid | Oxalic acid | Malonic acid |
|---|---|---|---|---|---|---|---|---|---|
| Orange | | | | | | | | | |
| Wash. Navel (I) | 145.9 | 99.0 | 41.5 | 11.8 | 0.40 | 0.01 | 0.02 | 0.11 | 0.02 |
| Wash. Navel (II) | 145.1 | 101.2 | 40.5 | 11.5 | .41 | .01 | .02 | .12 | .01 |
| Wash. Navel (III) | 143.3 | 102.8 | 36.1 | 10.3 | .41 | .01 | .02 | .10 | .03 |
| Valencia | 115.8 | 88.2 | 26.1 | 7.7 | .44 | Trace | .02 | .13 | .03 |
| Grapefruit | | | | | | | | | |
| Calif. (Marsh) | 286.2 | 193.3 | 90.5 | 20.3 | .32 | .01 | .03 | .06 | .02 |
| Arizona | 361.1 | 243.7 | 112.8 | 28.3 | .59 | .03 | .10 | .12 | .02 |
| Texas (pink) | 395.4 | 311.7 | 80.2 | 18.8 | .53 | .01 | .08 | .08 | .02 |
| Lemon | | | | | | | | | |
| Eureka (I) | 97.8 | 69.2 | 27.0 | 5.5 | .60 | .04 | .04 | .15 | .03 |
| Eureka (II) | 92.5 | 64.5 | 26.7 | 5.5 | .53 | .03 | .02 | .12 | .04 |
| Lime | | | | | | | | | |
| Palestine Sweet | 137.7 | 115.7 | 21.5 | 4.9 | .46 | Trace | .04 | .05 | .05 |
| Tangerine | | | | | | | | | |
| Dancy (I) | 108.2 | 81.7 | 25.0 | 6.9 | .60 | .02 | .06 | .15 | .01 |
| Dancy (II) | 70.2 | 48.6 | 20.9 | 5.4 | .68 | .02 | .09 | .20 | .02 |

reactions characteristic of chlorogenic acid. However, it did not appear in peel effluents in sufficient amounts to give an acid reaction with bromophenol blue on paper chromatograms. The oxalate fractions also contained other compounds, though in relatively small amounts. Each of the peels produced at least fifteen different peaks, and a total of 25–30 components on paper chromatograms. No attempts were made to retain volatile acids, and, as with the juices, the slow-moving acids would not appear in the column effluent.

Table 2 shows that oxalate predominated in every peel except that of the Texas grapefruit, in which oxalate and malate appeared in equivalent amounts; malate content approached that of oxalate in the other grapefruit. The lemons and tangerine exhibited the highest total-anion and oxalate contents. Subsequent data (in preparation) show that the peel acids occur in considerably higher concentrations in flavedo than in albedo (approximately 2:1). Therefore, high acid content in tangerine peel may be a reflection of low albedo content in this fruit. It also suggests that lemon flavedo would demonstrate a relatively high acid content.

The values for oxalate, citrate, and malate in peels of orange, lemon, and grapefruit agree closely with those reported by Sinclair and Eny (1947). Those authors did not report malonate, although Biale and Young (1962) found malonic acid in lemon peel. In the present study, malonic acid content was exceeded only by that of oxalate (in several instances); malonate and oxalate appeared in equivalent amounts in the sample of Palestine sweet lime. Hatch and Stumpf (1962) reviewed the occurrence and possible roles of malonic acid in plants, and concluded from their own experiments that enzymes for the synthesis and breakdown of malonate are widely distributed among plants. Although the present study involved relatively limited sampling, it indicates that malonic acid is normally present in measurable amounts in the peels of mature citrus fruits. It does not appear to be present in the pulp, however. The role of malonate, as well as that of the other carboxylic acids which accumulate in these fruits, remains to be determined.

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# A simplified and sensitive method for citrate determination in biological samples

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COSTELLO, L. C., AND JOHN J. O'NEILL. *A simplified and sensitive method for citrate determination in biological samples.* J. Appl. Physiol. 27(1): 120–122. 1969.—A rapid and sensitive micromethod for the determination of citrate is described. The procedure is fluoroenzymatic and is based on the enzymatic conversion of citrate to malate by citrate lyase and malic dehydrogenase. The accompanying oxidation of NADH is measured fluorimetrically. The assay is linear in the range of $2 \times 10^{-7}$ to $10^{-5}$ M and specific for citrate. The method has been applied to citrate determination of urine, deproteinized plasma, and tissue extracts.

citrate assay; enzymatic assay; fluorimetric determination; citrate lyase

FOR RESEARCH PROGRAMS in our laboratories we required a simplified and sensitive assay procedure for the determination of citrate in blood, urine, and tissue extracts. The colorimetric procedures based on the conversion of citrate to pentabromoacetone (5) lacked the required specificity and sensitivity and were most tedious. The enzymatic assay using aconitase and isocitric dehydrogenase (4) had limitations owing to the low stability of aconitase and to the problem of substrate specificity. A better approach seemed to be offered by the use of citrate lyase (CL) as reported by Dagley (1). However, the enzyme preparation and assay procedure described did not afford the sensitivity and specificity required. The spectrophotometric method reported by Moellering and Gruber (3) afforded a satisfactory citrate assay procedure within the sensitivity limitations of the spectrophotometric assay of NADH. The procedure which we developed makes use of the fluorimetric determination of NADH and defines more ideal conditions of the enzymatic assay. The increased sensitivity permits the direct assay of citrate in urine, deproteinized plasma, and tissue extracts without any additional prior treatment of the biological material. The procedure is based on the following reaction:

$$\text{citrate} \xrightarrow{\text{CL}} \text{oxalacetate} + \text{acetate}$$
$$\text{oxalacetate} + \text{NADH} \xrightarrow{\text{MDH}} \text{malate} + \text{NAD}^+$$

The oxidation of NADH is determined fluorimetrically and is directly related to the concentration of citrate on a molar basis.

## METHODS

Initially, we prepared the CL from *Aerobacter aerogenes* by a modification of the method of Dagley (1) since it was essential to minimize the presence of interfering enzymes, particularly NADH oxidase. However, during the course of these studies, CL became commercially available from Boehringer Company and we found it to be quite satisfactory for this assay. The precise conditions for assay depend on the citrate concentration being analyzed. A typical protocol is presented below to demonstrate the procedure.

*Reagents.*

*a*) Buffer—20 mM tris(hydroxymethyl)aminomethane (Tris, pH 8.2) containing 1.0 mM $MgCl_2$.

*b*) NADH—approximately 1–10 mM prepared in a mixture of 0.133 M $Na_2CO_3$/0.067 M $NaHCO_3$. The NADH concentration is determined spectrophotometrically at 340 mμ. A NADH sample is diluted with 0.1 M phosphate (pH 7.0) in a cuvette to approximately 0.1 mM and the initial absorbancy is ascertained. By the addition of LDH and excess pyruvate all the enzymatically active NADH is oxidized and the final absorbancy is determined. The concentration of NADH is then calculated from the extinction coefficient ($6.22 \times 10^6$ cm²/mole).

*c*) Citrate—1–10 mM prepared in Tris buffer (pH 8.2).

*d*) Oxalacetate—1–10 mM prepared in 1.0 mM acetic acid.

*e*) Citrate lyase (Boehringer)—approximately 4.0 mg dry powder/ml prepared in 20 mM Tris buffer (pH 8.2) containing 1% bovine serum albumin. Such a preparation will contain about 1.0 mg citrate lyase/ml. As described below, the enzyme concentration should provide a reaction half-time ($t_{1/2}$) of approximately 1 min. We have observed that the dilute enzyme preparation can be stored for 4 months at −20 C with about 26% loss of activity and at −80 C with a loss of 19% activity.

*f*) Malic dehydrogenase (Boehringer)—approximately 1.0 mg/ml prepared as described in *e* above. The enzyme concentration must provide at $t_{1/2}$ less than that of CL rate.

The reaction is performed in culture tubes at 30 C and fluorescence is determined with a sensitive and stable fluorometer (e.g., Farrand photoelectric fluorometer model A-2).

The general fluorimetric assay procedures described by Lowry and Passonneau (2) are employed. The citrate assay is performed as follows: *a*) add 1.0 ml Tris buffer with $MgCl_2$; *b*) add sample (1–10 μl) containing citrate (final concn $10^{-5} - 2 \times 10^{-7}$ M); *c*) add 1–10 μl NADH (final concentration $10^{-5} - 2 \times 10^{-7}$ and record the initial fluorescence (Fi); *d*) add 1.0 μl MDH and 1.0 μl CL and determine the decreased fluorescence (Fc). With the conditions described the reaction is complete in 10 min; *e*) add 1.0 μl oxalacetate to oxidize the remaining NADH and record this blank fluorescence (Fb). The proportion of the fluorescence change due to sample (Fi-Fc) to the total fluorescence of the standardized NADH (Fi-Fb) is converted to concentration of citrate in the reaction. Multiplication by the dilution factor (total reaction volume ÷ sample volume) gives the citrate concentration of the sample.

## RESULTS AND DISCUSSION

The success of this assay procedure depends upon several conditions. The amount of MDH and CL to be used must be determined for each lot of enzyme. The amount of CL should provide a $t_{1/2}$ of approximately 1.0 min and the MDH should produce a $t_{1/2}$ of approximately 0.5 min (Fig. 1). Under these conditions, the assay

reaction is complete in 10 min. Also, these conditions insure that the oxalacetate formed from citrate does not accumulate and is immediately converted to malate by MDH. Such conditions virtually eliminate any interference by contaminating decarboxylase activity of the CL preparations. Under these conditions, even the addition of hydrazine ($10^{-3}$ M) did not interfere with the assay. The pH selected for this assay was 8.2. The optimal pH of CL is 7.8. However, CL preparations often contain some NADH oxidase activity which will interfere with the assay. As demonstrated in Fig. 2, approximately 10% of maximal NADH oxidase activity is observed at pH 8.2 as compared to about 75% of the maximal CL activity. The conditions for the spectrophotometric assays described earlier (1, 3) included a pH 7.4 which would not seem to be advantageous. The use of pH 8.2 virtually eliminates any possible interference by contaminating oxidase activity. Furthermore, under the conditions of this assay system, we have not observed any advantage to the addition of $Zn^{++}$ as previously described (3).

Under these conditions, the sensitivity of the citrate assay is linear throughout the range of $10^{-5}$ – $2 \times 10^{-7}$ M. All assays were prepared in triplicate and fluorescent values of replicates generally were within 3% of the mean. The mean standard deviation for the entire assay range as determined from several experiments (N = 48) was ± 2.3. Figure 3 shows the linearity over the range of $10^{-5}$ to $10^{-6}$ M. Figure 4 demonstrates the linearity obtained with increasing concentration of deproteinized plasma. Figure 5 demonstrates the recovery and linearity achieved with addition of citrate to brain homogenate, and Table 1 presents the recovery of added citrate to blood. All such results indicate the accuracy and sensitivity of this procedure. In addition the assay is specific for citrate as indicated by the absence of any interference by the addition of $10^{-3}$ M succinate, pyruvate, isocitrate, alpha-ketoglutarate, aspartate, and glutamate (Table 2) as well as by the specificity

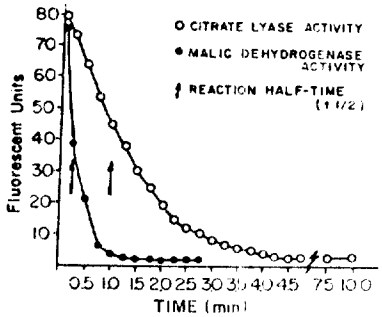


FIG. 1. Reaction rates of citrate lyase and malic dehydrogenase for citrate assay. Ideally the $t_{1/2}$ for malic dehydrogenase must be less than the $t_{1/2}$ for citrate lyase. Under the conditions illustrated the reaction is complete within 10 min.

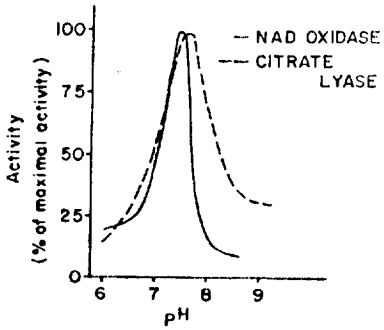


FIG. 2. pH activity curves for citrate lyase and NADH oxidase. Source of NADH oxidase was the contaminant of partially purified citrate lyase prepared essentially by the method of Dagley (1).

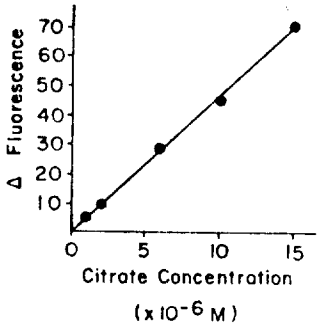


FIG. 3. Linear relationship between citrate concentration and decreased fluorescence of NADH. Blank fluorescence was equivalent to $4 \times 10^{-7}$ M citrate.

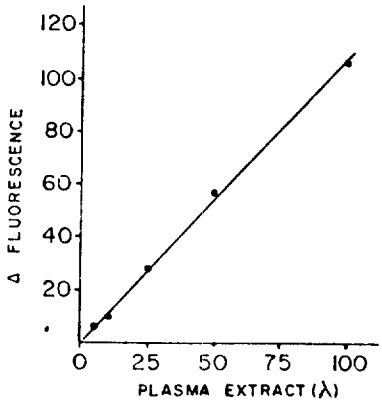


FIG. 4. Linear relationship of the citrate assay method with increasing concentrations of deproteinized plasma.

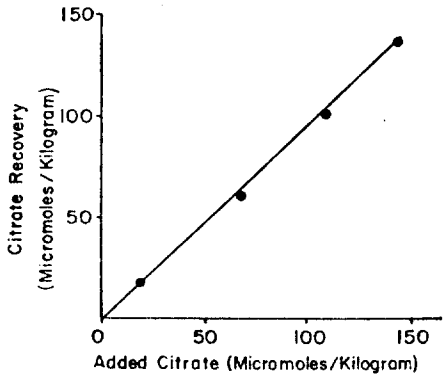


FIG. 5. Recovery of added citrate from brain homogenate.

studies of Moellering and Gruber (3). Furthermore, we have observed no interfering LDH activity in the enzyme preparations under these assay conditions. This is also indicated by the absence of any interference by added pyruvate. However, it is important to check the enzyme preparations for LDH contamination. Earlier procedures (1, 3) required the use of LDH in the assay. However, this would necessitate treatment of the sample to remove pyruvate and/or determination of the pyruvate level. We have not observed any need for the use of LDH with MDH in this assay as described by Moellering and Gruber (3). The absence of LDH provides the advantage of eliminating the problems of pyruvate interference. Should LDH contamination of the auxillary enzymes cause interference with the assay, the problem can be eliminated by the prior addition of hydrazine to the reaction system. In this way the citrate

TABLE 1. *Recovery of added citrate from plasma*

| | Fluorescent Units | |
|---|---|---|
| | *Exp 1* | *Exp 2* |
| Plasma | 31 | 31 |
| Added citrate | 78 | 37 |
| Plasma + added citrate | 108 | 70 |
| Calculated total | 109 | 68 |

In *exp 1* the amount of added citrate to blood was twice the amount of *exp 2*. After the addition of citrate, the plasma was deproteinized with trichloroacetic acid and neutralized prior to assay.

TABLE 2. *Effect of various substrates on citrate determination*

| Substrate | Initial Fluorescence | Final Fluorescence | Difference |
|---|---|---|---|
| Minus citrate | 73 | 73 | 0 |
| Citrate | 73 | 39 | 34 |
| Citrate + alpha-ketoglutarate | 71 | 35 | 36 |
| Citrate + succinate | 70 | 36 | 34 |
| Citrate + isocitrate | 70 | 34 | 36 |
| Citrate + aspartate | 70 | 35 | 35 |
| Citrate + glutamate | 71 | 36 | 35 |
| Citrate + pyruvate | 72 | 38 | 34 |

System contained citrate lyase, malic dehydrogenase, NADH ($1 \times 10^{-5}$ M), citrate ($0.5 \times 10^{-5}$ M), other substrates ($1 \times 10^{-}$ M), 20 mM Tris buffer (pH 8.2), 1.0 mM $MgCl_2$, in a final volume of 1.0 ml.

assay can be performed in the same reaction with no contribution to the NADH blanks.

We have had an opportunity to apply this assay procedure to a number of physiological and biochemical problems over the past 2 years. Urine citrate can be determined directly without any prior sample treatment. Plasma and tissue extracts must be deproteinized prior to assay. In this regard we have observed no differences with the use of trichloroacetic acid or perchloric acid treatment as deproteinizing agents. However, zinc precipitation of plasma resulted in only partial recovery (about 70%) of citrate. The assay procedure described should be applicable to essentially all types of biological materials where the level of citrate is to be determined.

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0516

# Tests on the Toxicity of Ascorbic Acid

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In the course of tests on the tumefaction of rat liver mitochondrias according to the technique described by Carvalho Guerra (1), associated with a polarographic study of the respiratory process with the Clark (2) electrode, our attention was directed to the possibility of checking alterations in a living animal which could be attributed to the toxic effect of ascorbic acid.

Electron microscope observations of sections of the liver and adrenal glands of rats fed high dosages of vitamin C or injected with large amounts of this drug showed the presence of alterations which would appear to justify an interesting line of study. It was precisely the first type of tests (oral feeding of vitamin C), which disclosed disturbances in the behavior and development of animals, that prompted the presentation of this report.

References to tests on the toxicity of ascorbic acid are seldom found in the literature; the latter contains numerous descriptions of deficiency phenomena, but the situation is different in regard to the after effects (consequences) of vitamin overdosage. In fact, this vitamin can be considered as nontoxic. Among the few observations encountered in this respect, we can refer to those of Stepp (3) and Velasquez (4). The latter author describes overdosage syndromes and cites Armijo who established a lethal dose of about 3.7 mg/g for small rats or mice, with death occurring as a result of respiratory and cardiac paralysis. On our part, we have injected rats intravenously with doses superior to 4 mg/g without any fatal results.

The results obtained after oral administration of vitamin C are listed in Tables III-VII. The development (growth) of animals fed a normal diet, and particularly of an animal series receiving a pulverized diet, is shown in Table I and II. This approach is justified to the extent that when drugs are added to food, the latter is always reduced in size in order to ensure a good distribution of the drugs in the food. On the other hand, while acknowledging the possibility of effects which we will have to check on the basis of acidity phenomena, we have submitted a batch of animals to a diet enriched with citric acid (Table VIII), and in two series of tests with ascorbic acid

we have added sodium bicarbonate to the water. As a basic food, we used a diet consisting of 41-B Inecil and water, in an unlimited amount.

We do not consider the results of the present tests sufficient to allow us to draw any causal conclusions. In the meantime, we are permitted to make some curious observations.

Animals which were fed "pellets" reduced to a powder showed a development inferior to that of animals receiving a product consisting of voluminous fragments. Since the composition of the diet does not vary as a result of pulverization, the only explanation we can provide for this phenomenon is the greater difficulty which the rats have in ingesting their food.

Small doses of ascorbic acid (of the order of 1%) do not exert any unfavorable effects on the development (growth) of rats; with 5% the weight drops initially, but later shows a tendency to restore itself, and with 10% the animals suffer a substantial physical deterioration which can result in death within a few days. It must be pointed out that there is no loss of appetite as long as the physical condition of the animals does not become seriously disturbed; on the contrary, these animals ingest, on the average, amounts of food superior to those taken by normal animals.

The simultaneous administration of sodium bicarbonate to animals treated with ascorbic acid has, in the dosage used, a clearly adverse (detrimental) effect.

Addition of 2.5% citric acid to the food leads to a stoppage or even a decrease of the weight of rats during the first few days. At the end of one week, when the animals are kept under the same feeding conditions, their growth shows a tendency to become normal. Similar conclusions can be drawn in regard to animals fed a diet with 5% ascorbic acid.

Autopsy did not disclose, in any group of animals, any macroscopically visible signs of visceral injury. However, a liquefaction of the bowel (colon) contents accompanied by gaseous distention was noted in animals treated with high doses of ascorbic acid, and especially in those animals which received simultaneously sodium bicarbonate. This finding agrees with the tendency to excrete liquid feces which was found in these animals. A laxative effect and disturbances in the absorption of food may, moreover, constitute the basis of the altered growth phenomena described here. This is a hypothesis which we intend to clarify.

Table 1 - Weight changes of rats fed a normal diet

| Weight, g \ Rat | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Initial | 72 | 70 | 80 | 65 | 70 | 65 |
| After 3 days | 90 | 85 | 87 | 88 | 100 | 83 |
| After 6 days | 100 | 100 | 104 | 100 | 117 | 100 |

Table 2 - Weight changes of rats fed a pulverized normal diet

| Weight, g \ Rat | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Initial | 65 | 75 | 65 | 85 | 70 | 90 |
| After 3 days | 70 | 70 | 70 | 80 | 70 | 90 |
| After 6 days | 70 | 67 | 70 | 80 | 75 | 95 |
| After 9 days | 70 | 68 | 80 | 82 | 80 | 105 |

Table 3 - Weight changes of rats fed a diet containing 1% ascorbic acid

| Weight, g \ Rat | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Initial | 80 | 80 | 80 | 80 | 80 | 80 |
| After 3 days | 80 | 85 | 78 | 80 | 80 | 78 |
| After 6 days | 85 | 85 | 87 | 85 | 83 | 87 |
| After 9 days | 90 | 90 | 90 | 90 | 95 | 95 |

Table 4 - Weight changes of rats fed a diet containing 5% ascorbic acid

| Weight, g \ Rat | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Initial | 85 | 75 | 85 | 75 | 85 | 55 |
| After 3 days | 80 | 75 | 82 | 70 | 80 | 55 |
| After 6 days | 75 | 75 | 90 | 70 | 80 | 55 |
| After 9 days | 85 | 80 | 100 | 68 | 84 | 61 |

Table 5 - Weight changes of rats fed a diet containing 10% ascorbic acid

| Weight, g \ Rat | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Initial | 60 | 65 | 68 | 65 | 65 | 80 |
| After 3 days | 52 | 64 | 68 | 52 | 64 | 80 |
| After 6 days | Died | 69 | 67 | Died | 57 | 69 |

Table 6 - Weight changes of rats fed a diet containing 5% ascorbic acid and 5% Na Bicarbonate

| Weight, g \ Rat | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Initial | 98 | 75 | 80 | 75 | 80 | 65 |
| After 2 days | 80 | 60 | 62 | 60 | 65 | 55 |
| After 4 days | 65 | 53 | 55 | -- | 57 | 45 |
| After 6 days | 57 | 45 | 45 | 52 | Died | Died |
| After 8 days | Died | Died | Died | Died | -- | -- |

Table 7 - Weight changes of rats fed a diet containing 10% ascorbic acid and 10% Na bicarbonate

| Weight, g \ Rat | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Initial | 109 | 65 | 73 | 80 | 55 | 67 |
| After 3 days | 87 | 54 | 61 | 66 | 48 | 59 |
| After 6 days | 85 | Died | 60 | 52 | Died | 55 |

Table 8 - Weight changes of rats fed a diet containing 2.5% citric acid

| Weight, g \ Rat | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Initial | 75 | 80 | 70 | 80 | 60 | 70 |
| After 3 days | 65 | 72 | 60 | 75 | 55 | 70 |
| After 6 days | 70 | 80 | 60 | 75 | 55 | 72 |
| After 9 days | 80 | 90 | 70 | 85 | 65 | 86 |

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0516

VOL. XX ★ 1970 ABRIL-JUNHO ★ N.º 2

# TRABALHOS ORIGINAIS

## ENSAIOS SOBRE A TOXICIDADE DO ÁCIDO ASCÓRBICO (*)

A. DE ALBUQUERQUE e M. ARMANDA HENRIQUES

No decurso de ensaios de tumefacção de mitocondrias de fígado de rato segundo técnica descrita por Carvalho Guerra [1], associando o estudo polarográfico do processo respiratório com o eléctrodo de Clark [2], a nossa atenção foi chamada para a possibilidade de se verificarem alterações no animal vivo que pudessem ser imputadas a um efeito tóxico do ácido ascórbico.

A observação, no microscópio electrónico, de fragmentos de fígado e supra-renal de ratos alimentados com doses elevadas de Vitamina C, ou injectados com fortes proporções do fármaco, mostrou alterações que parecem justificar uma interessante linha de trabalho. Foi precisamente o primeiro tipo de ensaios que, ao revelar perturbações do comportamento e do desenvolvimento dos animais, deu lugar à apresentação desta nota.

É raro encontrar na literatura referências a ensaios de toxicidade do ácido ascórbico; se aquela é pródiga na descrição de fenómenos de carência, outrotanto não acontece quando se trata de consequências de hiperdosagem. Na realidade, esta vitamina pode considerar-se como não tóxica. Dentro das poucas observações que a este respeito encontramos podemos referir as de STEPP [4], e de VELASQUEZ [5]. Este último autor descreve síndromes de hiperdosagem e cita ARMIJO que estabeleceu, no ratinho, uma dose letal de cerca de 3,7 mg por grama de ani-

(*) Trabalho apresentado às VII Jornadas Farmacêuticas.

0516



mal, occorrendo a morte por paralisia respiratória e cardíaca. Pela nossa parte injectamos, no rato, por via intravenosa, doses superiores a 4 mg por grama sem consequências fatais.

Os resultados da administração de Vitamina C por via oral encontram-se distribuídos nos quadros III, IV, V, VI e VII. Nos quadros I e II inscrevemos a evolução de animais alimentados normalmente, com a particularidade de uma série ter recebido o alimento pulverizado. Esta atitude justifica-se na medida em que sempre que se adicionaram fármacos aos alimentos, estes foram prèviamente reduzidos a pó para assegurar um boa distribuição. Admitindo, por outro lado, a possibilidade de os efeitos que verificamos terem por base fenómenos de acidez, submetemos um lote de animais a uma dieta enriquecida em ácido cítrico (quadro VIII) e em duas séries com ácido ascórbico adicionamos bicarbonato de sódio à água. Como alimentação básica utilizamos uma dieta composta 41-B Inecil e água, sem restrições.

Não consideramos os resultados dos presentes ensaios suficientes para se poderem tirar conclusões causais. Entretanto, permitem algumas observações curiosas:

— os animais alimentados com as «rolhas» reduzidas a pó acusam um desenvolvimento inferior aos que receberam o produto em fragmentos volumosos. Uma vez que a constituição da dieta não variou em consequência da pulverização, só encontramos como explicação para o fenómeno a maior dificuldade que os ratos encontrem em tomar o alimento;

— pequenas doses de ácido ascórbico (da ordem do 1 %) não tem efeitos desfavoráveis sobre o desenvolvimento dos ratos; com 5 % o peso cai inicialmente, mas mostra, depois, tendência para a recuperação e com 10 % os animais sofrem uma deterioração física notória que os pode levar à morte ao fim de poucos dias. Deve assinalar-se que não houve quebra de apetite, pelo menos enquanto o estado físico não ficou sèriamente perturbado; pelo contrário, estes animais ingeriram, em média, quantidades de alimento superiores aos animais normais;

— a administração simultânea de bicarbonato de sódio aos animais tratados com ácido ascórbico teve, nas dosagens utilizadas, um efeito marcadamente prejudicial;

—a adição de 2,5 % de ácido cítrico aos alimentos levou a uma estagnação ou mesmo quebra, de peso dos ratos nos primeiros dias. Ao fim de uma semana de tratamento e mantendo-se as mesmas condições alimentares, os animais mostraram tendência para normalizar o desenvolvimento. Conclusão semelhante se pode tirar dos animais alimentados com 5 % de ácido ascórbico.

A autópsia não mostrou, em qualquer grupo de animais, sinais de lesão visceral macroscòpicamente visível. Há, entretanto, nos animais tratados com doses elevadas de ácido ascórbico, e sobretudo nos que receberam simultâneamente bicarbonato de sódio, liquefacção do conteúdo cólico acompanhada de distensão gasosa. Este achado concorda com a tendência para a dejecção de fezes líquidas que se verifica nestes animais. Um efeito laxativo e perturbações da absorção dos alimentos poderiam, de resto, estar na base dos fenómenos de alteração de desenvolvimento descritos. É uma hipótese que tencionamos esclarecer.

0516



QUADRO I

Evolução do peso de ratos alimentados com dieta normal

| Rato / Peso g | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Inicial | 72 | 70 | 80 | 65 | 70 | 65 |
| Aos 3 dias | 90 | 85 | 87 | 88 | 100 | 83 |
| Aos 6 dias | 100 | 100 | 104 | 100 | 117 | 100 |

QUADRO II

Evolução do peso de ratos alimentados com dieta normal, pulverizada

| Rato / Peso g | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Inicial | 65 | 75 | 65 | 85 | 70 | 90 |
| Aos 3 dias | 70 | 70 | 70 | 80 | 70 | 90 |
| Aos 6 dias | 70 | 67 | 70 | 80 | 75 | 95 |
| Aos 9 dias | 70 | 68 | 80 | 82 | 80 | 105 |

0516



QUADRO III

Evolução do peso de ratos alimentados com dieta com 1 % de ácido ascórbi

| Rato / Peso g | 1 | 2 | 3 | 4 | 5 | 5 |
|---|---|---|---|---|---|---|
| Inicial | 80 | 80 | 80 | 80 | 80 | 80 |
| Aos 3 dias | 80 | 85 | 78 | 80 | 80 | 78 |
| Aos 6 dias | 85 | 85 | 87 | 85 | 83 | 87 |
| Aos 9 dias | 90 | 90 | 90 | 90 | 95 | 95 |

QUADRO IV

Evolução do peso de ratos alimentados com dieta com 5 % de ácido ascórbi

| Rato / Peso g | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Inicial | 85 | 75 | 85 | 75 | 85 | 55 |
| Aos 3 dias | 80 | 75 | 82 | 70 | 80 | 55 |
| Aos 6 dias | 75 | 75 | 90 | 70 | 80 | 55 |
| Aos 9 dias | 85 | 80 | 100 | 68 | 84 | 61 |

QUADRO V

Evolução do peso de ratos alimentados com dieta com 10 % de ácido ascórbico

| Rato / Peso g | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Inicial | 60 | 65 | 68 | 65 | 65 | 80 |
| Aos 3 dias | 52 | 64 | 68 | 52 | 64 | 80 |
| Aos 6 dias | Morreu | 69 | 67 | Morreu | 57 | 69 |

QUADRO VI

Evolução do peso de ratos alimentados com dieta com 5 % de ácido ascórbico e 5 % de bicarbonato de sódio

| Rato / Peso g | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Inicial | 98 | 75 | 80 | 75 | 80 | 65 |
| Aos 2 dias | 80 | 60 | 62 | 60 | 65 | 55 |
| Aos 4 dias | 65 | 53 | 55 | — | 57 | 45 |
| Aos 6 dias | 57 | 45 | 45 | 52 | Morreu | Morreu |
| Aos 8 dias | Morreu | Morreu | Morreu | Morreu | — | — |

QUADRO VII

Evolução do peso de ratos alimentados com dieta com 10 % de ácido ascórbic e 10 % de bicarbonato de sódio

| Rato / Peso g | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Inicial | 109 | 65 | 73 | 80 | 55 | 67 |
| Aos 3 dias | 87 | 54 | 61 | 66 | 48 | 59 |
| Aos 6 dias | 85 | Morreu | 60 | 52 | Morreu | 55 |

QUADRO VIII

Evolução do peso de ratos alimentados com dieta com 2,5 % de ácido cítric

| Rato / Peso g | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Inicial | 75 | 80 | 70 | 80 | 60 | 70 |
| Aos 3 dias | 65 | 72 | 60 | 75 | 55 | 70 |
| Aos 6 dias | 70 | 80 | 60 | 75 | 55 | 72 |
| Aos 9 dias | 80 | 90 | 70 | 85 | 65 | 86 |

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(Trabalho realizado no Centro de Estudos de Bioquimi da Faculdade de Farmácia do Porto)

0564

Proc. Am. Soc. Hort. Sci 86: 387-391 (1965), (Eng)

# Changes in Nonvolatile Organic Acid Constituents in Broccoli (*Brassica Oleracea* var. *Italica*) Following Post-Harvest $N^6$-Benzyladenine Treatment[1]

By H. C. Dostal,[2] R. R. Dedolph,[3] and V. Tuli, *Department of Horticulture, Michigan State University, East Lansing, Michigan*

*Abstract.* This study was directed toward broadening the biochemical basis of observed physiological activity of $N^6$-benzyladenine. To this end, the time course changes in nonvolatile organic acids and respiration in broccoli were concurrently measured in treated and control samples over the 5-day period following the day of harvest. Shikimic, quinic, galacturonic, glyceric, glycolic, succinic, citramalic, fumaric, citric, malic and phosphoric acids were detected with the methods used, but only the last 3 listed showed distinctive trends and were present in appreciable quantities.

Increases in citric acid levels of control tissues during early storage and of treated tissues in late storage are ascribed to an influx of organic acids into the tricarboxilic acid cycle from protein and amino acid degradation.

Increases in malic acid with time in both control and treated tissue were thought to be related partly or wholly to nonphotosynthetic carbon fixation. Malic acid was apparently not a major substrate for metabolism in broccoli.

Phosphoric acid trends support the concept that $N^6$-benzyladenine functions by effecting increases in organic phosphate fractions.

Respiration rates, which showed characteristic early storage inhibition and late storage stimulation in treated tissue, indicated that modifications of respiratory metabolism preceded changes in biochemical constituents. It was concluded that the primary effect of this phytokinin was alteration of the functional metabolism of the organism.

## Introduction

$N^6$-BENZYLADENINE, one of the phytokinins (4), extends the storage life of some commodities and enhances their market acceptability. The effects of pre- and post-harvest treatment on respiration (1, 3, 5, 6, 9) have received attention as have changes in chlorophyll (6, 11, 13), protein (12, 15) and nucleic acid contents (12). This study relates changes in organic acid contents to the changes in respiration rates.

## Materials and Methods

Six randomly selected freshly harvested 3-head samples of Spartan Early broccoli were treated by dipping in a 10 ppm aqueous solution of $N^6$-benzyladenine containing 0.1% Tween 20 as a wetting agent. A like number of similar control samples were dipped in water containing Tween 20. Samples were then placed in respirometers at 25° C. After 16 hours equilibration, $CO_2$ evolution was determined colorimetrically (2) at 12-hour intervals. At 16, 40, 64, 88, 112 and 136 hours after harvest, a control and a treated sample were removed from their respirometers, frozen over dry ice and subsequently held at −20° C.

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Organic acid analysis of these stored frozen samples was by modification (7, 10) of the procedure developed by Hulme and Wooltorton (8).

*Extraction.* Frozen samples were removed from cold storage and blended with 50 ml of boiling water at *ca.* 7500 rpm for 3 minutes. The slurry was boiled, cooled and filtered. The aqueous filtrate was concentrated *in vacuo,* pectins were removed by coagulation in ethanol, and lead sub-acetate was added to precipitate the organic acids. The precipitate was washed with ethanol and centrifuged. The acids were regenerated from their lead salts by bubbling $H_2S$ gas through the suspension. After washing, the lead sulfide pellet was discarded. The acid extract was concentrated and passed through a 0.7 × 20.0 cm column of Dowex 50 × 8 ion exchange resin (H+ form, 50–100 mesh) to remove all cations, brought to 50 ml volume with water and stored at 2° C.

*Separation and characterization.* Aliquots from these stored extracts containing *ca.* 1.5 meq were added to 0.7 × 30.0 cm column of Dowex 1 × 8 ion exchange resin (acetate form 200–400 mesh) and were removed by a concentration gradient elution (7). Two acid reservoirs were used and were connected to the mixing vessel by means of a 3-way stopcock. Sufficient air pressure was applied at the top of the system to maintain a flow rate of *ca.* 60 ml/hour over the resin column.

Six eluting solvents in order of increasing normality were added (7). Eighty fractions of 5.0 ml volume were collected per sample aliquot, in an automatic fraction collector.

The fractions were dried *in vacuo,* then reconstituted in hot water and titrated to the phenolphthalein endpoint with 0.01 *N* NaOH. After filtration, the residues were dissolved in 0.1 ml 50% ethanol and spotted on Whatman No. 1 filter paper sheets 46 × 57 cm, 2.5 cm apart and 7.5 cm away from the long edge of the sheet. The papers were placed in a chromatographic cabinet, the atmosphere of which was equilibrated with the lower phase of a mixture of 1-butanol, formic acid and water (4:1:5 v/v). The upper phase of this mixture was used in descending irrigation of papers. After development the papers were sprayed with color-specific reagents (14).

Standardization of the Dowex 1 × 8 column was accomplished according to procedures described by Dostal (7). These standardized elution profiles were then used to further characterize the unknown acids.

*Total acidity determination.* Three 1.0 ml aliquots, in addition to that used for separation and characterization, were removed from each stored extract sample and titrated to the phenolphthalein endpoint with 0.01 *N* NaOH to determine total acidity. From these values the total quantities of each individual acid per given quantity of tissue were calculated.

## Results and Discussion

Ten organic acids were detected with the analysis employed. In their order of emergence from the column these acids were: shikimic, quinic, galacturonic, glyceric, glycolic, succinic, citramalic, malic, citric and fumaric. Phosphoric acid, which is ubiquitous in plant extracts, was also detected (Fig. 1). Malic and citric were the only organic acids present in quantity.

Total acidity of control samples rose rapidly during the 64 hours after harvest and then declined precipitously during the next 2 days (Fig. 1). Thereafter little change was noted. Treated samples conversely showed little change in total acidity over the first 5 days after harvest, but from the fifth to the sixth day a marked increase in total acidity occurred. The differences in total acidity were due primarily to fluctuations in the levels of citric and malic acids, and of the phosphate fraction.

Relative rates of respiration in control and treated samples were essentially paralleled by relative total acidity contents over the time course of this experiment with the exception of the first 2 days following harvest. The precedence of respiration rate modification to total acidity differences in the period closely following harvest indicates that the modification in organic acid content is a consequence of modifications of respiratory metabolism rather than a cause.

Treated samples showed uniform and consistent decrease in citric acid content over the first 4 days of storage, followed by a slight increase. In the control samples, citric acid content changed

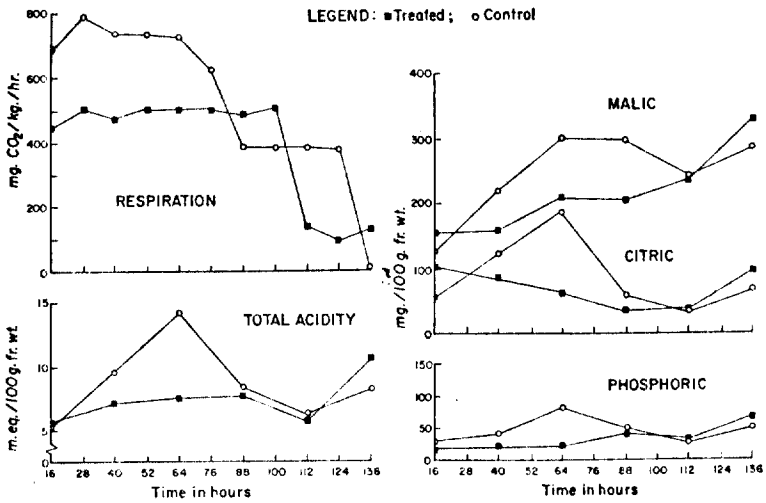


*Fig. 1.* The time course changes in respiration rates and various acid fractions in broccoli treated with $N^6$-benzyladenine and corresponding control samples.

in pattern and magnitude exactly as did the total acidity. The initial build up in citric acid in control samples, accompanied by a relative steady rate of $CO_2$ evolution during this period, suggest that the source of this citric acid was from the degradation of amino acids with acid products being fed into the Krebs cycle. The rapid decrease in citric acid and concomitant decrease in $CO_2$ evolution from the third to the fourth day in these control samples indicate a decrease in availability of organic acids from free amino acids, probably coupled with a decrease in available intermediary metabolites from glycolysis. The rise in citric acid content during the last day of storage was probably due to a relatively rapid decrease in functional activity of the tricarboxilic acid cycle evidenced by lower $CO_2$ evolution rates, coupled with a marked increase in organic acids derived from free amino acids of protein degradation.

An over-all gradual increase in malic acid content of the treated samples occurred throughout the entire experimental period. In the control samples, a rather rapid increase in malic acid content occurred from harvest through the third day of the experiment. No material change was noted thereafter. As the $CO_2$ content of the respiration chambers fluctuated markedly, concomitant variations in non-photosynthetic carbon fixation may account for the observed trends and differences. Failure of the malic acid levels to closely parallel respiration strongly indicates that there was no highly functional system in broccoli utilizing malic acid as a substrate.

Respiration rates of the control samples were materially higher than those of the treated samples only during that period when the phosphate levels within the control samples were likewise higher than those of the treated samples. This condition provides supporting evidence for the hypothesis that $N^6$-benzyladenine is functional by means of effecting an increase in the amount of organic phosphate, particularly in the adenosine triphosphate fraction. Decreased respiration in treated tissue was probably due to limitations on the supply of functional sites of dissipation for adenosine triphosphate.

Remaining organic acid fractions were not sufficiently great in magnitude nor consistent enough in trend to justify extended discussion.

The trends observed in organic acids hardly support a "maintenance hormone" concept in relation to the phytokinins; rather these data indicate that the phytokinins effect basic changes in the functional metabolism of the organism, which are reflected in respiration rates.

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# INVESTIGATION OF AEROBIC METABOLISM IN SURGICAL PATIENTS: CITRATE TOLERANCE STUDIES

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## INTRODUCTION

Previous studies have shown that surgery causes a decreased rate of assimilation of glucose, pyruvate, and lactate but no significant impairment in the assimilation of fructose. These results have been interpreted as indicating that surgery produces a disturbance in anaerobic metabolism. Since fructose does not share in the postoperative alteration of metabolism, it has been suggested that enzymatic processes are not uniformly disturbed; rather, that specific metabolic pathways are inhibited. The present study was undertaken to determine the effect of surgery on aerobic metabolism. Citric acid was selected for study because of its vital role in energy production.

## METHODS

Citrate tolerance tests were performed by the intravenous administration of a 2½% solution of sodium citrate for a period of 1 hr., on the basis of 0.065 gm./kg. body weight. In confirmation of other in-

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vestigations the infusion of citrate did not cause an alteration in electrocardiographic pattern or in the amount of estimated ionized calcium. The tolerance tests were performed 3 times: a day or 2 prior to surgery, during surgery, and during the first postoperative day. A series of 5 patients was studied. One operation was performed under a spinal anesthesia, and 4 operations were performed under general anesthesia (pentothal, nitrous oxide-oxygen, and cyclopropane). None of the 5 cases required a blood transfusion during surgery. Samples of venous blood were drawn through an indwelling Cournand needle prior to the citrate infusion and every 30 min. for 3 hr. thereafter. The samples were analyzed for glucose, pyruvic and lactic acids, and inorganic phosphorus by methods previously described. Citrate was determined as citric acid by the pentabromacetone method of Taussky modified by Natelson.

## RESULTS

The average results of the 5 patients who were studied are summarized in Table I. No significant alteration was found in citrate

*Table I*

| TIME | CONTROL | | | | DURING SURGERY | | | | POSTOPERATIVE | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MINUTES | CA* | PA | LA | G | CA* | PA | LA | G | CA* | PA | LA | G |
| 0 | 2 | .7 | 9.8 | 91 | 2 | .8 | 13.9 | 109 | 2 | 1.3 | 11.9 | 102 |
| 60 | 8 | .7 | 9.1 | 90 | 11 | 1.2 | 15.6 | 118 | 10 | .9 | 10.2 | 106 |
| 120 | 4 | .7 | 7.5 | 88 | 4 | .9 | 14.9 | 100 | 3 | 1.1 | 9.5 | 104 |
| 180 | 3 | .7 | 6.9 | 87 | 4 | .6 | 15.6 | 94 | 2 | 1.1 | 9.4 | 97 |

CA–citric acid; PA–pyruvic acid; LA–lactic acid; G–glucose.
* All citric acid values in comparison with control $p > .4$.

tolerance either during or after surgery in comparison with the preoperative tests ($p > 0.4$). The significant alterations in the response of both glucose and lactic acid to citrate infusion during surgery ($p < 0.05$) became less marked in the postoperative tests. The results for the patient who was studied under spinal anesthesia did not differ from those of the 4 patients who were given general anesthesia.

## DISCUSSION

Although the amount of citrate given in this study (4.5 for a 70 kg. patient) was relatively small in comparison to that which would be administered with massive transfusions, the results indicate that surgery does not cause a disturbance in the body's ability to assimilate



citrate rapidly. It is unlikely, therefore, that operative trauma or general anesthesia will increase the possibility of citrate toxicity.

The significant changes in glucose and lactic acid but no change in citrate tolerance in the tests during surgery as compared with the preoperative tests suggest that pathways of aerobic metabolism are intact at a time when surgery has produced an alteration in anaerobic pathways. The role of citrate as an energy substrate during surgery remains to be explored more fully.

## CONCLUSIONS

1. Surgery does not alter the utilization of exogenous sodium citrate.

2. Infusion of sodium citrate is ordinarily associated with a fall in blood glucose and lactic acid; but during surgery it is associated with a rise in blood glucose and lactic acid.

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# EFFECT OF METABOLIC FACTORS ON THE SUSCEPTIBILITY OF ALBINO MICE TO EXPERIMENTAL TUBERCULOSIS

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(Received for publication, September 15, 1954)

Clinical and epidemiological observations strongly suggest that the resistance of man to tuberculosis can be decreased by physiological disturbances resulting from causes as varied as malnutrition, uncontrolled diabetes, or subtotal gastrectomy. There are many reasons to believe that this adverse influence on host-parasite relationship is the outcome of changes in the metabolism of infected tissues, rather than in their immunological state—humoral or cellular. It is known, for example, that resistance to infection in starved or diabetic individuals usually becomes normal again when these patients are returned to a satisfactory nutritional state, or when their diabetes is brought under control by adequate insulin therapy. It appears that susceptibility to infection in these cases is the expression of a reversible disorder, probably biochemical in nature.

There have been published many reports showing that the resistance of experimental animals to tuberculosis can be decreased by feeding them diets deficient in one or several nutritional factors—particularly proteins and certain vitamins (1–11). The present study has a different purpose, not being primarily concerned with the role played in tuberculosis by specific nutritional deficiencies. Rather it is part of an attempt to determine the effect of changes in the physicochemical environment *in vivo* on the fate of microbial pathogens, and hence on the course of infectious processes. This study—which involves experimental infections caused by several bacterial species—has been pursued along two independent but related approaches. On the one hand, experiments *in vitro* have dealt with the effect exerted on the multiplication and survival of microorganisms by substances and conditions which are presumed to occur at the site of the lesion. On the other hand, efforts have been made to modify the resistance of mice to infection by procedures designed to alter their metabolic processes. The present paper deals with the latter phase of this program.

The design of the *in vivo* experiments was much influenced by results obtained *in vitro*. It has been found that the addition of sodium lactate to artificial culture media is inimical to tubercle bacilli, particularly at the slightly acidic reactions known to prevail in inflammatory areas, whereas

several polycarboxylic acids and keto compounds favor the multiplication and survival of the bacilli under the same conditions (12–14). It appeared possible therefore that physiological disorders could affect resistance to infectious diseases through the pathological production of certain metabolites, concentrated perhaps at the site of the inflammatory response. (For a more extensive presentation of this hypothesis, see reference 15.) The present report is limited to a description of some effects exerted on mouse tuberculosis by treatments and nutritional regimens presumed to decrease the glycogen stores of the body, to interfere with the glycolytic activity of tissue cells, or to increase the concentration of acetone bodies and polycarboxylic acids in the body fluids.

## EXPERIMENTAL

### *Materials and Methods*

The experiments were carried out with four virulent and six attenuated strains of tubercle bacilli, of human or bovine origin. As the pattern of results was the same in all cases, it will suffice to describe the findings with two strains, one representative of the virulent, and one of the attenuated group: MV, a bovine strain (Vallée) maintained in a state of high virulence for white mice by frequent recovery from the spleens of these animals 2 weeks after infection; BCG-P, a strain of BCG obtained through the courtesy of Dr. J. Aronson, of the Henry Phipps Institute in Philadelphia. The cultures were grown at 37°C. in tween-albumin medium, and were used when 10 to 12 days old.

The animals were albino mice bred at the Rockefeller Institute. They were received in the laboratory the week after weaning, at 4 weeks of age. They were kept in groups of 2 (except as noted) in metal cages on a wire screen without bedding throughout the duration of the experiments. Efforts were made to minimize variations in temperature in the animal room, but it was only during the last 4 months of experimentation that air conditioning of the room was available. The temperature was maintained from then on at approximately 78°F.

The diets were as indicated in each individual experiment. When "pellets" were used, they were Purina laboratory chow, obtained from Ralston Purina Company. According to the producer, these pellets contain 25 per cent protein and 6 per cent fat. Some experiments were carried out with the synthetic diet 191 (16) which contains 18 per cent casein and 5 per cent fat. The other experimental diets consisted of mixtures of dried skim milk (containing 36.8 per cent protein and 0.8 per cent fat) and wheat flour to which other ingredients were added in the desired proportions.

In the case of both synthetic diet 191, and the skim milk–wheat flour diets, 1 kg. of the dry food mixture was resuspended in 1 liter of 7.5 per cent gelatin dissolved in tap water at 45°C. The food-gelatin suspension was allowed to set in a cold room and was preserved there until used. Fresh food was prepared every week.

Food was given *ad lib.*, unless otherwise noted. It was supplied in the form of pellets, or of solid gelatin cakes cut just before use. The temperature of the animal room was such that the gelatin cakes remained set while in the animal cage.

All food remnants were removed, and cages cleaned, once weekly.

In general, the mice were infected within 10 days after weaning, *i.e.*, at approximately 29 to 32 days of age; their weights at that time averaged 15 to 18 gm. Infection was always by the intravenous route, the inoculum being diluted in a final volume of 0.2 ml. of 0.1 per cent bovine albumin in 0.8 per cent NaCl. The infective dose was 0.2 ml. of culture for BCG-P and varied as noted from 0.1 to 0.001 ml. for MV.

*Validity of the Results.*—Because of the very large numbers of animals used, it was rarely possible to carry out autopsies or cultural tests to confirm the cause of death. In practically all cases however, and in particular with the dietary regimens which did not allow for maximum growth of the mice, groups of 6 to 10 animals on each experimental diet were kept uninfected for the duration of the tests in the same room and under exactly the same conditions as the infected ones. The results of experiments in which deaths occurred in the uninfected control groups have not been used in the present report.

The experiments were carried out with groups of 10 to 20 animals. In general, the differences in survival times between groups were so striking as to give a high degree of significance to the results. However no attempt was made to assess the findings in statistical terms. Instead, all experiments were repeated numerous times (at least 3 and up to 10 times). The results obtained were considered significant—and have been reported here—only in cases in which all experiments without exception gave differences which were obvious and of the same order of magnitude.

*Effect of Limiting the Amount of Food:*

In general, the effect of malnutrition on experimental tuberculosis has been studied by the use of diets deficient in some essential growth factor—particularly vitamins or protein. None of the diets used in the following experiments was grossly deficient in any of the essential dietary factors. The regimens were designed to establish, not deficiency states, but rather different levels of undernutrition—produced either by short periods of acute fasting or by a chronic shortage of food. The effect of these two kinds of regimens will now be considered separately.

The response of tuberculous mice to acute but transient fasting was studied by depriving the infected animals completely of any source of food for periods of 30 consecutive hours.

Mice were given *ad lib.* either pellets, or the synthetic diet 191, or a mixture of wheat flour (66), skim milk (33), and salt (1). Duplicate groups of animals were also fed these diets *ad lib.* except that the food was entirely removed from their cages for periods of 30 hours on the 4th, 11th, and 18th day after infection (water remained available all the time *ad lib*). In other words, the animals in this second group were fasted for 30 hours every week. Ten mice in each of the groups were infected with 0.01 ml. MV. Their survival time is presented in Table I and Fig. 1, which summarize the results of four consecutive experiments carried out over a total period of 5 months. Table I also indicates the weight gain of uninfected mice kept on the experimental regimens for 3 weeks (average of ten mice).

As appears from the results presented in Table I and Fig. 1, mice fed *ad lib.* without interruption survived as a group much longer than those also fed *ad lib.* except that they were deprived of food for 30 consecutive hours every week. The results were essentially the same whether the animals received pellets, or a skim milk–wheat flour mixture, or synthetic diet 191.

The preceding experiments dealt with the effect of *acute* but transient fasting on resistance to tuberculosis. In contrast, the following tests were designed to determine the survival time of tuberculous mice maintained in a *chronic* state of undernutrition throughout the period of observation.

TABLE I

(see Fig. 1)

*Effect of Acute Fasting on Mouse Tuberculosis*

| Fig. No. | Regimen | Weight gain* | Cumulative number of mice (out of 10) dead at | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 3 wks. | 4 wks. | 5 wks. | 6 wks. | 7 wks. | 8 wks. |
| | | *gm.* | | | | | | |
| 1-a | Skim milk 33, wheat flour 66, *ad lib.*, uninterrupted | 6.2 | 0 | 1 | 5 | 10 | | |
| 1-as | Skim milk 33, wheat flour 66, *ad lib.*, withdrawn 30 hrs. weekly | 4.5 | 4 | 7 | 9 | 10 | | |
| 1-b | Pellets, *ad lib*, uninterrupted | 9.8 | 0 | 1 | 3 | 4 | 7 | |
| 1-bs | Pellets, *ad lib.*, withdrawn 30 hrs. weekly | 5.7 | 3 | 5 | 5 | 8 | 10 | |
| 1-c | Pellets, *ad lib.*, uninterrupted | 8.4 | 0 | 1 | 1 | 3 | 6 | 10 |
| 1-cs | Pellets, *ad lib.*, withdrawn 30 hrs. weekly | 4.2 | 0 | 2 | 2 | 6 | 10 | |
| 1-d | Synthetic diet 191, *ad lib.*, uninterrupted | Not weighed | 3 | 5 | 6 | 7 | 10 | |
| 1-ds | Synthetic diet 191, *ad lib.*, withdrawn 30 hrs. weekly | Not weighed | 3 | 7 | 8 | 8 | 10 | |

* Average of 10 uninfected mice over a total period of 3 weeks.

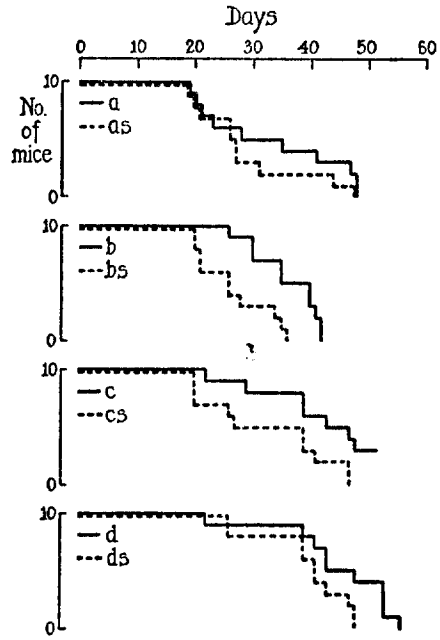



FIG. 1. Effect of fasting on mouse tuberculosis. Ordinates indicate number of mice (out of 10) surviving at different periods of time after infection (abscissae). For experimental details, see Table I.

When food was supplied *ad lib.*, the average daily consumption per mouse (30 to 40 days of age) was found to be slightly less than 5 gm. of Sherman diet, a mixture consisting of: whole wheat flour (66), dried whole milk (33), NaCl(1). On this regimen (with water supplied *ad lib.*,) the animals gained approximately 8 to 10 gm. weight during the first period of 3 weeks. The effect exerted by limitation of the quantity of food on susceptibility to infection was determined by the following technique:

Mice were kept singly in metal cages. Some were fed the Sherman mixture *ad lib.* Others received 3.8 gm. of it daily, corresponding to approximately 75 per cent of the maximum food intake. The animals on the restricted regimen gained little if any weight during the 2 months of observation, but otherwise appeared healthy. A third group received Sherman diet *ad lib.* except that the food was entirely removed from their cages for periods of 30 hours on the 4th, 11th, and 18th day after infection (water remained available all the time *ad lib*).

Groups of 10 animals each were infected with 0.01 ml. of the culture MV, 2 days after being placed on either of the three food regimens (Table I A).

The mean survival time was 27.4 days (23 to 40) for the mice fed *ad lib.*, and 28.7 days (19 to 43) for those receiving only 75 per cent of the maximum

TABLE 1 A

*Effect of Chronic Undernutrition on Mouse Tuberculosis*

| Regimen | Weight gain* | Cumulative number of mice (out of 10) dead at | | | | |
|---|---|---|---|---|---|---|
| | | 3 wks. | 4 wks. | 5 wks. | 6 wks. | 7 wks. |
| | *gm.* | | | | | |
| Sherman diet *ad lib.*, uninterrupted | 9.8 | 0 | 6 | 9 | 10 | |
| Sherman diet *ad lib.*, withdrawn 30 hrs. weekly | 5.8 | 4 | 10 | | | |
| Sherman diet 3.8 gm. daily, uninterrupted | 1.0 | 1 | 6 | 9 | 9 | 10 |

* Average of 10 uninfected mice over a total period of 3 weeks.

food intake. The results are presented in Table I A. Thus, there was no evidence that the susceptibility of mice to tuberculous infection was modified to an appreciable extent by limitation of the food intake, even though it was of such a degree as to reduce drastically the increase in weight of uninfected animals.

*Effect of Content of Diet in Skim Milk:*

One of the nutritional factors most commonly assumed to play an essential role in resistance to tuberculosis is the protein content of the food. In none of the tests reported here was the percentage of protein the only variable in the composition of the diet. However, its content in skim milk was varied from 4 to 33 per cent in several experiments designed for other purposes. The weight balance in the diet was then made up either with wheat flour, cerelose, peanut oil, sodium citrate, or mixtures of these materials.

Table II presents the results obtained (in three consecutive experiments) with some of these mixtures in comparison with those obtained with synthetic

diet 191 or with pellets. Food and drinking water were provided *ad lib.* The mice (in groups of 10) were infected with 0.01 ml. of culture MV the morning before being placed on the experimental diets. Other groups of 6 were kept as uninfected controls and weighed every week. The average weight gain per mouse for the first 3 weeks in each non-infected group is recorded in Table II, as well as the time of death of the infected animals.

The results of the three experiments presented in Table II show that, under the conditions of the tests, mice fed diets containing only 4 or 5 per cent skim milk (supplemented with peanut oil) were no more susceptible to tuberculosis than those receiving either pellets (containing 25 per cent protein), or diets containing 15, 33, or 34 per cent skim milk, or synthetic diet 191 with 18 per cent casein. This observation is particularly striking in view of the fact that the protein content of the dried skim milk used was only 36.8 per cent. It must be pointed out, however, that other evidence indicates that mice on low protein regimens are particularly sensitive to the infection-enhancing effect of certain other components of the diet. This is apparent in the low resistance to infection of mice fed diets containing either 4 or 5 per cent skim milk, in which peanut oil had been replaced by cerelose (see Tables II, III, IV and Fig. 2). A similar deleterious effect was observed when sodium citrate or large amounts of cocoa butter were added to diets low in protein.

TABLE II

*Effect of Concentration of Protein and Fat in the Diet on Mouse Tuberculosis*

| Percentage composition of diets* | | | | Weight gain | Cumulative number of mice (out of 10) dead at | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Skim milk | Wheat flour | Cerelose | Peanut oil | | 1 wk. | 5 wks. | 7 wks. | 9 wks. | 11 wks. |
| | | | | *gm.* | | | | | |
| 4 | 45 | 30 | 20 | 3.8 | 0 | 1 | 2 | 3 | 3 |
| 4 | 45 | 50 | — | 3.0 | 1 | 7 | 10 | | |
| 34 | 65 | — | — | 8.8 | 0 | 0 | 4 | 4 | 4 |
| 5 | 74 | — | 20 | 11.0 | 0 | 0 | 1 | 3 | 8 |
| 5 | 74 | 20 | — | 8.2 | 0 | 0 | 3 | 7 | 10 |
| 15 | 64 | — | 20 | 10.1 | 0 | 0 | 1 | 4 | 6 |
| 15 | 64 | 20 | — | 10.8 | 0 | 0 | 2 | 8 | 8 |
| 33 | 66 | — | — | 10.1 | 0 | 0 | 1 | 6 | 7 |
| 5 | 74 | 20 | | 4.6 | 1 | 2 | 8 | 9 | 9 |
| Synthetic diet 191 (18 per cent casein, 5 per cent fat)..... | | | | 8.4 | 0 | 1 | 6 | 9 | 9 |
| Pellets (24 per cent protein, 5 per cent fat)..... | | | | 9.6 | 0 | 3 | 8 | 8 | 9 |

* 1 per cent salt added.

*Comparative Effects of Cerelose and Various Fats in the Diets:*

There have been many conflicting reports concerning the role of dietary fats in experimental tuberculosis (17, 18). Experimentation *in vitro* also yields a confusing picture of the effects of lipids on tubercle bacilli, the results ranging from marked enhancement to complete inhibition of growth depending upon the conditions under which the culture tests were carried out (19 to 21). In the course of the present study many experiments have been carried out to determine the effect on mouse tuberculosis of addition to the diet of various glycerides or fatty acids. Although the results do not yet appear in a clear pattern, they leave no doubt that the effect of dietary fats on infection is determined to a very large extent by the presence or absence of other substances in the diet. It is very likely furthermore that many of the effects observed are not caused directly by the fats themselves, but rather indirectly through the products of their intermediary metabolism. Out of the welter of results yielded by infection tests carried out over several years under a wide range of conditions, a few will be selected to illustrate the effect exerted on mouse tuberculosis by the replacement of cerelose by equal weights of plant or animal fats.

The percentage composition of some of the diets used is presented in Table III (three consecutive experiments). The fat (or cerelose) was mixed thoroughly by hand with the skim milk and wheat flour. Each batch of food was resuspended in an equal weight of 7.5 per cent gelatin in water. Food and drinking water were supplied *ad lib.* All animals were infected with 0.01 ml. of culture MV the day that they were placed on the experimental diets. The time of death of infected animals and the average weight gains of uninfected controls (over a total period of 3 weeks) are recorded in Table III.

The results presented in Table III do not reveal any obvious effect of the replacement of cerelose by either peanut oil, cocoa butter, or lard in diets containing 15 or 20 per cent skim milk. It is clear, however, that mice proved particularly susceptible to tuberculous infection when they were fed diets containing only 4 or 5 per cent skim milk, with cerelose but no added fat beyond that which was present in the skim milk and wheat flour. This infection-enhancing effect could be counteracted by adding peanut oil to the low skim milk diet, but not so well with cocoa butter. Table IV and Fig. 2 present the results of three consecutive experiments which illustrate the comparative effects of equal weights of cerelose or of peanut oil, added to diets containing only 4 or 5 per cent skim milk. The protective effect of peanut oil under these particular conditions is obvious.

*Effect of Addition of Sodium Citrate to the Diet:*

Two independent reasons prompted a study of the effect of adding sodium citrate to the diet on the course of tuberculosis. (*a*) On the one hand, it is

known that citric acid enhances the growth of tubercle bacilli *in vitro*, and can furthermore protect them against the toxic effects of lactic acid at slightly acidic reactions (13, 14). It seemed likely that these effects would also obtain *in vivo* and that addition of citrate to the diet might in consequence accelerate the course of tuberculosis. (*b*) On the other hand, it appeared probable that the presence *in vivo* of high concentrations of citric acid can modify some of the reactions of the Krebs cycle in the tissues, and thereby affect the accumulation of some metabolites associated with this cycle. Some of these

TABLE III

*Effect on Mouse Tuberculosis of Addition to Diets of Equal Weights of Cerelose or Various Fats*

| Percentage composition of diets* | | | | | | Weight gain | Cumulative number of mice (out of 10) dead at | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Skim milk | Wheat flour | Cerelose | Peanut oil | Cocoa butter | Lard | | 5 wks. | 7 wks. | 9 wks. | 11 wks. |
| | | | | | | *gm.* | | | | |
| 4 | 45 | 50 | — | — | — | 3.0 | 7 | 10 | | |
| 4 | 45 | 30 | 20 | — | — | 3.8 | 1 | 2 | 3 | —‡ |
| 4 | 45 | 30 | — | 20 | — | 3.1 | 3 | 4 | 6 | —‡ |
| 5 | 74 | 20 | — | — | — | 8.2 | 0 | 3 | 7 | 10 |
| 5 | 74 | — | 20 | — | — | 11.0 | 0 | 1 | 7 | 8 |
| 5 | 74 | — | — | 20 | — | 8.9 | 0 | 5 | 8 | 10 |
| 15 | 64 | 20 | — | — | — | 10.8 | 0 | 2 | 7 | 8 |
| 15 | 64 | — | 20 | — | — | 10.1 | 0 | 1 | 5 | 7 |
| 15 | 64 | — | — | 20 | — | 10.5 | 0 | 2 | 4 | 6 |
| 20 | 50 | 30 | — | — | — | Not Weighed | 0 | 2 | 5 | 9 |
| 20 | 50 | 10 | — | 20 | — | Not Weighed | 0 | 4 | 7 | 9 |
| 20 | 50 | 10 | — | — | 20 | Not Weighed | 0 | 4 | 7 | 9 |

* 1 per cent salt added.

‡ —, experiment discontinued.

metabolites have been found to favor the multiplication and survival of tubercle bacilli (13, 14). The effect on mouse tuberculosis of diets supplemented with citric acid is illustrated in Tables V, VI, and VII and Figs. 3 A, 3 B, and 4.

The results plotted in Figs. 3 A (four experiments) and 3 B (two experiments) were obtained with diets containing 4, 5, 15, or 19 per cent skim milk. Their composition is outlined in Tables V and VI. All food mixtures were incorporated in an equal volume of 7.5 per cent gelatin in tap water and fed *ad lib.* to mice which had been infected intravenously the same day with 0.01 ml. culture MV.

TABLE IV

(see Fig. 2)

*Effect of Peanut Oil on Tuberculosis of Mice Fed Diets Containing 4 or 5 Per Cent Skim Milk*

| Fig. No. | Percentage composition of diets* | | | | Weight gain | Cumulative number of mice (out of 10) dead at | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Skim milk | Wheat flour | Cerelose | Peanut oil | | 3 wks. | 5 wks. | 7 wks. | 9 wks. | 11 wks. |
| | | | | | *gm.* | | | | | |
| 2-ac | 4 | 45 | 50 | — | 3.0 | 1 | 7 | 10 | | —‡ |
| 2-ap | 4 | 45 | 30 | 20 | 3.8 | 0 | 1 | 2 | 3 | —‡ |
| 2-bc | 5 | 74 | 20 | — | 8.2 | 0 | 0 | 3 | 7 | 10 |
| 2-bp | 5 | 74 | — | 20 | 11.0 | 0 | 0 | 1 | 7 | 8 |
| 2-cc | 5 | 74 | 20 | — | Not Weighed | 3 | 9 | 10 | | |
| 2-cp | 5 | 74 | — | 20 | Not Weighed | 1 | 2 | 8 | —‡ | |

* 1 per cent salt added.

‡ —, experiment discontinued.

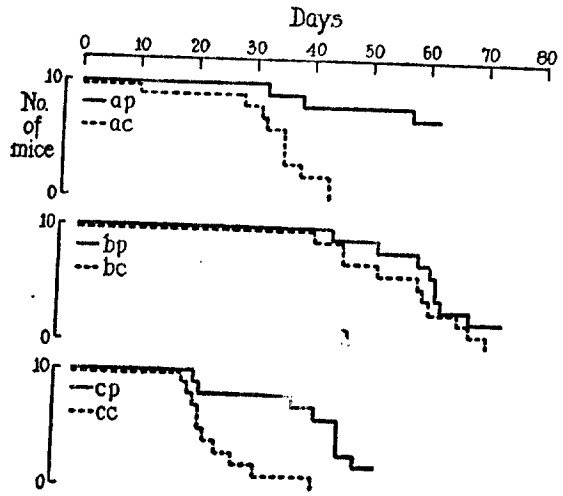



FIG. 2. Effect of peanut oil on tuberculosis of mice fed diets low in milk. Ordinates indicate number of mice (out of 10) surviving at different periods of time after infection (abscissae). For experimental details, see Table IV.

TABLE V

(see Fig. 3 A)

*Effect of Citrate in Diets Containing 4 to 5 per Cent Skim Milk*

| Fig. No. | Percentage composition of diets* | | | | | | Weight gain | Cumulative number of mice (out of 10) dead at | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Skim milk | Wheat flour | Cerelose | Peanut oil | Cocoa butter | Citrate | | 3 wks. | 5 wks. | 7 wks. | 9 wks. | 11 wks. |
| | | | | | | | *gm.* | | | | | |
| 3 A-a | 4 | 45 | 50 | — | — | — | 3.0 | 1 | 7 | 10 | | |
| 3 A-ac | 4 | 45 | 40 | — | — | 10 | −0.5 | 3 | 8 | 9 | 10 | |
| 3 A-b | 4 | 45 | 30 | 20 | — | — | 3.8 | 0 | 1 | 2 | 3 | —‡ |
| 3 A-bc | 4 | 45 | 20 | 20 | — | 10 | 0.4 | 0 | 9 | 10 | | |
| 3 A-c | 4 | 45 | 30 | | 20 | — | 3.1 | 0 | 4 | 5 | 6 | —‡ |
| 3 A-cc | 4 | 45 | 20 | | 20 | 10 | 0 | 3 | 10 | | | |
| 3 A-d | 5 | 74 | 20 | — | — | — | 8.2 | 0 | 0 | 3 | 7 | 10 |
| 3 A-dc | 5 | 66 | 20 | — | — | 8 | 2.5 | 0 | 0 | 6 | 10 | |
| 3 A-e | 5 | 74 | — | 20 | — | — | 11.0 | 0 | 0 | 1 | 7 | 8 |
| 3 A-ec | 5 | 66 | — | 20 | — | 8 | 6.7 | 0 | 0 | 3 | 10 | |
| 3 A-f | 5 | 74 | — | — | 20 | — | 8.9 | 0 | 0 | 5 | 9 | 10 |
| 3-A-fc | 5 | 66 | — | — | 20 | 8 | 5.8 | 0 | 1 | 9 | 10 | |
| 3 A-g | 5 | 74 | — | — | 20 | — | Not weighed | 0 | 3 | 9 | 10 | |
| 3 A-gc | 5 | 66 | — | — | 20 | 8 | Not weighed | 3 | 7 | 9 | 10 | |
| 3 A-h | 5 | 74 | — | 20 | — | — | Not weighed | 1 | 2 | 8 | 10 | |
| 3-A-hc | 5 | 66 | — | 20 | — | 8 | Not weighed | 2 | 6 | 9 | 10 | |

* 1 per cent salt added.

‡ — experiment discontinued.

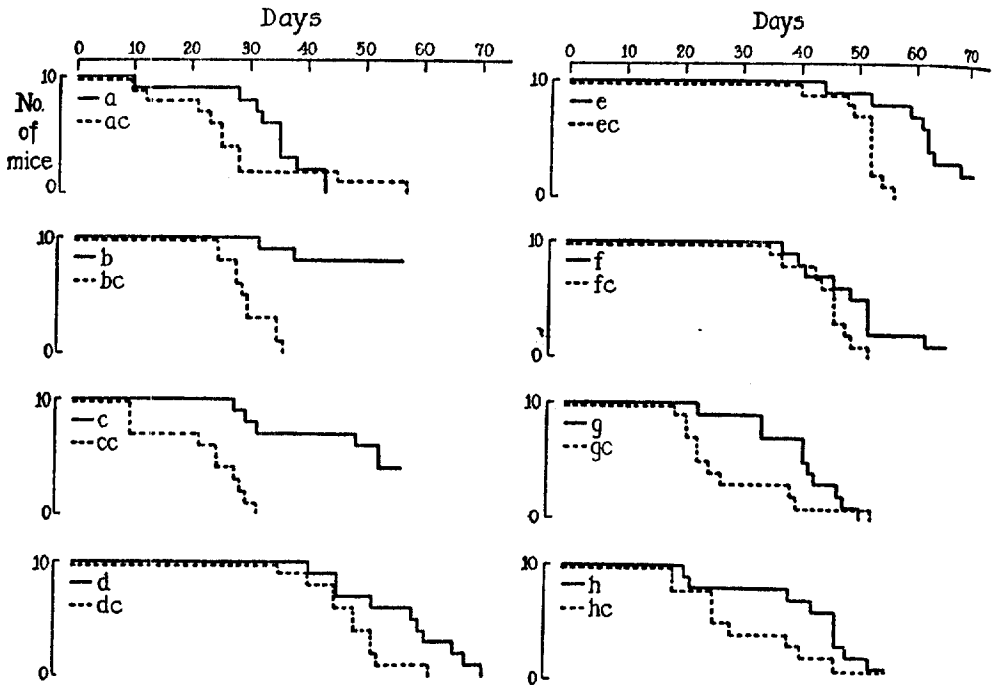



FIG. 3 A. Effect of citrate on mouse tuberculosis. Ordinates indicate number of mice (out of 10) surviving at different periods of time after infection (abscissae). For experimental details, see Table V.

TABLE VI

(see Fig. 3 B)

*Effect of Citrate in Diets Containing 15 to 19 per Cent Skim Milk*

| Fig. No. | Percentage composition of diets* | | | | | | | Weight gain | Cumulative number of mice (out of 10) dead at | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Skim milk | Wheat flour | Cerelose | Peanut oil | Cocoa butter | Lard | Citrate | | 3 wks. | 5 wks. | 7 wks. | 9 wks. | 11 wks. |
| | | | | | | | | *gm.* | | | | | |
| 3 B-i | 15 | 64 | 20 | — | — | — | — | 10.8 | 0 | 0 | 2 | 7 | 8 |
| 3 B-ic | 15 | 56 | 20 | — | — | — | 8 | 6.1 | 1 | 1 | 4 | 10 | |
| 3 B-j | 15 | 64 | — | 20 | — | — | — | 10.1 | 0 | 0 | 1 | 4 | 6 |
| 3 B-jc | 15 | 56 | — | 20 | — | — | 8 | 7.3 | 0 | 1 | 3 | 8 | 9 |
| 3 B-k | 15 | 64 | — | — | 20 | — | — | 10.5 | 0 | 0 | 2 | 4 | 6 |
| 3 B-kc | 15 | 56 | — | — | 20 | — | 8 | 5.3 | 0 | 1 | 2 | 6 | 8 |
| 3 B-l | 19 | 50 | 30 | — | — | — | — | Not weighed | 0 | 0 | 2 | 5 | 9 |
| 3 B-lc | 19 | 50 | 20 | — | — | — | 10 | Not weighed | 1 | 3 | 5 | 8 | 10 |
| 3 B-m | 19 | 50 | 10 | — | 20 | — | — | Not weighed | 0 | 0 | 4 | 7 | 9 |
| 3 B-mc | 19 | 50 | — | — | 20 | — | 10 | Not weighed | 0 | 1 | 6 | 10 | |
| 3 B-n | 19 | 50 | 10 | — | — | 20 | — | Not weighed | 0 | 0 | 2 | 8 | 10 |
| 3 B-nc | 19 | 50 | — | — | — | 20 | 10 | Not weighed | 0 | 2 | 6 | 10 | |

* 1 per cent salt added.

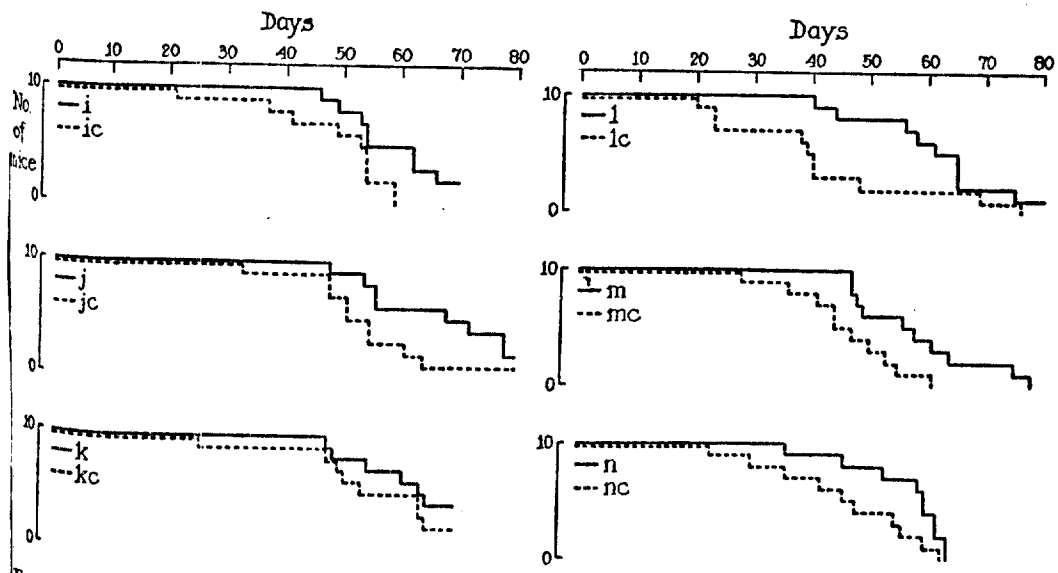



FIG. 3 B. Effect of citrate on mouse tuberculosis. Ordinates indicate number of mice (out of 10) surviving at differ- periods of time after infection (abscissae). For experimental details, see Table VI.

Fig. 4 and Table VII present the results of an experiment in which all mice were fed dry pellets *ad lib.* and were given various solutions as drinking fluid (also *ad lib.*). Ten mice received 0.8 per cent NaCl; ten received a solution of 2 per cent citric acid adjusted to pH 6.0 with sodium hydroxyde; ten received a solution of 1 per cent glutaric acid also at pH 6.0; and ten, a solution of 5 per cent alcohol in 0.8 per cent sodium chloride.

The results presented in Tables V, VI, and VII and in Figs. 3 A, 3 B, and 4 show that addition of citric acid to any of the various diets tested shortened

TABLE VII

(see Fig. 4)

*Effect on Mouse Tuberculosis of Citrate, Glutarate, or Alcohol Added to Drinking Fluid*

| Fig. No. | Diet | Drinking fluid | Cumulative number of mice (out of 10) dead at | | | |
|---|---|---|---|---|---|---|
| | | | 3 wks. | 4 wks. | 5 wks. | 6 wks. |
| 4-a | Pellets *ad lib.* | 0.8 per cent NaCl | 0 | 1 | 5 | 10 |
| 4-b | " " " | 2 per cent Na citrate (pH6.0) | 2 | 7 | 9 | 10 |
| 4-c | " " " | 1 per cent Na glutarate (pH6.0) | 1 | 7 | 9 | 10 |
| 4-d | " " " | 5 per cent alcohol in saline | 0 | 2 | 7 | 9 |

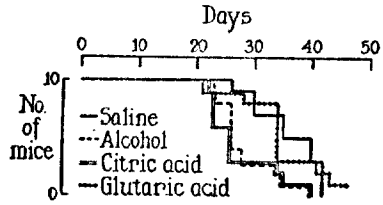



FIG. 4. Effect of citrate, glutarate, or alcohol on mouse tuberculosis. Ordinates indicate number of mice (out of 10) surviving at different periods of time after infection (abscissae). For experimental details, see Table VII. The symbols for glutaric acid and alcohol have been inadvertently reversed in Fig. 4 (The data in Table VII are correct).

the expectancy of life of tuberculous mice. It will be noted in Table VII and Fig. 4 that 1 per cent glutarate administered in the drinking fluid enhanced infection as much as did citrate (only one experiment performed), whereas 5 per cent alcohol had no significant effect on susceptibility. In many tests to be reported in a subsequent paper, addition of lactic acid to the diet failed to enhance infection and indeed appeared to retard somewhat the death of tuberculous mice. Other experiments are under way to determine the effect of other organic acids on infection, as well as the minimum effective dose of citric acid. In two experiments, designed to test the latter point, no significant difference could be noted between the infection enhancing effects of 3, 5, 7, and 10 per cent citrate in the diet.

Acceleration of the course of experimental tuberculosis by citrate has been observed whether the mice were fed pellets, or diets containing 4, 5, 15, or 20 per cent skim milk, or 18 per cent casein. It must be pointed out, however, that the results with diets containing 30 or 33 per cent skim milk have been less consistent—as appears from the two experiments presented in Table VIII. Since the tests with high concentrations of skim milk have not yet been extended, it is not possible to discuss further the bearing of these early findings on the infection-enhancing effect of citrate.

*Weight Gain and Susceptibility to Infection:*

In order to determine whether there was any relation between growth curve and susceptibility to infection, groups of 6 to 10 mice on most of the diets

TABLE VIII

*Effect of Citrate on Tuberculosis of Mice Fed Diets Containing 30 or 34 Per Cent Skim Milk*

| Percentage composition of diets* | | | | | Weight gain | Infective dose ml. MV | Cumulative number of mice (out of 10) dead at | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Skim milk | Wheat flour | Peanut oil | Cerelose | Citrate | | | 3 wks. | 5 wks. | 7 wks. | 9 wks. |
| | | | | | *gm.* | | | | | |
| 30 | 60 | — | 9 | — | Not weighed | 0.1 | 7 | 10 | | |
| 30 | 60 | — | — | 9 | Not weighed | 0.1 | 6 | 10 | | |
| 30 | 60 | — | 9 | — | Not weighed | 0.01 | 0 | 0 | 5 | 6 |
| 30 | 60 | — | — | 9 | Not weighed | 0.01 | 0 | 0 | 5 | 6 |
| 34 | 65 | — | — | — | 8.8 | 0.01 | 0 | 0 | 4 | 4 |
| 34 | 15 | 20 | 20 | 10 | 0 | 0.01 | 0 | 0 | 1 | 5 |
| 34 | 15 | — | 40 | 10 | −0.6 | 0.01 | 0 | 7 | 8 | 10 |

* 1 per cent NaCl added.

tested were kept as non-infected controls and weighed at weekly intervals. The average weight gains (per uninfected mouse during the initial period of 3 weeks on each regimen) are given in the tables or figures presenting the results of individual experiments.

Examination of weight gains in relation to the length of survival following infection reveals that all the regimens tested which proved capable of shortening the life expectancy of tuberculous mice also decreased the rate of growth of uninfected animals. It must be emphasized, however, that several regimens which caused marked retardation of growth failed to increase in a detectable manner the susceptibility of mice to tuberculosis. This was the case with chronic undernutrition brought about by maintaining the daily food intake to a constant level low enough to reduce drastically the weight gain of normal animals. Similarly, mice fed diets supplemented with peanut oil but low in

protein (4 or 5 per cent skim milk), gained weight only slowly (3.5 gm. over a period of 3 weeks) yet were fully as resistant to infection as mice which received *ad lib.* either pellets (25 per cent protein), or the synthetic diet 191 (18 per cent casein), or Sherman diet (33 per cent dried milk)—three high protein diets that allowed a much more rapid increase in weight. Furthermore, the mice on low protein–peanut oil diets were much more resistant than mice fed either pellets or diet 191, but deprived of food 30 consecutive hours every week, even though the two latter groups gained as much weight as did the

TABLE IX

(see Fig. 5)

*Effect of Weight Gain of Mice on Resistance to Tuberculosis*

| Fig. No. | Percentage composition of diets* | | | | | Weight gain | Cumulative number of mice (out of 10) dead at | | |
|---|---|---|---|---|---|---|---|---|---|
| | Skim milk | Wheat flour | Cerelose | Peanut oil | Citrate | | 3 wks. | 5 wks. | 7 wks. |
| | | | | | | *gm.* | | | |
| 5-a | 4 | 45 | 30 | 20 | — | 3.8 | 0 | 1 | 2 |
| 5-b | 4 | 45 | 50 | — | — | 3.0 | 1 | 7 | 10 |
| 5-c | 4 | 45 | 40 | — | 10 | 0 | 3 | 8 | 10 |
| 5-d | 34 | 65 | — | — | — | 8.8 | 0 | 0 | 4 |

* 1 per cent salt added.

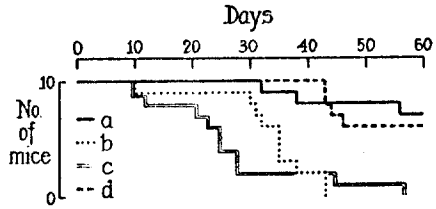



FIG. 5. Weight gain and resistance to tuberculosis in mice. Ordinates indicate number of mice (out of 10) surviving at different periods of time after infection (abscissae). For experimental details, see Table VIII.

former. Many similar discrepancies which are revealed by a comparison of mortality curves among tuberculous mice and of weight curves among non-infected groups, make clear that the susceptibility to infection cannot be predicted from the effects exerted by the dietary regimens on the growth curve. This lack of correlation appears strikingly from the results of the experiment selected for illustration in Table IX and Fig. 5.

*Effect of Dinitrophenol and Thyroid Extract:*

It is known that dinitrophenol can interfere with the synthesis of glycogen by tissue cells and that prolonged excessive feeding of thyroid extract reduces

greatly the store of glycogen in the liver (22, 23). There is evidence on the other hand that leucocytes can convert this polysaccharide into lactic acid and use it as a source of energy in some phases of phagocytosis (24, 25). These facts suggested that treatment with dinitrophenol or thyroxine might affect unfavorably the resistance of mice to tuberculosis by depleting their glycogen reserves.

Preliminary experiments were conducted to determine the minimum quantity of dinitrophenol, or of thyroid extract, that had to be added to an otherwise normal diet to reduce by approximately two-thirds the rate of growth of normal mice. As shown in Table X, addition of 90 mg. of dinitrophenol to 100 gm. of a wheat flour–skim milk diet reduced drastically the weight gain of normal mice. The corresponding dose for the preparation of thyroid extract used was 200 mg. per 100 gm. of diet.

TABLE X

*Effect of Dinitrophenol on Weight Curves of Uninfected Mice*

| Percentage composition of diet* | | | Weight‡ after following times on experimental diets | | | | Total change at day 15 |
|---|---|---|---|---|---|---|---|
| Skim milk | Wheat flour | Dinitrophenol | Initial | 5 days | 10 days | 15 days | |
| | | | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* |
| 33 | 66 | 0.27 | 93 | 90 | 83 | 91 | −2 |
| 33 | 66 | 0.09 | 89 | 93 | 93 | 97 | +8 |
| 33 | 66 | 0.03 | 84 | 91 | 94 | 100 | +16 |
| 33 | 66 | 0 | 84 | 92 | 97 | 104 | +20 |

* 1 per cent salt added.

‡ total for four mice weighed together.

The effect of dinitrophenol and thyroid extract on mouse tuberculosis was determined in several experiments which are presented in Tables XI, XII, XIII, and XIV, and in Figs. 6, 7, 8, 9, and 10.

In the experiment corresponding to Table XI, and Figs. 6 and 7, seven groups of 10 mice each were infected with 0.01 ml. culture MV and fed pellets *ad lib.* One group of 10 mice received 0.8 per cent NaCl as drinking fluid (control); three groups were given solutions of dinitrophenol (in saline) in concentrations of 0.1, 0.05, or 0.025 per cent; the three other groups received solutions (also in saline) of thyroid extract in concentration of 0.03, 0.01, or 0.003 per cent. (Thyroid U.S.P. powder No. 58—Eli Lilly and Co.—stated to contain not less than 0.17 nor more than 0.23 per cent iodine.) All experimental regimens were started on the day of infection.

It is clear from the results presented in Table XI and Figs. 6 and 7 that infected mice receiving the lowest concentrations of dinitrophenol (0.025 per cent) or of thyroid extract (0.003 per cent) survived at least as long as those

receiving saline. An infection-enhancing effect was apparent only with the higher concentrations of these drugs.

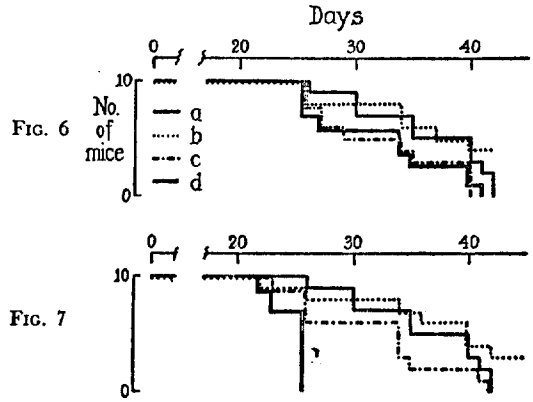
In the experiment corresponding to Table XII and Fig. 8 half of the mice were infected intravenously with 0.004 ml. and the other half with 0.1 ml. of culture MV. Immediately after infection, they were placed, and maintained thereafter, on a diet consisting of wheat

TABLE XI

(see Figs. 6 and 7)

*Effect of Dinitrophenol and Thyroid Extract on Mouse Tuberculosis*

| Fig. No. | Diet | Drinking fluid containing (*per cent*) | | Cumulative number of mice (out of 10) dead at | | |
|---|---|---|---|---|---|---|
| | | Dinitrophenol | Thyroid extract | 4 wks. | 5 wks. | 6 wks. |
| 6-a, 7-a | Pellets *ad lib.* | 0 | 0 | 1 | 5 | 10 |
| 6-b | " " " | 0.0025 | 0 | 2 | 4 | 6 |
| 6-c | " " " | 0.005 | 0 | 4 | 7 | 10 |
| 6-d | " " " | 0.01 | 0 | 4 | 7 | 10 |
| 7-b | " " " | 0 | 0.003 | 2 | 3 | 7 |
| 7-c | " " " | 0 | 0.01 | 4 | 8 | 10 |
| 7-d | " " " | 0 | 0.03 | 10 | — | — |




FIGS. 6 and 7. Effect of dinitrophenol and thyroid extract on mouse tuberculosis. Ordinates indicate number of mice (out of 10) surviving at different periods of time after infection (abscissae). For experimental details, see Table XI.

flour (66), skim milk (33), and salt (1)—with or without dinitrophenol (100 mg. per 100 gm. diet). The diets were resuspended in an equal weight of 7.5 per cent gelatin in tap water and fed *ad lib.* The results are presented in curves $A_1$, $D_1$, $A_2$, $D_2$ of Fig. 8.

In similar experiments, dinitrophenol in concentration of 0.1 per cent of the food (dry weight) was added to either the skim milk-white flour diet, or to synthetic diet 191. All animals were infected with 0.01 ml. MV and placed on the experimental diets on the day of infection or 2 days later (curves A, AD, B, and BD of Fig. 9, Table XIII).

As can be seen from the results presented in Tables XI, XII, XIII, and Figs. 6, 7, 8, and 9 (curves A, AD, B, and BD) the infection-enhancing effect of dinitrophenol, or of thyroid extract, was apparent only in the mice that died approximately 4 weeks after infection or later. No effect could be detected when a large infective dose was used (0.1 ml. of culture MV), most of the animals in this case dying within 24 days after infection. Another experiment was therefore designed to test whether the effect of dinitrophenol could be made evident earlier by using a smaller infective dose and beginning administration of the drug a few days before infection.

TABLE XII

(see Fig. 8)

*Effect of Infective Dose on Enhancement of Mouse Tuberculosis by Dinitrophenol*

| Fig. No. | Percentage composition of diet* | | | Infective dose (ml. MV) | Cumulative number (out of 10) of mice dead at | | | |
|---|---|---|---|---|---|---|---|---|
| | Skim milk | Wheat flour | Dinitrophenol | | 3 wks. | 5 wks. | 7 wks. | 9 wks. |
| 8-A1 | 33 | 66 | — | 0.1 | 7 | 9 | 10 | 10 |
| 8-D1 | 33 | 66 | 0.1 | 0.1 | 8 | 10 | 10 | 10 |
| 8-A2 | 33 | 66 | — | 0.004 | 0 | 0 | 5 | 7 |
| 8-D2 | 33 | 66 | 0.1 | 0.004 | 0 | 5 | 8 | 10 |

* 1 per cent salt added.

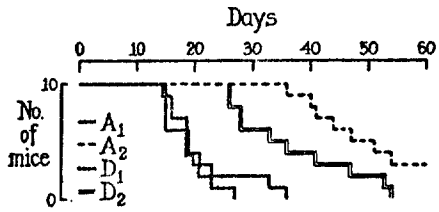



FIG. 8. Effect of dinitrophenol on mouse tuberculosis. Ordinates indicate number of mice (out of 10) surviving at different periods of time after infection (abscissae). For experimental details, see Table XII.

In the experiment corresponding to curves C and CD of Fig. 9 (Table XIII), the mice were infected with a very small dose (0.001 ml. of culture MV). They were fed a diet consisting of wheat flour (66), skim milk (33), and salt (1) with or without dinitrophenol (100 mg. per 100 gm., of diet); these food mixtures were incorporated in an equal weight of 7.5 per cent gelatin in tap water and fed *ad lib.* The animals were placed on the experimental diets 5 days before infection.

The infection-enhancing effect of dinitrophenol illustrated in curves C and CD of Fig. 9 became apparent on the 18th day after infection, approximately 1 week earlier than had been observed in the preceding tests. Taken together, the results presented in Figs. 6, 7, 8, and 9 suggest therefore that the effects

exerted by dinitrophenol and thyroid extract on infection are not immediate, but have a latent period of at least several days. In only one case was it pos-

TABLE XIII

(see Fig. 9)

*Effect of Time of Administration of Dinitrophenol on Mouse Tuberculosis*

| Fig. No. | Percentage composition of diet* | | | Infective dose (ml. MV) | Diet started at following time | Cumulative number of mice (out of 10) dead at | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Skim milk | Wheat flour | Dinitrophenol | | | 3 wks. | 5 wks. | 7 wks. | 9 wks. |
| 9-A | 33 | 66 | — | 0.01 | 2 days after infection | 3 | 3 | 4 | —‡ |
| 9-AD | 33 | 66 | 0.1 | 0.01 | | 3 | 7 | 10 | —‡ |
| 9-B | (Diet 191) | | — | 0.01 | Same day as infection | 0 | 0 | 4 | 6 |
| 9-BD | (Diet 191) | | 0.1 | 0.01 | | 0 | 0 | 9 | 10 |
| 9-C | 33 | 66 | — | 0.001 | 5 days before infection | 0 | 1 | 1 | —‡ |
| 9-CD | 33 | 66 | 0.1 | 0.001 | | 3 | 7 | 10 | —‡ |

* 1 per cent salt added.

‡ —, experiment discontinued.

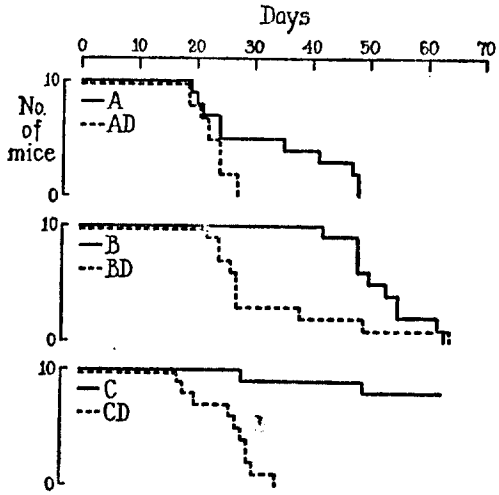



FIG. 9. Effect of dinitrophenol on mouse tuberculosis. Ordinates indicate number of mice (out of 10) surviving at different periods of time after infection (abscissae). For experimental details, see Table XIII.

sible to detect a very early effect of dinitrophenol, namely in mice which had been previously vaccinated with BCG.

Three groups of ten mice each were vaccinated intraperitoneally with 0.02 ml. of culture BCG-P grown in liquid tween-medium containing 0.1 per cent human albumin. They were

challenged 3 weeks later by the intravenous injection of 0.2 ml. of culture MV, grown in bovine albumin. On the same day, they were placed on experimental diets (*ad lib.*) consisting of wheat flour (66), skim milk (33), salt (1) with or without thyroid extract or dinitrophenol in a final concentration of 80 mg. per 100 gm. of dry food. The results are presented in Table XIV and Fig. 10.

Here again, treatment with thyroid extract and dinitrophenol shortened the life expectancy of the tuberculous mice; the results with thyroid extract were less striking than in the experiments presented in Fig. 7, perhaps because the concentration of hormone used was much smaller. In contrast with what had been observed in preceding experiments (Figs. 6, 8, and 9), the effect of

**TABLE XIV**

(see Fig. 10)

*Effect of Dinitrophenol and Thyroid on Tuberculosis in Vaccinated Mice*

| Fig. No. | Percentage composition of diets* | | | | Vaccination (0.2 ml.) | Infection (0.2 ml. MV) | Cumulative number of mice (out of 10) dead at | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Skim milk | Wheat flour | Dinitro-phenol | Thyroid extract | | | 2 wks. | 3 wks. | 4 wks. | 5 wks. |
| 11-A | 33 | 66 | — | — | BCG | 3 wks. after | 0 | 3 | 4 | 8 |
| 11-D | 33 | 66 | 0.08 | — | BCG | vaccina- | 4 | 5 | 7 | 10 |
| 11-T | 33 | 66 | — | 0.08 | BCG | tion | 0 | 7 | 10 | 10 |

* 1 per cent salt added.

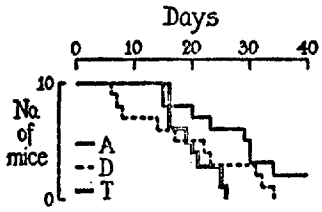



FIG. 10. Effect of dinitrophenol on tuberculosis of vaccinated mice. Ordinates indicate number of mice (out of 10) surviving at different periods of time after infection (abscissae). For experimental details, see Table XIV.

dinitrophenol became apparent very soon after infection. However, the significance of this observation is obscure since the mice certainly harbored large numbers of BCG organisms in their organs at the time that they were placed on the experimental diets. It is possible that the physiological effects associated with BCG infection had rendered the vaccinated animals more susceptible to dinitrophenol. Moreover, as will be presently shown, administration of this drug allows BCG to establish a fatal infection in mice.

*Effect of Metabolic Disturbances on the Susceptibility of Mice to BCG:*

All the infection-enhancing effects which have been described in the preceding parts of this paper were observed by using a mouse virulent myco-

bacterium (MV) for the infection tests. Many similar experiments have been carried out with attenuated cultures of mammalian tubercle bacilli (R1Rv and several substrains of BCG) as well as with staphylococci. Detailed description of the findings with these microorganisms will be presented in subsequent reports (26, 27). Suffice it to outline here the results obtained with the culture BCG-P.

Mice were infected with 0.2 ml. of 10 day old culture BCG-P in tween-albumin medium and immediately placed on experimental diets containing citrate or dinitrophenol. The composition of the diets and concentrations of the additives are indicated in Table XV.

The results presented in Table XV make it clear that most of the mice infected with BCG-P developed a slowly progressive fatal disease when fed

TABLE XV

*Effect of Addition to Diet of Citrate or Dinitrophenol on Susceptibility of Mice to BCG**

| Percentage composition of diets‡ | | | | | Dinitrophenol | Cumulative number of mice (out of 10) dead at | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Skim milk | Wheat flour | Peanut oil | Cerelose | Citrate | | 5 wks. | 7 wks. | 9 wks. | 11 wks. | 13 wks. |
| 20 | 50 | 20 | 10 | — | — | 0 | 0 | 0 | 1 | 1 |
| 20 | 50 | 20 | — | 10 | — | 2 | 3 | 4 | 7 | 8 |
| 20 | 50 | 20 | 10 | — | 0.1 | 1 | 3 | 5 | 6 | 7 |

* 0.2 ml BCG-P injected intravenously.
‡ 1 per cent salt added.

diets containing citrate or dinitrophenol, substances which have been shown to accelerate the course of virulent tuberculous infection.

## DISCUSSION

The findings described in the present report leave no doubt that it is possible to decrease at will the resistance of albino mice to tuberculous infection by simple manipulations of the dietary regimens. However, the effects obtained do not appear to have been the direct result of nutritional deficiencies *per se*, but rather the indirect expression of metabolic disturbances set in motion by the various experimental procedures.

The lack of correlation between enhancement of infection and effect of the dietary regimen on the weight curves of the uninfected animals was to be expected. It had already been found by other investigators that mice fed a diet enriched with a variety of natural food products (cereals, yeast, cod liver oil, fish, meat, and bone meat, etc.) showed higher resistance to tuberculosis than mice receiving Sherman diet, although they grew approximately as well on the latter as on the former regimen. Of six natural diets fed mice infected

0574



with *Salmonella* or tubercle bacilli "the best diet for reproduction, which was also a good diet for growth, was the poorest for resistance to infection" (4, 5, 11). In the present study, it was found that mice gained weight on some of the citrate-containing diets which caused them to die rapidly of tuberculosis. In contrast, the disease was little if at all aggravated in animals which were prevented from gaining weight by being maintained in a state of chronic undernutrition by limiting to a low, but constant level their total food intake or the protein content of their diet. On the other hand, animals completely deprived of food for 30 consecutive hours every week, and fed optimum diets *ad lib.* at other times, did gain weight but became highly susceptible to infection. Recent experiments with staphylococci and Friedländer bacilli have revealed that the resistance of fasted animals to infection returns to normal within a short time after feeding has been resumed (28). The contrast between chronic undernutrition and acute starvation with regard to infectious agents had already been observed in rats and mice infected with *Salmonella typhimurium*. Whereas progressive reduction of the food intake during this experimental disease stimulated the rate of bacterial multiplication *in vivo*, restriction down to 80 per cent of the optimum amount proved to have no influence provided the restricted regimen was maintained at a constant level from day to day (29).

In many tests not described in this report, the resistance of mice to infection appeared to be unaffected by addition to the diet of any of the vitamins (including vitamin B12 and large amounts of fat-soluble vitamins). This finding may be of little significance since the diets used contained wheat flour and skim milk which probably provided a fairly adequate vitamin complement. More surprising was the failure to observe any deleterious effect of protein limitation. The level of resistance observed with some of the diets containing only 4 per cent skim milk was as high as that observed with any of the other experimental regimens tested, however high their protein content. Thus, tuberculous mice died at the same rate whether they received high protein diets (pellets containing 25 per cent of mixed protein; synthetic diet 191 with 18 per cent casein; wheat flour supplemented with 15, 20, or 33 per cent skim milk) or a diet containing only 7 per cent organic nitrogen (1.5 per cent supplied in the form of 4 per cent skim milk, and 5.5 per cent supplied by the wheat flour). It must be emphasized however that susceptibility to infection on this low protein diet was exceedingly high when the caloric balance was made up exclusively with cerelose. Resistance became normal only when part of the cerelose was replaced by peanut oil, or some other fat.

Despite common belief, published information does not provide a clear picture of the role of protein intake on resistance to infectious diseases. In general, manipulation of the protein level in the diet has been found to have but little effect on the outcome of experimental infections, unless depletion

was so complete and prolonged as to impair profoundly the general health of the animal. The results obtained in experimental tuberculosis of the rat, guinea pig, and hamster have been contradictory and provide little support for the view that the level of dietary protein plays an important part in resistance to this disease (1, 4–9, 11, 29–33).

Whereas the experiments reported in the present paper provided no indication that protein deficiency *alone* could accelerate the course of tuberculosis there was some evidence that mice fed diets containing only small amounts of skim milk became more sensitive to certain infection-enhancing effects resulting for example from excessive intake of cerelose, cocoa butter, or citrate. In man, the circumstances (individual or epidemiological) which lead to a decrease in protein consumption are usually conducive to other simultaneous nutritional aberrations. It may be worth noting in this respect that investigators who have tried to correlate deficiency in nutritional factors with susceptibility to tuberculosis on the basis of epidemiological studies of the human disease have emphasized the importance of proteins of *animal source.* As deficiency in foods of animal origin is almost always accompanied by other dietary limitations, it seems possible that these, rather than low protein intake, are responsible for the increase in susceptibility to tuberculosis which has been observed in certain epidemiological situations, for example during war time.

As mentioned in the introduction, the design of many of the experiments reported in the present paper was based on the working hypothesis that resistance to infection is conditioned by the relative concentration in the infected tissues of lactic acid on the one hand, and of certain polycarboxylic acids and acetone bodies on the other (12–15). It need not be pointed out that, because of their essentially biological nature, the observations reported here do not provide evidence for or against this view. The most that can be said is that the results obtained were compatible with some of the consequences to be expected from the working hypothesis.

It is known for example that in man, and large animal species, ketosis is present during the early phase of acute fasting, but is usually absent in chronic undernutrition. Since keto acids favor the multiplication of tubercle bacilli under certain circumstances, it appears possible that the ketosis which occurs during the early period of acute fasting may cause a temporary stimulation of bacillary metabolism. Experiments in progress indicate that mouse tuberculosis can be rendered more severe by the addition to the diet of certain fatty acids with short carbon chains, the oxidation of which is known to lead to the formation of acetone bodies *in vivo.*

Whatever the diet used, addition to it of sodium citrate reduced consistently the life expectancy of mice infected with tubercle bacilli. Definite effects were obtained with daily intakes of 0.1 gm. citrate per mouse. Addition

of alcohol or sodium lactate to the diet even in large amounts had no apparent effect on the course of the disease. Worth mentioning here is the fact that daily injections of citric acid into mice has been shown by others to increase the susceptibility of these animals to infection with *Salmonella typhimurium* (34, 35).

Two hypotheses can be considered to explain the enhancement of tuberculosis by citrate. On the one hand, citric acid is known to favor the multiplication of tubercle bacilli *in vitro*, and it may be surmised that it favors also their multiplication *in vivo*. In addition to this direct effect on the bacilli, citric acid may influence the course of infection in an indirect manner by modifying tissue metabolism, perhaps at some stage in the Krebs cycle. It has been found in fact that addition of citrate to many of the experimental diets reduced markedly the rate at which uninfected animals gained weight. Available information does not seem to warrant any further discussion of the actual mechanism by which citrate aggravated the course of tuberculosis in the present experiments.

One of the first consequences of acute fasting is the exhaustion of the glycogen reserves of the body. Since phagocytic cells utilize glycogen as source of energy (24, 25), their behavior is most probably altered during certain phases of fasting. It can also be presumed that glycogen depletion will bring about a decrease in lactic acid production by tissue cells and thus jeopardize one of the normal tissue reactions in the inflammatory areas (12–15). A similar effect may follow prolonged treatment with dinitrophenol and thyroid extract, since these substances interfere with the accumulation of glycogen by decreasing the efficiency with which phosphorylations are coupled to oxidations (22, 23).

Any attempt to explain the mechanisms by which thyroxine and dinitrophenol accelerate the course of mouse tuberculosis brings into sharp focus the superficiality of the present discussion. It is obvious that the administration of either of these two metabolic stimulants sets in motion a large number of complex processes with a multiplicity of ill defined secondary effects. Similarly, any modification of the nutritional status has repercussions far removed from the initial metabolic disturbance that it causes—involving in many cases various components of the hormonal system. Stimulation of the adrenal cortex for example, might account for many of the phenomena observed, by bringing about an overproduction of cortisone. (Let it be mentioned in passing that cortisone, by reducing the inflammatory reaction, most likely decreases the production of lactic acid at the site of the lesion). Many other mechanisms should be considered as susceptible of playing some role in the decrease in resistance to infection which has been observed in the present experiments. Thus, it is possible that, instead of providing a more favorable environment for the multiplication of tubercle bacilli, the procedures used merely served

to render the animal tissues more susceptible to the bacterial toxins. It is clear that mere observation of survival time and weight curves cannot provide an answer to these questions. New techniques will be required to analyze in other terms the complex relationships between host and parasite under various metabolic conditions.

Irrespective of their mechanism, the infection-enhancing effects observed in the present study may contribute to the understanding of certain problems of the pathogenesis of infection. It has been found that the resistance of mice to tuberculosis can be markedly decreased by metabolic disturbances of short duration (for example as caused by the removal of food for 30 hours); a similar effect obtains in the case of infections with Friedländer bacilli and staphylococci as will be shown in other publications (27, 28). The fact that a *transient* physiological upset can increase so markedly the receptivity to infection bids fair to throw light on some obscure epidemiological situations. It has been shown furthermore, and will be illustrated more extensively elsewhere (26), that the procedures which increase susceptibility to virulent tubercle bacilli can also break down the resistance which mice normally display against attenuated organisms like BCG. This fact gives support to the belief that the physicochemical environments provided by the phagocytic cells and by the inflammatory response, will prove to be decisive factors in determining the ability of mycobacteria to behave as pathogenic agents.

## SUMMARY

Mice maintained on various types of diets were found to become more susceptible to tuberculosis when deprived of food for periods of 30 hours shortly after infection. In contrast, the susceptibility of the animals to the disease was unaffected by undernutrition resulting from limitation of food intake to a low but constant daily level.

The resistance of mice to tuberculosis appeared to be independent—within wide limits—of the protein content of the diet. It is true that mice fed a diet very low in protein and high in carbohydrate proved highly susceptible, but resistance was normal if part of the carbohydrate was replaced by fat (peanut oil)—without any change in the protein content of the food.

Resistance to tuberculosis could be consistently and markedly decreased by adding sodium citrate (or glutarate) to a variety of diets.

The survival time following infection was greatly shortened if dinitrophenol or thyroxine were administered *per os* in amounts sufficient to limit the weight gains of non-infected controls. There was usually a lag period of several days before the infection-enhancing effect of these metabolic stimulants became manifest.

The procedures which increased the susceptibility of mice to infection with

virulent tubercle bacilli also made it possible to establish in these animals a fatal infection with BCG.

There was no constant relation between weight gains of uninfected mice on the various regimens, and the effect of the latter on susceptibility to tuberculosis.

These findings appear compatible with, but do not prove, the hypothesis that a decrease in resistance to infection can be brought about by metabolic disturbances which cause either a depletion of the glycogen reserves of the body, or a reduction in the glycolytic activity of inflammatory cells, or an increase in the concentration of certain polycarboxylic acids and ketones in the tissues.

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Food Protection Committee, 1972

Comprehensive GRAS Survey, Usage Levels -- infant formulas and baby foods

Nat. Acad. Sci., Nat. Res. Coun., Washington, D.C.
Table 3, p. 2

Food Protection Committee, 1972

Comprehensive GRAS Survey, usage levels — citric acid

Nat. Acad. Sci., Nat. Res. Coun., Washington, D.C.
Table 2, p. 13

The quantities retained were calculated by dividing the specific activity of the substrate in the media by the radioactivity retained by the washed mitochondria. This calculation leads to an overestimation to the extent to which the substrates are converted to other materials. The results to be considered next (Fig. 2) indicate that the products of metabolism of citric and malic acids account for less than 10 to 20% of the retained radioactivity, when the incubations were limited to 10 minutes.

TABLE I

*System of column and paper chromatography used for isolation and identification of Krebs citric acid cycle intermediates*

| Elution fraction | Formic acid | Ammonium formate | Eluate volume | Appearance of known acids | |
|---|---|---|---|---|---|
| | | | | Acid | $R_F$ value* |
| | *M* | *M* | *ml* | | |
| I | 1.5 | | 10 | | |
| II | 2.5 | | 10 | Succinic | 0.78 |
| | | | | Malic | 0.63 |
| III | 2.5 | | 4 | | |
| IV | 4.0 | | 14 | Citric | 0.57 |
| V | 4.0 | 0.15 | 4 | Fumaric | 0.86 |
| VI | 4.0 | 1.0 | 4 | α-Ketoglutaric | 0.67 |

* Refers to results obtained subsequently with paper chromatography.

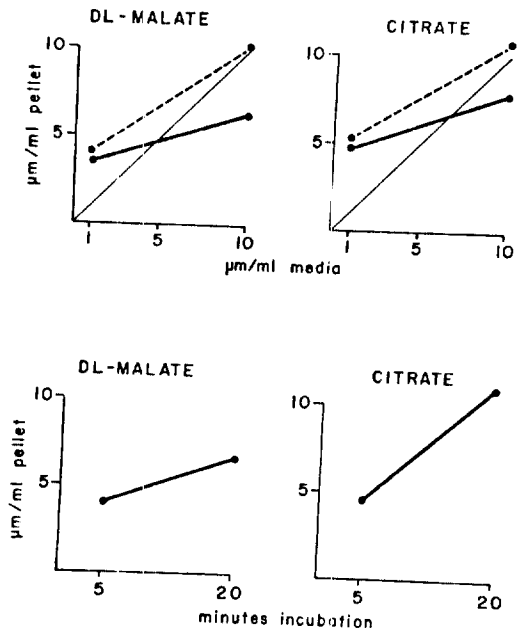


FIG. 1. Accumulation of DL-malate and of citrate by mitochondria. *Upper curves*, the samples were incubated for 6 minutes in the presence of sodium DL-malate and sodium citrate at concentrations of 1 and 10 μmoles per ml. *Lower curves*, the samples were incubated in the presence of these substances at a concentration of 4 μmoles per ml for 5 and for 20 minutes. The incubations were carried out in volumes of 6 ml containing sucrose, 0.25 M; KCl, 0.05 M; and Tris-HCl, 0.003 M, pH 7.5. Malate and citrate were labeled with 0.5 μC of DL-malate-3-$^{14}$C and of citrate-1,5-$^{14}$C. Accumulation was calculated on the basis of retained radioactivity. The *dashed lines* of the *upper graphs* refer to concentrations of malate and citrate in unwashed mitochondrial pellets. The *solid lines* refer to concentrations measured after washing.

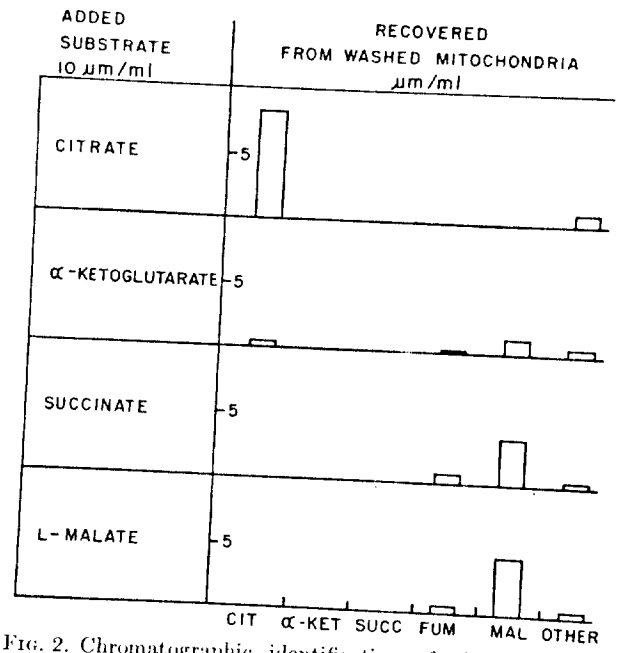


FIG. 2. Chromatographic identification of citric acid cycle components retained by washed mitochondria. In four experiments, mitochondria containing 3 to 5 mg of nitrogen were incubated for 10 minutes at 23° in volumes of 6 ml with one of the substrates listed on the *left*. These substrates were labeled with $^{14}$C and were present at a concentration of 0.01 M. The mitochondria were washed twice after incubation, and the various components were extracted, identified, and measured according to the procedure described in the section on methods. The concentration of potassium in the washed pellets in the four experiments were (reading down) 38, 33, 30, and 30 μmoles per ml, respectively.

*Chromatographic Identification of Materials Retained by Washed Mitochondria*—An analysis of the labeled materials retained by washed mitochondria was carried out with column and paper chromatography. The results, depicted in Fig. 2, indicate that the citrate and malate were accumulated, as such, with minimal conversion to other products. In contrast, succinate and α-ketoglutarate were not accumulated. When the mitochondria were incubated with these substrates, most of the radioactivity was recovered with material which had the chromatographic characteristics of malic acid. Fumaric acid was identified in smaller amounts, equivalent to 10 to 25% of the quantities recovered as malic acid.

*Relationships between Accumulation of Citrate, Release of Phosphate, and Retention of Potassium*—The diagrams of Fig. 3 depict the accumulation of citrate under a variety of conditions and show relationships to the release of phosphate and the retention of potassium. The *bar graphs* describe the changes with reference to a control sample incubated in the cold without citrate. The first group of results (*Sample 1*) was obtained from a second control experiment, in which the incubation was carried out without citrate, to show the effects of raising the temperature to 23°. The mitochondria contained 5.8 μmoles of potassium or 0.1 μmole less than the sample kept in the cold. The value for citrate was essentially unchanged, and a small increase in the loss of phosphate was observed. When citrate was added to the incubation medium, 1.3 μmoles were retained, and 0.95 μmole of phosphate was released into the medium during the incubation. The content of potassium was greater

by 0.4 μmole than that of the control sample kept at 0°. The inclusion of L-malate in the incubation medium of the third sample resulted in a moderate stimulation of citrate incorporation and in a small additional increase in the retention of potassium.

In the last four experiments the accumulation of citrate was

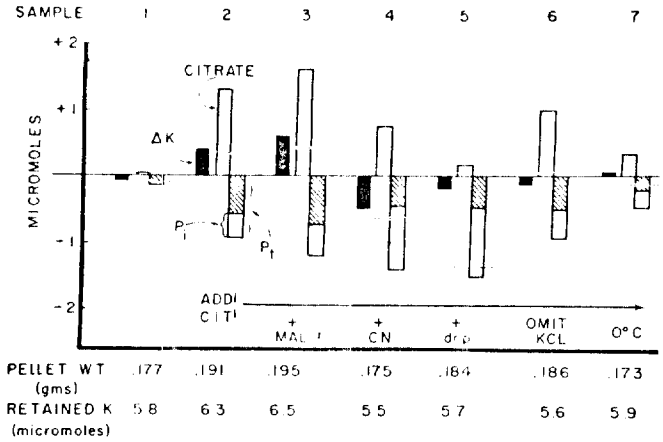


FIG. 3. Relationships between gain or loss of potassium (*solid bars*), accumulation of citrate (*open bars*), and loss of phosphate (*partially filled bars*). Citrate and potassium were measured after washing. Phosphate was determined in the supernatant of the postincubation centrifugation. Except when indicated otherwise, equal aliquots of the mitochondria were incubated at 23° for 10 minutes in 6 ml of sucrose, 0.25 M, containing KCl, 0.05 M. The concentrations of the other reagents were as follows: sodium citrate, 0.01 M; sodium L-malate, 0.01 M; sodium cyanide, $10^{-3}$ M; 2,4 dinitrophenol, $10^{-4}$ M. The *bar graphs* describe the changes with reference to a control sample which was incubated at 0° for 10 minutes without citrate. The colorimetric reading for citrate in this sample was equivalent to 0.2 μmole; and this quantity, which includes the tissue blank, was subtracted from the other measurements. Similarly, 0.15 μmole of phosphate was released into the incubation medium by the mitochondria in the control sample; and this amount, as a base-line rate of loss, was subtracted from the other measurements. The *horizontal dashed lines* indicate the values obtained when the micromoles of phosphate released are subtracted from the micromoles of citrate retained.

TABLE II

*Accumulation of L- and D-malate with associated release of organic and inorganic phosphate*

Mitochondria, containing 6.1 mg of nitrogen, were incubated for 10 minutes in 6 ml of sucrose, 0.25 M, containing KCl, 0.05 M, and D- or L-malate, 0.01 M. The phosphate data refer to the amounts released into the incubation media. Citrate and potassium were measured after washing. When the mitochondria were incubated in the absence of malate, the fluorometric reading was equivalent to 0.4 μmole of malate, and this value, which includes the tissue blank, was subtracted from the two subsequent determinations.

| Substrate | Pellet weight | Retained potassium | Malic acid accumulation | Phosphate release | |
|---|---|---|---|---|---|
| | | | | Total | Inorganic |
| | *g* | *μmoles* | | *μmoles* | |
| None | 0.221 | 7.9 | | 0.18 | 0.10 |
| L-Malate | 0.222 | 7.9 | 0.93 | 0.91 | 0.57 |
| D-Malate | 0.225 | 7.7 | 1.32 | 1.35 | 1.05 |

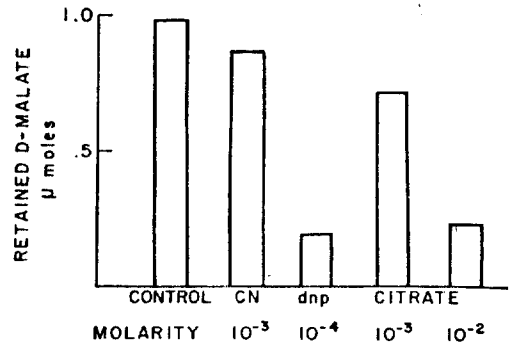


FIG. 4. Inhibition of accumulation of D-malate. Mitochondria (4.1 mg of nitrogen) were incubated for 10 minutes in 6 ml of isotonic sucrose containing D-malate, 0.01 M. The results show effects of cyanide, $10^{-3}$ M; 2,4-dinitrophenol, $10^{-4}$ M; and citrate, $10^{-3}$ and $10^{-2}$ M. Mitochondrial D-malate was measured after washing.

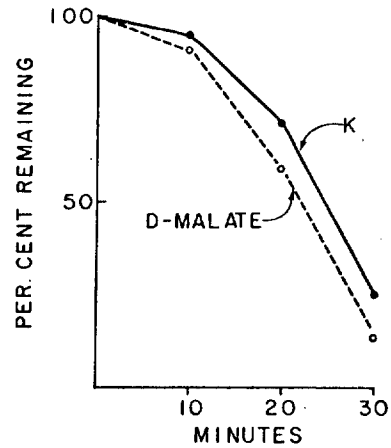


FIG. 5. Release of D-malate and potassium with aging. Mitochondria were preincubated with sodium D-malate (0.01 M) for 5 minutes at 23°. They were washed in the cold twice and were reincubated at 23° in isotonic sucrose without added KCl as the aging procedure. Aliquots of mitochondria were removed at the start and at the times indicated. The first aliquot contained 2.86 μmoles of potassium and 0.48 μmole of D-malate.

inhibited in various ways. Dinitrophenol ($10^{-4}$ M) proved a potent inhibitor, whereas cyanide ($10^{-3}$ M) was much less effective. The loss of phosphate and potassium was increased by both of these agents. When potassium was omitted from the incubation media and when its accumulation was precluded, the accumulation of citrate was reduced and was balanced more closely by the loss of phosphate. When the temperature was lowered to 0°, the uptake of citrate and the release of phosphate were reduced markedly. Nonreciprocity between the gain in citrate and the loss of phosphate was observed after treatment with dinitrophenol. In a previous study of the effects of this inhibitor, a loss of phosphate was associated with an accumulation of chloride (13).

The *horizontal dashed lines* shown in Fig. 3 give the values obtained by subtracting molar quantities of released phosphate from those of accumulated citrate. When there was a net gain of these anions, there was increased retention of potassium, and conversely, with a net loss of the anions, potassium retention was decreased. Mitochondrial volume, as indicated by the

values for pellet weight (Fig. 3), did not change by more than 5 to 10%.

Sodium is retained only in trace amounts through washing procedures with isotonic sucrose (2, 13). In the experiment of *Sample 6* (Fig. 3) the mitochondria were incubated in a potassium-free solution which contained 30 μmoles of sodium per ml (sodium citrate, 0.1 M). After they were washed, these mitochondria contained 5.6 μmoles of potassium (33 μmoles per ml) and 0.2 μmole of sodium (1.2 μmoles per ml).

*Accumulation of L- and D-Malate and Release of Phosphate*—Table II summarizes the relation between the accumulation of L- and D-malate and the release of phosphate. Somewhat larger quantities of the unnatural D-malate were retained. With both isomers, the phosphate released into the incubation media was nearly equal to the amount of malate accumulated. The phosphate released after incubation with D-malate contained a larger proportion of inorganic phosphate.

*Inhibition of Accumulation of D-Malate*—The effects of cyanide, dinitrophenol, and citrate upon the accumulation of D-malate are described in Fig. 4. Accumulation was reduced by 12% in the presence of cyanide ($10^{-3}$ M) and by 80% when incubated with dinitrophenol ($10^{-4}$ M). Citrate also provided strong inhibition when present at a concentration equal to that of the malate. In supplementary experiments, citrate was found to inhibit accumulation of L-malate to the same degree. Mercuric chloride, which has been studied previously with respect to its inhibition of retention of mitochondrial potassium (14), also inhibited accumulation of malate. Minimally effective concentrations (1 to 3 × $10^{-5}$ M) produced proportional reductions in retention of potassium and in accumulation of malate.

*Loss of Potassium and of D-Malate from Mitochondria with Aging*—The *curves* shown in Fig. 5 describe nearly proportional rates of loss of potassium and of D-malate from mitochondria undergoing decomposition with aging. For this experiment the mitochondria had been preloaded with malate during a previous incubation.

## DISCUSSION

Citrate and malate were accumulated rapidly by isolated mitochondria and were retained in such a way that they were not removed by treatments with cold, isotonic sucrose. When the mitochondria were incubated in solutions containing 1 μmole per ml of citrate or malate, these substances were found to be concentrated to 4 to 5 μmoles per ml in the mitochondria. With higher levels in the media, the quantities in the mitochondria were increased but appeared to reach maximal values of 8 to 12 μmoles per ml (Fig. 1). The materials were retained against concentration gradients of more than 1000:1 during the washing procedures. Respiration was not required for accumulation. Citrate was incorporated in the presence of cyanide (Fig. 3), and D-malate was taken up in the absence of an added oxidizable substrate (Fig. 4 and Table II). However, accumulation of citrate was greater in the absence of cyanide (Fig. 3) and was stimulated by addition of L-malate, which, as an intermediate in the citric acid cycle, would be expected to stimulate respiration.

Citrate and malate were accumulated as such, with little conversion to other materials; but when α-ketoglutarate or succinate was the initial substrate, the materials retained consisted almost entirely of the conversion products, primarily malate with smaller amounts of fumarate and citrate (Fig. 2). Plaut and Plaut (15) and Chappell (16) have commented upon the slow metabolism of citrate obtained with washed mitochondria. The present observations indicate a rapid accumulation in spite of the minimal metabolic breakdown. The relative quantities of the materials retained must reflect differences in steady state levels of accumulation and differences in affinity or permeability. A high affinity for succinate would be masked by a rapid conversion to fumarate and malate; but, on the other hand, accumulation of the nonmetabolizable D-malate (Table II and Fig. 4) points to an affinity for this substance. Furthermore, there must be a still greater affinity for citrate since it inhibits accumulation of malate (Fig. 4). This inhibitory effect of citrate upon malate uptake contrasts with the stimulatory action of L-malate upon citrate incorporation (Fig. 3).

Earlier studies (5, 17) have shown that potassium and phosphate are released together from aging mitochondria as though both were deriving from the same compartment, and the results as indicated in Fig. 3 and Table II are compatible with the view that citrate and malate enter such a potassium-containing compartment and cause displacement of the phosphate. This concept is supported by the results of the experiment of Fig. 5 which show proportional rates of loss of potassium and of previously accumulated D-malate from aging mitochondria. On the other hand, there are significant differences in the diffusion characteristics of potassium and of citrate and malate. The results of a previous study (3) have indicated that potassium is unrestrained in 55 to 70% of the volume of centrifuged pellets and is present in this portion at the concentrations of the surrounding fluids. Presumably this freely diffusible potassium is removed with washing, leaving the more tightly bound potassium in a smaller and more impermeable portion. As described in Fig. 1, the additional quantities of malate and citrate in the unwashed pellets produced concentrations that were higher only by 0.4 to 3.5 μmoles per ml of pellet as the concentrations in the incubation media were increased from 1 to 10 μmoles per ml, respectively. These data may be interpreted to indicate free diffusion of malate and citrate in 30 to 40% of the volume of the pellets. This figure is significantly smaller than that given above for potassium, and it can be accounted for chiefly as interparticle space. As defined by $^{14}$C-labeled polyglucose (4, 18) interparticle space includes 25 to 29% of the volume of the pellet.

When mitochondria from kidney were incubated with succinate in the presence of potassium, a net inward movement and increase in retention of potassium were obtained (3). When mitochondria from liver were used, this response was not seen with succinate as the substrate; but as shown in Fig. 3, it was obtained after incubation with citrate. The inward movement of potassium may be secondary to an increased retention of anion. As shown in this figure, larger amounts of potassium were retained when the gain in citrate was greater than the loss of phosphate. The changes in the content of potassium were small, however, and were not sufficient to compensate for the net changes in the equivalence of the multivalent anions. A close coupling with potassium is ruled out by the demonstrated accumulation of citrate from a potassium-free medium (Fig. 3, *Sample 6*). The increased retention of potassium in the presence of an oxidizable substrate may be related to the maintenance or resynthesis of certain polyphosphate compounds. The phos-

phate appearing in the incubation media during active respiration consisted primarily of organic phosphate; whereas, in the presence of cyanide or when incubated with D-malate, the inorganic phosphate was released in the larger amount (Fig. 3 and Table II).

For both the potassium and the organic acids retention appears an example of a specific attraction with a fixed binding capacity. This might involve, for example, a "steric fit" in a phospholipid or lipoprotein matrix. The fixed type of retention differs from the process of cellular accumulation of potassium which is linked to sodium extrusion, and from mitochondrial accumulation of calcium which is coupled to respiratory activity (19) and is associated with deposition of calcium phosphate (20). The biochemical role of mitochondrial accumulation of these citric acid cycle intermediates is not understood at this time. Accumulation does not appear to be a prerequisite for oxidation since dinitrophenol inhibits accumulation at concentrations which are known to stimulate respiration. The inhibition by dinitrophenol suggests involvement of components of the mechanism of oxidative phosphorylation; however, the effect of this inhibitor may be upon a more general aspect of mitochondrial function or organization.

## SUMMARY

Isolated mitochondria have the capacity to accumulate malate and citrate and to retain them through washing procedures with isotonic sucrose. These substrates were concentrated 2- to 3-fold in the mitochondria after incubation for 20 minutes at 23°. The accumulation was inhibited by 10 to 30% in the presence of cyanide and by 70 to 90% in the presence of dinitrophenol. The unnatural D-malate was rapidly accumulated. The incorporation of citrate and malate was associated with the release of nearly equal amounts of phosphate. These carboxylic acids appear to be contained in a portion of the mitochondrial substance that contains potassium as the chief cation and that initially contains various phosphate compounds as the predominant anions.

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0802

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# CITRIC ACID MILK IN INFANT FEEDING*

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Because of the strikingly beneficial results obtained by the use of lactic acid milk in the artificial feeding of infants since its introduction in 1923 by Marriott and Davidson,[1] the need of a modified milk of similar qualities but of easier preparation and more constant acceptability to the infant has become apparent. The need of an acid milk that does not have to be chilled in the course of its preparation has been felt, especially by those dealing with families too poor to afford ice and with families living, as do those in farming communities, in places too remote to obtain a regular supply of ice. Even though no one type of artificial feeding can be expected to act as a panacea for all infants, an acid milk of constant acceptability is desirable. It is true that the number of infants who cannot take lactic acid milk because of vomiting is not large; nevertheless, if an acid milk that in itself never causes vomiting and yet fulfils the same requirements as lactic acid milk could be found, it would at least be an acceptable type of milk with which to feed infants.

With these ideas in mind, an investigation of citric acid as an acid with which to modify cow's milk for infant feeding was started at the Wisconsin General Hospital in September, 1928, and has continued up to the present time. This investigation has been directed at the following items: the ease of preparation; the hydrogen ion concentration of the gastric contents at the height of digestion; the size of curds at the height of digestion; the acceptability of the milk to the infant; the general progress of the infant; the character of the stools, and the presence or absence of signs of kidney irritation as expressed by urinary and blood conditions.

Although the use of orange juice milk has been in common practice in infant feeding for the past several years, no one in this country

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1. Marriott, W. H., and Davidson, L. J.: Acidified Whole Milk as a Routine Infant Food, J. A. M. A. **81**:2007 (Dec. 15) 1923.

seems to have tried the effect of commercial, dehydrated citric acid in modifying cow's milk for infant feeding. In Germany, however, citric acid milk has been used by Weissenberg,[2] Meyer[3] and Behrens.[4] Originally in 1926, and later in 1928, Weissenberg reported excellent results from the use of citric acid milk as a routine feeding mixture in 900 cases (1928). In both reports he emphasized the low incidence of rickets in infants fed by such means. Meyer, in 1927, reported good results from the use of citric acid milk in the treatment of 7 infants suffering from diarrheal disorders. Behrens, in 1929, reported observations on 109 infants fed on citric acid milk to the effect that normal infants thrive on it, gaining in weight as rapidly as do the breast-fed infants, that infants afflicted with nutritional disturbances respond well to it and that the incidence of rickets in infants unprotected by the use of cod liver oil, irradiated ergosterol or special exposure to sunlight is much less in those fed on citric acid milk than in those fed on other artificial mixtures. The present observation duplicates several aspects of Weissenberg's work, but in addition covers other points untouched by him.

Knowing that orange juice is much easier to prepare than lactic acid milk, it was anticipated that pure citric acid could be added to boiled milk cooled to room temperature without the formation of large curds. It was found that when 4 Gm. of dehydrated citric acid is added to a quart of milk which has been boiled for five minutes and then cooled to room temperature by placing the container in a basin of running cold water, a mixture identical in appearance with lactic acid milk is obtained. The milk does not have to be chilled first, and the citric acid does not have to be added slowly in order to obtain a smooth, homogeneous mixture.

When less than 4 Gm. of dehydrated citric acid is added to a quart of milk, there is apt to be some recoagulation of the curds in the infant's stomach at the height of digestion. Because of this, 4 Gm. was taken as the ideal amount of citric acid to add to the quart of milk. Such a mixture has an average hydrogen ion concentration of 4.8 (sufficient markedly to inhibit bacterial growth of the colon, of the dysentery and of the typhoid group)[5] as compared to the average of 4.2 of lactic acid milk made by the addition of 4 cc. of lactic acid to a pint of milk. This fact indicates that the buffer substance of cow's milk is not so completely saturated by the addition of 2 Gm. of dehy-

2. Weissenberg, H.: Arch. f. Kinderh. **73**:276, 1925; **84**:200, 1928.
3. Meyer, Selma: Arch. f. Kinderh. **82**:241, 1927.
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0802



drated citric acid to a pint of milk as it is by the addition of 4 cc. of lactic acid to a pint of milk. However, the difference in the saturation of the buffer substance as between the two milks is not great. That this is true is shown by the fact that in order to increase the acidity of 100 cc. of citric acid milk from a $p_H$ of 4.2 to a $p_H$ of 3.75, the average $p_H$ of the stomach contents of normal breast-fed infants at the height of digestion, it is only necessary to add 40 cc. of tenth-normal hydrochloric acid as compared to the 30 cc. of tenth-normal hydrochloric acid necessary in the case of lactic acid milk. As will be seen, the hydrogen ion concentration and the size of the curds of the gastric contents at the height of digestion of infants fed on citric acid

Fig. 1. Photomicrograph of the stomach contents that were removed two hours after ingestion of whole citric acid milk prepared by adding 2 Gm. of dehydrated citric acid to each pint of milk.

milk are practically the same as in infants fed on lactic acid milk. Consequently it appears that none of the advantages of lactic acid milk in respect to these factors is lost by the difference in degree of saturation of the buffer substance as between the two milks. In addition, one might even consider it an advantage to have almost the same effects brought about by a smaller amount of foreign substance added to the milk for the infant's consumption.

By determining the hydrogen ion concentration of the gastric contents at the height of digestion of infants fed on citric acid milk, a total of 60 tests was made on 35 infants, an average of almost 2 tests on each. The average concentration in normal infants was

0802



found to be 3.72, and in abnormal infants, 4.15. That the hydrogen ion concentration of the gastric contents at the height of digestion is a matter of as much importance as the size of the curds at the height of digestion in indicating the desirability and digestibility of any milk mixture has recently been questioned, though not denied, by Brennemann.[6] The pros and cons of this debated question are intentionally avoided here, except to mention the fact that in aspirating the stomachs of infants fed on citric acid milk, a fair amount of gastric contents can be obtained two hours after the ingestion of the milk; but the amount that can be obtained after two hours rapidly diminishes and between two and one-quarter and two and one-half hours none can

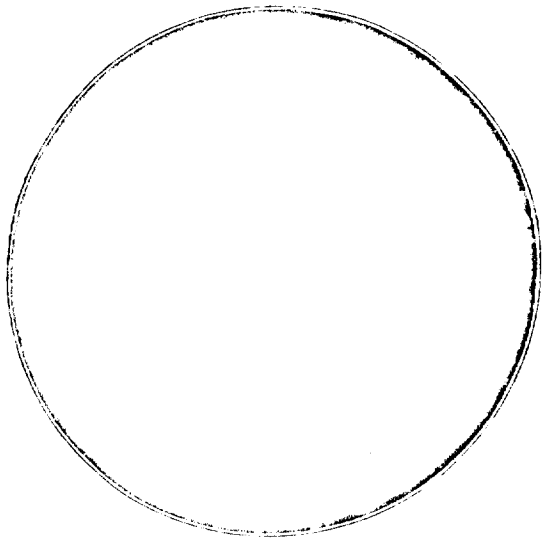

Fig. 2.—Photomicrograph of the stomach contents that were removed two hours after ingestion of whole lactic acid milk prepared by adding 4 cc. of lactic acid to each pint of milk.

be obtained, thereby probably indicating the influence of the gastric acidity in bringing about a rapid emptying of the stomach.

As already stated, 4 Gm. of dehydrated citric acid to 1 quart of milk was decided on as the optimal amount to use in acidifying cow's milk for infant feeding, because such an amount was found to be the least quantity that could be depended on in all cases to keep the curds in the infant's stomach small at the height of digestion. When the gastric contents are removed two hours after the ingestion of citric

6. Brennemann, Joseph: The Curd and the Buffer in Infant Feeding, J. A. M. A. **92**:364 (Feb. 2) 1929.

0802



acid milk, the majority of the curds are found to measure less than 1 mm. in diameter and the largest curds, few in number, to measure not over 3 mm. in diameter. All of the curds are so soft and small that the individual curds cannot be distinguished by grasping between the fingers. As will be seen in the accompanying figures, the curds of citric acid milk at the height of digestion remain separate, and although there is some tendency for the grouping of the curds, there is no actual fusion into large curds whereas those of lactic acid milk, 4 cc. to 1 pint, are here and there definitely fused into solid curds considerably larger than the largest citric acid milk curd, the main mass of the curds, however, being about the same size as the citric acid milk curds. Figure 3

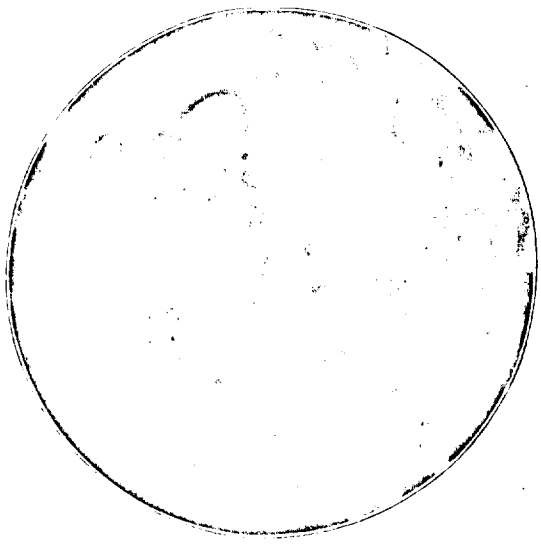

Fig. 3.—Photomicrograph of the stomach contents that were removed two hours after ingestion of whole lactic acid milk prepared by adding 3 cc. of lactic acid to each pint of milk.

shows the curds removed from a normal infant two hours after the ingestion of lactic acid milk, 3 cc. to each pint of milk, and it demonstrates strikingly that such an amount of lactic acid is not nearly so effective in preventing curd recoagulation as is either citric acid milk, 2 Gm. to 1 pint of milk, or lactic acid milk, 4 cc. to 1 pint of milk. Here again the relative merits of the case of the size of the curd in the infant's stomach are not argued except to recall the fact, as originally stated by Brennemann,[7] that all methods used in the past for the modification

7. Brennemann, Joseph, in Abt, I. A.: Pediatrics, Philadelphia, W. B. Saunders Company, 1923, vol. 2, p. 651.

0802



of cow's milk for infant feeding have either wittingly or unwittingly resulted in a reduction in the size of the curd in the infant's stomach.

In water, citric acid has a decided lemon flavor, and with the addition of sugar makes a nice lemonade, but in milk the lemon flavor is much less noticeable. With amounts of corn syrup as small as 1½ ounces to a quart of citric acid milk, a mild acid tinge is momentarily left in the mouth after swallowing. With 3 ounces of corn syrup to 1 quart of citric acid milk, the flavor is extremely pleasant as the taste of acid completely obscured.

As to the acceptability of citric acid milk to the infant, the general statement may be made that citric acid milk is well taken by the general run of infants and that, in itself, it never causes persistent vomiting. Citric acid milk has been fed to 150 infants, including 10 new-born infants, in the wards of the Wisconsin General Hospital, and in all cases, in which pathologic conditions characterized by vomiting have been absent, the feedings have been well taken and retained. A few new-born infants have vomited part of the first few feedings, but even in such instances the feedings have become well tolerated and completely retained within from twelve to twenty-four hours after beginning them. To such new-born infants a mixture of 12 ounces of whole citric acid milk, 8 ounces of water and ⅔ ounce of corn syrup has been used to complement the breast feedings; and in those cases remaining under observation in which artificial feedings have been continued beyond the age of 2 weeks either because of insufficient breast milk or because of weaning, the milk mixture has been gradually increased to whole citric acid milk by the age of 1 month. Previously a similar dilution of lactic acid milk, 4 cc. to a pint of milk, had been stopped as a routine measure because so many normal infants were unable to retain it well even after several days' trial. When citric acid milk was started, the difference in acceptability between the two milks to the infant was pronounced. No normal infant persisted in vomiting the citric acid milk beyond the first two or three feedings, and few infants vomited it at all. As with the new-born infants fed on lactic acid milk, older infants every now and then have been seen in the pediatric ward of the hospital who, although free from pathologic causes of vomiting, regularly vomited whole lactic acid milk as often as it was fed to them. No such trouble has been experienced with whole citric acid milk.

Beginning in September, 1928, all infants admitted to the pediatric ward of the Wisconsin General Hospital and all infants born on the obstetric service who needed complemental feedings were put on citric acid milk feedings. Aside from the observations on the acceptability of citric acid milk to the new-born infant, the chief concern of this report is with the observations on the infants admitted to the pediatric ward.

0802



There were 50 of these infants. In age there were 4 infants under 14 days, 9 under 1 month, 6 under 2 months, 5 under 3 months, 4 under 4 months, 8 under 5 months, 5 under 8 months, 4 under 12 months, 4 under 15 months and 1 under 18 months. The primary diagnoses in this group of infants were: harelip and cleft palate, 18; club-feet, 4; other congenital deformities, 3; simple regulation of feeding, 14; athrepsia, 2; congenital syphilis, 1; cerebrospastic paralysis, 2; acute respiratory infections, 3, and acrodynia, 3. Secondary diagnoses consisted of acute respiratory infection in 20 (including 15 cases of otitis media); diarrhea (secondary to parenteral infection) in 4; pyelitis in 1; severe secondary anemia in 7; simple dystrophy in 5; athrepsia in 4; rickets in 1; anorexia in 2; erysipelas in 1; cervical adenitis in 1, and eczema in 1.

Observation of these infants was continued for a period of at least ten days, and in several instances, such as in cases of harelip and cleft palate, through repeated admittances to the hospital, they were observed for almost a year. The average duration of observation on the ward was fifty days, and for that of 19 infants at home after discharge from the hospital, four months. Three of the infants having congenital deformities requiring frequently repeated treatments remained in the hospital for as long as from six to nine months. The routine feeding of the infants over 1 month of age consisted of whole citric acid milk with the addition of corn syrup. The amount of milk per pound of body weight taken in twenty-four hours naturally varied considerably, but under ordinary circumstances it ran between 2 and 2½ ounces. In general the infants were allowed to take whatever amounts they desired. In the case of markedly malnourished infants, amounts as great as 3 ounces per pound of body weight were taken in twenty-four hours. Infants less than 2 weeks of age were given diluted citric acid milk mixtures so as to prevent an intake of milk in excess of 2 ounces per pound in twenty-four hours. When the infant was 2 weeks old, the concentration of the formula was gradually strengthened to whole citric acid milk by the age of 1 month. The amount of carbohydrate in the form of corn syrup added to the mixture varied according to the age of the infants and according to the character of the feeding before admittance to the hospital. The initial day's feeding contained at least one tenth of the body weight in pounds expressed in ounces of carbohydrate, and the subsequent feedings contained an increasing amount of carbohydrate up to 3 ounces per quart of milk. Twenty (40 per cent) of the infants received 3 ounces per quart of milk and the remainder amounts somewhat between 1½ and 3 ounces per quart.

The feedings were invariably well taken, and the regularity with which many of the infants gained weight was unusual for hospital

0802



patients. For example, 10 of the infants who gained weight most rapidly showed an average daily gain of 1.28 ounces during an average hospitalization period of thirty-three days. Even in the presence of acute illness, such as acute respiratory infections complicated by otitis media, and under circumstances of postoperative conditions in cases of harelip and cleft palate many continued to gain weight, whereas no actual loss of weight of any marked degree did not occur in any case. Of the entire group of 50 infants, 46 (92 per cent) gained in weight and 2 (4 per cent) remained stationary; 2 (4 per cent) lost weight. The average gain in the hospital of the 46 infants who showed a gain in weight was 0.73 ounce per day. The average gain of 19 of the infants under observation at home after discharge from the hospital ward was 0.9 ounce per day. This satisfactory rate of gain in weight at home has been most strikingly emphasized by the fine state of nutrition of infants readmitted to the hospital for further plastic surgery after long periods at home between operations. In previous years, in spite of control of the feeding at home, it was usual to find extremely poor nutrition in infants readmitted for the repair of a cleft palate after a six months' interval at home following an operation for harelip. Ordinarily, weeks had to be spent in improving their general physical status before it was dared to subject the patient to the rigors of an operation for cleft palate. During the past six months, it has been most gratifying to find practically all patients in an excellent state of nutrition on their second admittances after periods of from four to eight months at home.

Throughout the study special attention was paid to the stools, and certain fundamental facts concerning their character and frequency determined. The most obvious fact observed is that the stools of infants fed on citric acid milk differ in character or frequency according to various conditions. New-born infants and those under 3 months of age have tended to develop loose frequent stools when fed on whole citric acid milk containing large amounts of corn syrup, such as 3 ounces to a quart of milk. On the other hand, such infants have taken from 1¾ to 2½ ounces of citric acid milk per pound of body weight in the twenty-four hours containing one tenth of the body weight in pounds expressed in ounces of corn syrup and have had from 1 to 3 yellow, smooth, soft, pasty or firm stools daily. Six infants, all of whom were less than 6 weeks of age, have taken 3 ounces of citric acid milk per pound with the addition of from ¾ to 1½ ounces of corn syrup in the twenty-four hours without any signs of indigestion and with steady gain in weight. Infants over 3 months of age and especially those more than 4 months of age have taken from 2 to 3 ounces of corn syrup to a quart of whole citric acid milk and have had no unusual increase in the number or looseness of the stools. A few of

0802



them actually have become constipated on such mixtures. In general, however, the stools of the infants fed on whole citric acid milk plus from 7 to 10 per cent of corn syrup have been more frequent and softer than those of infants fed on the ordinary simple dilution mixtures of cow's milk, but no more, and usually much less, frequent and soft than those of infants fed on evaporated milk mixtures containing from 7 to 10 per cent of corn syrup. The actual number of stools has varied from 1 to 3 daily, occasionally going as high as 4 stools a day in the young infant. The average daily number of stools of all infants under hospital observation during the past year, except for 4 infants who developed diarrhea for a few days secondary to acute respiratory infections, has been 1.5 stools a day. Although the number of stools has been in excess of the old standard of 1 stool a day for the artificially fed infant, there have been no evidences of the intestinal conditions being inconsistent with the general health and progress of these infants. Symptoms of dyspepsia such as vomiting, variable appetite, flatulence and colic have not been present. Aside from a few infants in whom the carbohydrate tolerance was overstepped, the stools have shown no signs of intestinal indigestion such as looseness, curds, mucus, green discoloration and excoriations of the buttocks. Whenever the tolerance for corn syrup has been exceeded, as indicated by the appearance of increased frequency or looseness of the stools, the reduction in the amount of corn syrup to the previous level has always resulted in a reduction of the frequency and an improvement in the consistency of the stools. Not infrequently a seeming intolerance to an increased amount of corn syrup has corrected itself without any reduction in the syrup. During the period of study, and especially for about four months (December to March, inclusive), acute respiratory infections, including otitis media, were often epidemic in the ward, and during this time, diarrhea developed in 4 of the 23 infants so affected. All 4 infants promptly recovered from the diarrhea following the reduction or entire removal of the corn syrup from the citric acid milk. Of the 19 infants remaining free from diarrhea, 7 were receiving and continued to receive throughout the course of the respiratory infection whole citric acid milk that contained 3 ounces of corn syrup per quart of milk. From these facts and from the fact that Meyer reported favorable results in the treatment of infants with diarrheal disorders by the use of whole citric acid milk, it appears that citric acid acts similarly to lactic acid in preventing a loss in tolerance for food, and, when lost, in restoring tolerance for it.

At the onset of the investigation the question naturally arose as to whether citric acid, in the amount used, might exert any toxic action on the kidneys. Such a possibility was considered unlikely, as Blather-

wich and Long[8] had shown by experiments with large amounts of orange juice taken by young adults over a period of four days that it was impossible to overreach the organism's ability to oxidize the contained citric acid though the amounts drunk in one day were equivalent of about 48 Gm. of acid." However, the authors assume, by a process of deduction, that "approximately 6 per cent of the ingested citric acid appears to have escaped oxidation," even though their chemical study of the urine seem "to render it improbable that the ingestion of large amounts of citrous fruits or fruit juices is capable of causing the production of an acid urine by overstepping the organism's ability to oxidize the organic acid or acids contained therein." Because of the assumed loss of citric acid to the extent of 6 per cent of the ingested acid, it

TABLE 1.—*Citric Acid Content of Urine Before and After Ingestion of Citric Acid*

| Name and Weight in Pounds | Day of Experiment | Volume of Urine | Mg. Citric Acid Recovered from Urine | |
|---|---|---|---|---|
| F. B. 82 (37.2 Kg.) | 1 | 440 | 245 | Basal diet |
| | 2 | 580 | 251 | Basal diet |
| | 3 | 520 | 12 | Basal diet + 22 Gm. dehydrated citric acid in 1,000 cc. water |
| | 4 | 920 | 5 | Basal diet + 22 Gm. dehydrated citric acid in 1,000 cc. water |
| | 5 | 490 | 5 | Basal diet + 22 Gm. dehydrated citric acid in 1,000 cc. water |
| G. B. 77¾ (35 Kg.) | 1 | 575 | 176 | Basal diet |
| | 2 | 1,800 | 6 | Basal diet |
| | 3 | 1,125 | 900 | Basal diet + 21 Gm. dehydrated citric acid in 1,000 cc. water |
| | 4 | 1,250 | 8 | Basal diet + 21 Gm. dehydrated citric acid in 1,000 cc. water |
| | 5 | 1,100 | 4 | |
| W. S. 41¾ (19 Kg.) | 1 | 430 | 2 | Basal diet |
| | 2 | 625 | 5 | Basal diet |
| | 3 | 1,100 | 30 | Basal diet + 11 Gm. dehydrated citric acid in 1,000 cc. water |
| | 4 | 1,025 | 4 | Basal diet + 11 Gm. dehydrated citric acid in 1,000 cc. water |
| | 5 | 1,025 | 4 | |
| F. S. 48¾ (22 Kg.) | 1 | 410 | 34 | Basal diet |
| | 2 | 430 | 60 | Basal diet |
| | 3 | 620 | 30 | Basal diet + 13 Gm. dehydrated citric acid in 1,000 cc. water |
| | 4 | 610 | 41 | Basal diet + 13 Gm. dehydrated citric acid in 1,000 cc. water |

was considered advisable to determine by direct examination by the methods of McClure[9] the actual amount of citric acid lost in the urine of children taking large amounts of pure citric acid. Consequently, 4 normal children, ranging in age from 7 to 12 years, after being placed on a basal diet for two days, were given 4 Gm. of dehydrated citric acid per 15 pounds (6.8 Kg.) of body weight daily for three days and the results tabulated in table 1 were obtained. This amount of citric acid, 4 Gm. per 15 pounds (6.8 Kg.) of body weight, was regarded as a proper figure in representing an amount in the older children analogous to the

8. Blatherwich, N. R., and Long, M. Louisa: Studies of Urinary Acidity. I. Some Effects of Drinking Large Amounts of Orange Juice and Sour Milk, J. Biol. Chem. **53**:103, 1922.

9. McClure, W. B.: The Adaption of the Pentabromoacetone Method to the Quantitative Determination of Citric Acid in the Urine, J. Biol. Chem. **53**:957, 1922.

0802



infant intake, as an age of 5 months, at which time the average weight is 15 pounds (6.8 Kg.), is the earliest average age at which the infant fed on citric acid milk receives 4 Gm. of citric acid daily. Although there is some question as to the absolute accuracy of McClure's method of determination of the citric acid in the urine, the foregoing results, at least, show that there is no increase in the amount of citric acid in the urine after the ingestion of the amounts used in the experiment. It also indicates that older children taking citric acid in amounts proportionate to the infant intake over a period of three days are able to oxidize large as well as small amounts of citric acid. In addition, the conclusion seems logical that under such circumstances no kidney damage could possibly occur.

However, the possibility that citric acid given over a long period of time might be different in effects than if given over a short period was considered important enough to necessitate careful study of the infants

TABLE 2.—*Results of Feeding Five Infants on Citric Acid Milk*

| Name | Months of Citric Acid Milk Feeding | Diagnosis | Nonprotein Nitrogen, Mg. | Creatinine, Mg. |
|---|---|---|---|---|
| W. M. | 9 | Club foot | 30.8 | |
| R. I. | 8 | Congenital syphilis | 31.4 | |
| J. R. | 24 | Harelip, cleft palate | 32.3 | 1.48 |
| A. I. | 6 | Simple dystrophy | 29.2 | 1.35 |
| J. S. | 9 | Harelip, cleft palate | 27.4 | 1.26 |

under observation for evidences of kidney damage. Consequently, routine chemical and microscopic urinalyses were made regularly on all infants, and determinations of the blood nonprotein nitrogen were made on a few from time to time. The routine urinalyses failed to reveal any consistently abnormal conditions common to all of the infants. The reactions were uniformly acid. As expected, a faint trace of albumin was found now and then, especially in the infants suffering from acute respiratory infections. The microscopic examinations showed no abnormality. Special attention was paid to the search for red blood cells, but none was found. Study of the blood chemistry showed no changes in the nonprotein nitrogen or creatinine after either short or long periods of citric acid milk feedings. The observations in the case of 5 infants fed on citric acid milk for long periods are shown in Table 2.

These normal urinary and blood observations seem to indicate the absence of any nephropathic action of citric acid in the amounts used and would consequently lead to the conclusion that citric acid given over an extended period is as readily oxidized as Blatherwich and Long found it to be over a short period of time.

0802



SUMMARY AND CONCLUSIONS

1. Citric acid milk is easy to prepare.

2. The hydrogen ion concentration of the gastric contents at the height of digestion of infants who are fed on whole citric acid milk averages 3.72 in the normal infant, and 4.15 in the abnormal infant.

3. The gastric curds at the height of digestion of infants fed on whole citric acid milk are uniformly small, the average individual curd being less than 1 mm. in diameter, and the largest being less than [illegible] mm. in diameter.

4. Citric acid milk is well liked by infants and never, in its use, causes persistent vomiting.

5. Infants fed on whole citric acid milk, both in the hospital and at home, gain weight with better than normal expectancy.

6. The stools of infants fed on whole citric acid milk are of a smooth pasty or firm consistency, and are somewhat more frequent in number than in infants fed on simple milk dilution mixtures.

7. Citric acid in the amounts recommended of 4 Gm. to a quart of milk is free from toxic action on the kidneys.

8. Consequently, since citric acid milk appears to equal lactic acid milk in the beneficial results, and because of its constant acceptability and ease of preparation, it is especially fitted for use in rural districts and in all homes in which ice cannot be obtained.

0850

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# A FLUORIMETRIC ENZYMIC METHOD FOR THE ASSAY OF MIXTURES OF ORGANIC ACIDS

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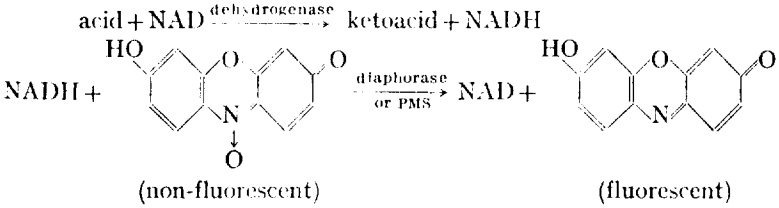
Most analytical methods for the determination of macro quantities (> 10 mg) of organic acids depend on the acidic properties of the carboxy group. Simple mixtures of organic acids that differ in acidity can be titrated potentiometrically in aqueous or nonaqueous solution. Many proposals have been made for the separation of acids by physical methods, *i.e.*, partition between immiscible solvents, azeotropic distillation and gas chromatography[1-4]. The latter does not yield satisfactory separations unless the acids are first esterified. There are no good analytical methods available for the direct analysis of several organic acids present in microconcentrations (0.1-100 $\mu$g) in a mixture.

Enzymic methods have been proposed for the determination of various single component organic acids, such as lactic, malic and glutamic. Surveys of these methods can be found in reviews by GUILBAULT[5,6], and a book by BERGMEYER[7].

Recently GUILBAULT AND KRAMER[8] described a simple, rapid fluorimetric method for measuring the activity of dehydrogenases. The method was based on the conversion of the non-fluorescent material, resazurin, to the highly fluorescent compound resorufin, in conjunction with the nicotinamide adenine dinucleotide-reduced nicotinamide adenine dinucleotide (NAD-NADH) system. An increase in sensitivity of 1-2 orders over conventional fluorimetric or colorimetric procedures involving NADPH or NADH formation was demonstrated.




In this study six dehydrogenase systems were used for the detection and determination of 21 organic acids. With malate, glutamate, $\beta$-hydroxybutyrate, isocitrate and lactate (types II and IV) dehydrogenases, coupled with NAD, phenazine methosulfate (PMS) and resazurin, procedures were developed for 0.1-500 $\mu$g of acetic, adipic, benzilic, butyric, D-$\alpha$- and D-$\beta$-hydroxybutyric, chloroacetic, DL-citric, formic, L-glutamic, glutaric, glycolic, threo-D-isocitric, L-lactic, L-malic, malonic, oxalic, phthalic, DL-succinic and L-tartaric acids, and mixtures thereof. The rate of production of the highly fluorescent compound, resorufin, is equated to the concentration

...id. An increase in sensitivity of at least one order of magnitude over fluori-... methods based on NADH or NADPH formation was obtained in the assay ... acids[6,7,9].

## EXPERIMENTAL

### *Reagents*

*Substrates. Resazurin (Eastman Organics Co., Rochester, N.Y.).* A stock $2 \cdot 10^{-4}$ *M* solution was prepared in methyl cellosolve. This solution should be non-fluorescent, with a bluish-red hue. It is stable indefinitely.

*Nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP)*, obtained from Calbiochem Co., were 86.3% and 70.1% pure, respectively. Stock $2 \cdot 10^{-4}$ *M* solutions were prepared in triply distilled water. If refrigerated these solutions are stable for one week.

*Phenazine methyl sulfate.* An aqueous stock $10^{-3}$ *M* solution of the purified substance (Sigma Chemical, St. Louis) is stable for 4 days if kept refrigerated in a dark bottle.

Solutions of all acids were prepared by dissolving the C.P. acid in triply distilled water to a concentration of 0.1 *M*. The solutions were adjusted to pH 7 with sodium hydroxide before use. All optically active acids were obtained from Sigma (St. Louis) and were the natural isomers in most cases: threo-D(+)-isocitric, L(+)lactic, L(+)malic, L(−)tartaric, L(+)glutamic, D-α- and D-β-hydroxybutyric. Citric acid (Sigma) was a DL-mixture. Other non-optically active acids (acetic, formic, butyric, etc.) were obtained in the highest available purity from Eastman (Rochester) or Sigma.

### *Enzymes*

All enzymes were assayed by standard conventional spectrophotometric methods, and the activities found are reported below.

*β-Hydroxybutyrate dehydrogenase, β-OH-BuDH (Boehringer Mannheim Corp., Rhodopseudomonas spheroides).* A 1 mg/ml solution was prepared in 33% ammonium sulfate. Activity = 3 units/mg (one unit is that amount which catalyzes the oxidation of 1 μmole of D-β-hydroxybutyrate per min at pH 7.3 and 37°).

*Glutamate dehydrogenase, GDH (Sigma Chemical, bovine liver, Type II).* A 1 mg/ml solution was prepared in triply distilled water. Activity = 2.7 units/mg (one unit catalyzes the oxidation of 1 μmole of L-glutamate at pH 7.6 and 25°).

*Lactate dehydrogenase, LDH (Sigma Chemical, Type II from rabbit muscle, activity 400 units/mg, and Type IV from Baker's yeast, 10 units/mg).* A 1 mg/ml solution of Type II was prepared in 33% ammonium sulfate; a 1 mg/ml solution of Type IV was prepared in water. Definition of units: one unit of Type II converts 1 μmole of pyruvate to lactate per minute at pH 7.5 and 37°; one unit of Type IV oxidizes 1.0 μmole of L-lactate to pyruvate at pH 8.4 and 37°.

*Malic dehydrogenase, MDH (Sigma, pig heart, Type I).* A 1 mg/ml solution was prepared in 33% ammonium sulfate, activity 50 units/mg. One unit converts 1 μmole of oxalacetate and β-NADH to malate and β-NAD at pH 7.5 and 25°.

*Isocitrate dehydrogenase, ICDH (Sigma, pig heart, Type IV).* A 1 mg/ml solution was prepared in water, activity 3 units/mg. One unit converts 1 μmole of

threo-D-isocitrate to α-ketoglutarate at pH 7.[illegible]

*Diaphorase (Type III, Sigma).* A solution was prepared by adding 1 ml of diaphorase to 10 ml with 33% ammonium sulfate, pH 7.7.

All enzyme solutions were stable for 2 days if refrigerated when not in use.

*Buffers*

*Tris buffer.* Tris(hydroxymethyl)aminomethane (Sigma). A 0.5 *M* solution, pH 9.5 and a 0.1 *M* solution, pH 7.0, were prepared. These solutions are stable indefinitely.

*Phosphate buffer, 0.1 M, pH 6.5.* This solution is stable indefinitely.

*Hydrazine-glycine buffer.* 1 *M* glycine-0.4 *M* hydrazine, pH 9.5. This solution is stable for 2 weeks.

*Apparatus*

All fluorescence measurements were made with an Aminco Bowman spectrophotofluorimeter (SPF), equipped with a thermoelectric cooler to maintain a constant temperature. The resorufin produced was measured at excitation and emission wavelengths of 560 and 580 nm. Although the excitation and emission wavelengths are close together, scattered radiation was not found to be a problem as was pointed out in a previous paper[8].

*Procedures*

*Determination of acids with GDH, LDH-II, MDH and β-OH-BuDH.* Place 1 ml of the acid to be determined (neutralized to pH 7) in a clean dry cuvette, then add 1 ml of glycine-hydrazine buffer (tris buffer for analysis of L-lactic acid with LDH and L-glutamic acid with GDH), pH 9.5, 0.1 ml of $2 \cdot 10^{-4}$ *M* NAD, 0.1 ml of $2 \cdot 10^{-4}$ *M* resazurin and 1 ml of $10^{-3}$ *M* phenazine methyl sulfate. (Diaphorase for benzilic acid.) Place the cuvette in the instrument, adjust the temperature to 30° and zero the instrument. Withdraw the cuvette, add 0.1 ml of the appropriate dehydrogenase (Table I), invert to mix, place the cuvette back in position, and record the change in fluorescence with time, $\Delta F$/min. Determine the concentration of acid present from a calibration plot of $\Delta F$/min *vs.* acid concentration.

*Determination of D-isocitric acid.* To 1 ml of a neutral solution of the threo-D-isocitric acid to be determined, add 1 ml of phosphate buffer, pH 6.5, 0.5 ml of magnesium chloride ($1 \cdot 10^{-2}$ *M*), 0.1 ml of NADP ($10^{-3}$ *M*), 0.1 ml of resazurin ($2 \cdot 10^{-4}$ *M*) and 1 ml of phenazine methyl sulfate (PMS) ($10^{-3}$ *M*). Adjust the temperature of the fluorimeter to 35° and zero the instrument. At time zero add 0.2 ml of a 0.3 mg/ml solution of isocitric dehydrogenase and record the change in fluorescence with time, $\Delta F$/min. The concentration of isocitric acid is calculated from a calibration plot as described above.

*Determination of L-lactic acid with LDH-IV.* To 1 ml of a solution of unknown L-lactic acid concentration (neutralized to pH 7) add 1.0 ml of tris buffer, pH 7.0, and 0.3 ml of resazurin ($2 \cdot 10^{-4}$ *M*). Adjust the fluorescence to read zero and adjust the temperature to 37°. Add 0.1 ml of a 22 mg/ml solution of LDH-IV and record the rate of change in fluorescence, $\Delta F$/min. The concentration of L-lactic acid present is calculated as described above.

*Determination of isocitric dehydrogenase.* The activity of isocitric dehydrogenase

ODS DETERMINABLE

| | Enzyme (buffer)[a] | Range (μg/ml) | Interferences |
|---|---|---|---|
| Acetic | MDH | 10–200 | Adipic (> 100 μg), benzilic, butyric, D-α-OH and β-OH-butyric (> 100 μg), chloroacetic (> 100 μg), DL-citric (> 150 μg), formic, glutaric (> 100 μg), glycolic, D-isocitric, L-malic, oxalic, phthalic, DL-succinic, D-tartaric (> 200 μg) |
| Adipic | MDH | 110–2500 | Same as acetic |
| Benzilic[b] | MDH | 5–500 | Same as acetic |
| Butyric | MDH | 10–250 | Same as acetic |
| D-α-Hydroxybutyric | MDH | 100–1500 | Same as acetic |
| | LDH-IV* (T,7) | 2–50 | L-Lactic acid |
| D-β-Hydroxybutyric | β-OH-BDH | 1–75 | DL-Citric (> 10 μg) |
| Chloroacetic | MDH | 100–2500 | Same as acetic |
| DL-Citric | MDH | 150–600 | Same as acetic |
| | LDH-II | 50–500 | L-Glutamic, glycolic, D-isocitric, L-lactic, L-malic (> 300 μg), oxalic |
| | GDH | 25–300 | L-Glutamic, glycolic, D-isocitric, L-lactic (> 100 μg), L-malic, oxalic (> 100 μg) |
| | β-OH-BDH* | 10–110 | D-β-OH-Butyric |
| Formic | MDH | 10–500 | Same as acetic |
| L-Glutamic | GDH (T, 9.5) | 1–100 | None[c] |
| Glutaric | MDH | 100–2500 | Same as acetic |
| Glycolic | GDH | 20–300 | Same as citric |
| | LDH-II* | 2–75 | Same as citric |
| | MDH | 20–400 | Same as acetic |
| D-Isocitric | ICDH* (P) | 0.02–1.0 | None |
| | GDH, LDH-II | 10–250 | Same as citric |
| | MDH | 20–400 | Same as acetic |
| L-Lactic | LDH-II (T, 9.5) | 4–175 | None[c] |
| | LDH-IV* (T,7) | 0.2–10 | D-α-OH-Butyric |
| L-Malic | GDH | 10–200 | Same as citric |
| | MDH* | 0.1–2.0 | Same as acetic |
| D-Malic | MDH | 50–800 | Same as acetic |
| Malonic | MDH | 20–550 | Same as acetic |
| Oxalic | MDH | 30–600 | Same as acetic |
| | LDH-II* | 5–100 | Same as citric |
| Phthalic | MDH | 50–1000 | Same as acetic |
| D-Tartaric | MDH | 200–3000 | Same as acetic |

GDH = glutamate dehydrogenase; LDH = lactate dehydrogenase; II = type II; IV = type IV; MDH = malic dehydrogenase; ICDH = isocitric dehydrogenase; β-OH-BDH = β-hydroxybutyric dehydrogenase.

[a] All glycine hydrazine buffer except as listed: T,7 = tris, pH 7; T, 9.5 = tris, pH 9.5; P = phosphate buffer, pH 6.5.

[b] Diaphorase used instead of phenazine methosulfate.

[c] Tris buffer, no hydrazine added.

* Best system for analysis.

can be determined under the conditions described [illegible] acid assay, except that a $10^{-3}$ $M$ isocitric acid concentration is used. The activity of enzyme can be calculated from a plot of $\Delta F$/min *vs.* enzyme concentration.

## DISCUSSION AND RESULTS

### *Specificity of enzyme systems*

Initial experiments were conducted to determine the specificity of various acid dehydrogenases. Some typical results obtained are indicated in Table II, which gives data on the relative rates of dehydrogenation (oxidation) of various acids using three different enzyme systems. The first two of these, lactic dehydrogenase, type II (rabbit muscle), and glutamate dehydrogenase (bovine liver) behave similarly with different acids, having no reaction with acetic, adipic, benzilic, butyric, D-α- and D-β-hydroxybutyric, chloroacetic, formic, glutaric, L-malic, malonic, phthalic, salicylic, DL-succinic, L-tartaric and amino acids (leucine, tryptophane, tyrosine, etc.). The order of decreasing reaction rate with LDH-II is glycolic acid > L-lactic

TABLE II

RELATIVE RATES[a] OF LACTIC, GLUTAMIC AND MALIC DEHYDROGENASE SYSTEMS

(Acid = 100 μg/ml; enzyme = 0.03 mg/ml)

| *Acid* | *LDH-II system*[b] | *GDH system*[c] | *MDH system*[d] |
|---|---|---|---|
| Acetic | 0 | 0 | 0.10 |
| Adipic | 0 | 0 | 0 |
| Benzilic | 0 | 0 | 0.12 |
| Butyric | 0 | 0 | 0.11 |
| D-α-Hydroxybutyric | 0 | 0 | 0.02 |
| D-β-Hydroxybutyric | 0 | 0 | 0.035 |
| Chloroacetic | 0 | 0 | 0.05 |
| DL-Citric | 0.03 | 0.04 | 0 |
| Formic | 0 | 0 | 0.15 |
| L-Glutamic | 0.16 | 0.35 | 0 |
| Glutaric | 0 | 0 | 0 |
| Glycolic | 0.53 | 0.12 | 0.18 |
| D-Isocitric | 0.16 | 0.18 | 0.23 |
| L-Lactic | 0.25 | 0.070 | 0 |
| L-Leucine | 0 | 0 | 0 |
| L-Malic | 0.01 | 0.13 | 0.25 |
| D-Malic | 0 | 0 | 0.052 |
| Malonic | 0 | 0 | 0.12 |
| Oxalic | 0.20 | 0.10 | 0.06 |
| Phthalic | 0 | 0 | 0.033 |
| Salicylic | 0 | 0 | 0 |
| DL-Succinic | 0 | 0 | 0.04 |
| D-Tartaric | 0 | 0 | 0 |
| Tryptophan, tyrosine | 0 | 0 | 0 |

[a] Expressed as $\Delta F$/min.
[b] Glycine–hydrazine buffer; with tris buffer only L-lactic acid reacts.
[c] Glycine–hydrazine buffer; with tris buffer only L-glutamic acid reacts.
[d] Glycine–hydrazine buffer.

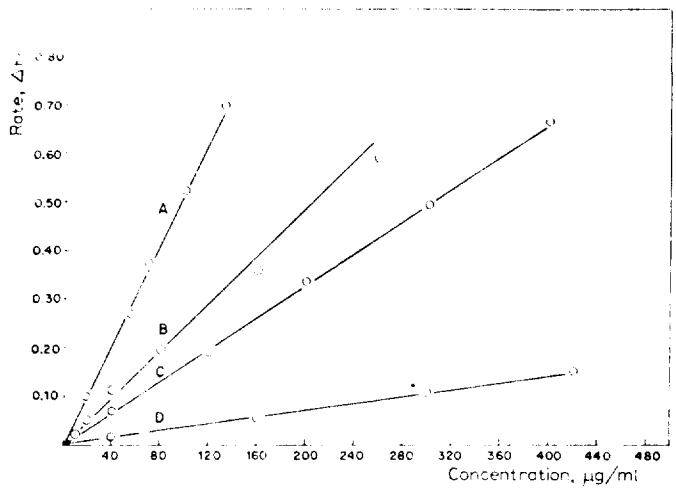



Fig. 1. Plot of $\Delta F$/min *vs.* concentration of organic acid. Lactate (H) dehydrogenase system. All conditions optimum. (A) Glycolic acid; (B) L-lactic acid; (C) D-isocitric acid; (D) DL-citric acid.

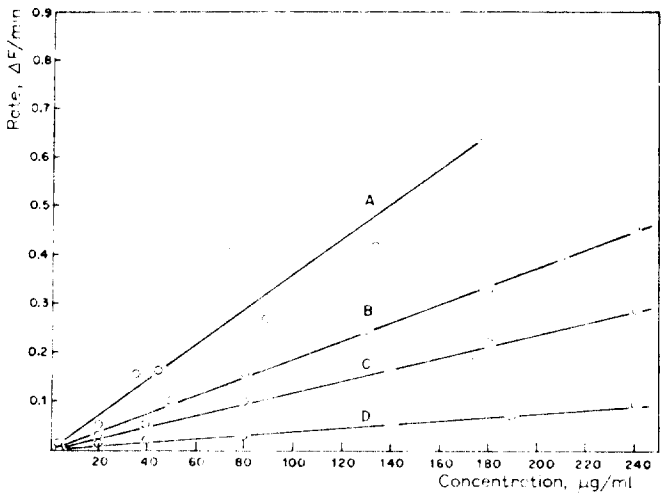
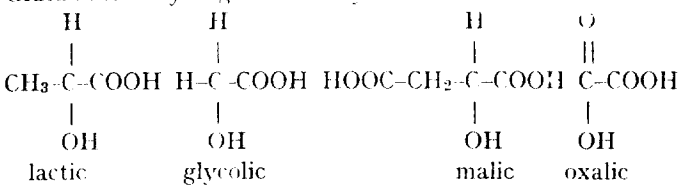



Fig. 2. Plot of $\Delta F$/min *vs.* concentration of organic acid. Glutamate dehydrogenase system. All conditions optimum. (A) L-Glutamic acid; (B) D-isocitric acid; (C) glycolic acid; (D) DL-citric acid.

acid > oxalic acid > L glutamic = D-isocitric acid > DL-citric acid > L-malic acid (Fig. 1 and Table II). Glycolic acid has a structure identical with lactic except for substitution of a hydrogen for a methyl group. The lack of specificity of lactate dehydrogenase is not surprising. MEISTER[10] has reported that a number of α-keto acids are reduced by rabbit muscle LDH: α-ketoglutamate, oxaloacetate, phenylpyruvate, α-ketobutyrate, α-ketocaproate, α-ketovalerate, etc.

Glutamate dehydrogenase catalyzes oxidation ...




rates of the order L-glutamic > D-isocitric > L-malic > glycolic > oxalic > L-lactic > DL-citric acid (Fig. 2 and Table II). If phenylhydrazine is not added to react with the α-keto acid formed and thus ensure a more complete reaction from hydroxy- to keto-acid, then only L-lactic acid reacts in the LDH-II system and only L-glutamic acid in the GDH system. Thus, with tris buffer of pH 9.5, L-lactic and L-glutamic acids can be specifically determined with their respective dehydrogenases. Malic dehydrogenase (MDH) is a less specific enzyme, and many acids can be determined with this dehydrogenase. Good rates were obtained with all acids regardless of the buffer used: tris, glycine, or glycine with hydrazine added (Fig. 3 and Table II).

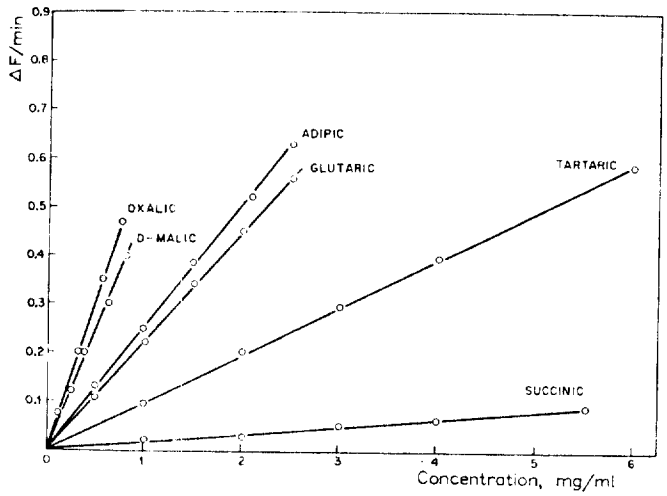



Fig. 3. Plot of $\Delta F$/min *vs.* concentration of organic acid. Malate dehydrogenase system. All conditions optimum.

Lactate dehydrogenase Type IV from Baker's yeast is an enzyme which does not require the use of NAD or NADH. Electron acceptors such as hexacyanoferrate-(II) or methylene blue can be used, and are directly reduced. Initial experiments indicated that resazurin is a good electron acceptor and is reduced to the fluorescent resorufin:

$$\text{L-lactate} + \underset{\text{(non-fluorescent)}}{\text{resazurin}} \xrightarrow{\text{type IV LDH}} \underset{\text{(fluorescent)}}{\text{resorufin}} + \text{pyruvate}$$

This enzyme is more specific than the type II; only L-lactic acid and D-α-hydroxybutyric acids react, two acids which are not generally found together. Greater

TABLE III

TYPICAL DETERMINATIONS OF ACIDS

| D-β-Hydroxybutyric acid[a] | | | L-Glutamic acid[b] | | | L-Lactic acid[c] | | |
|---|---|---|---|---|---|---|---|---|
| Added (μg/ml) | Found (μg/ml) | Rel. error (%) | Added (μg/ml) | Found (μg/ml) | Rel. error (%) | Added (μg/ml) | Found (μg/ml) | Rel. error (%) |
| 1.00 | 1.01 | + 1.0 | 5.00 | 4.95 | 1.0 | 0.500 | 0.510 | + 2.0 |
| 5.00 | 4.90 | 2.0 | 15.0 | 14.7 | 2.0 | 1.50 | 1.47 | − 2.0 |
| 10.0 | 10.0 | 0.0 | 25.0 | 25.0 | 0.0 | 3.0 | 3.08 | + 2.7 |
| 50.0 | 51.0 | + 2.0 | 75.0 | 77.0 | + 2.2 | 5.00 | 4.85 | − 3.0 |
| 75.0 | 74.0 | 1.5 | 100.0 | 102.0 | + 2.0 | 10.0 | 10.0 | 0.0 |
| Av. rel. error | | ± 1.3 | | | ± 1.4 | | | ± 1.9 |

[a] Analysis with β-hydroxybutyric dehydrogenase in the presence of 1 mg/ml of L-glutamic, L-lactic, L-malic and D-α-hydroxybutyric acids.
[b] Analysis with glutamate dehydrogenase in the presence of 1 mg/ml of acetic, D-tartaric and D-β-hydroxybutyric acids.
[c] Analysis with lactate dehydrogenase Type IV in the presence of 1 mg/ml L-malic, L-glutamic and D-β-hydroxybutyric acids.

sensitivity was found with the type IV enzyme, also. As little as 0.2 μg/ml of L-lactic acid can be determined with LDH IV compared to 4 μg/ml with the type II enzyme.

Isocitric dehydrogenase, Type IV from pig heart, is a highly specific enzyme; of all the acids tried only threo-D-isocitric acid reacted with this enzyme. Thus a completely specific analysis of D-isocitric acid is possible.

β-Hydroxybutyrate dehydrogenase from Rhodopseudomonas spheroides is also a highly specific enzyme. Of all the acids tried (those listed in Table II) only D-β-hydroxybutyric and DL-citric acids reacted and were determinable. Even the D-α-hydroxy acid and butyric acid do not react. Since D-isocitric acid does not react, a specific method for DL-citric acid in the presence of D-isocitrate is possible.

Table I lists all the acids that can be determined by means of these 6 enzyme systems together with the range of concentrations determinable, and the interferences. An asterisk indicates the best enzyme system for analysis. Some typical results for the analysis of acids are shown in Table III. With β-hydroxybutyrate dehydrogenase, 1–75 μg/ml of D-β-hydroxybutyrate were analyzed in the presence of 1 mg/ml concentrations of L-glutamic, L-lactic, L-malic and D-α-hydroxybutyric acids with an accuracy of ±1.3% and a precision of 2%. DL-Citric acid is the only interference (Table I), being determinable in the range 10–110 μg/ml with a precision and accuracy of about 2%.

L-Glutamic acid, in concentrations of 5–100 μg/ml, was determined with a precision and accuracy of about 1.5% in the presence of acetic, D-tartaric and D-β-hydroxybutyric acids. No interferences can be anticipated if tris buffer, pH 9.5, is used. Higher rates are observed with glycine hydrazine buffer, however, and if the interfering acids (Table I) are known to be absent, analysis should be performed with this buffer system.

With lactate dehydrogenase, type IV, 0.4–10 μg/ml of L-lactic acid were determined in the presence of 1 mg/ml of L-malic, L-glutamic and D-β-hydroxybutyric acids with a precision and accuracy of 1.9%. Only D-α-hydroxybutyric acid interferes. With type II LDH, 4–175 μg/ml of L-lactic acid can be specifically determined in the presence of all acids including D-α-hydroxybutyric.

0850



TABLE IV

ANALYSIS OF A MIXTURE OF CITRIC, ISOCITRIC, LACTIC AND GLUTAMIC ACIDS

| Added (μg/ml) | | | | Found (μg/ml) | | | |
|---|---|---|---|---|---|---|---|
| DL-Citric | D-Isocitric | L-Lactic | L-Glutamic | DL-Citric | D-Isocitric | L-Lactic | L-Glutamic |
| 0.0 | 1.00 | 10.0 | 10.0 | 10.1 | 1.00 | 9.90 | 10.2 |
| 0.0 | 1.00 | 5.00 | 50.0 | 10.0 | 1.01 | 5.10 | 49.2 |
| 0.0 | 1.00 | 10.0 | 50.0 | 9.9 | 0.980 | 10.1 | 51.0 |
| 0.0 | 0.500 | 10.0 | 50.0 | 49.2 | 0.495 | 10.0 | 51.0 |

| Rel. error (%) | | | |
|---|---|---|---|
| DL-Citric | D-Isocitric | L-Lactic | L-Glutamic |
| + 1.0 | 0.0 | − 1.0 | − 2.0 |
| 0.0 | + 1.0 | + 2.0 | − 1.6 |
| 1.0 | − 2.0 | + 1.0 | + 2.0 |
| − 1.0 | − 1.0 | 0.0 | + 2.0 |

TABLE V

ANALYSIS OF ISOCITRIC DEHYDROGENASE

| Enzyme (units/ml) | | Rel. error (%) |
|---|---|---|
| Added | Found | |
| 0.000100 | 0.000103 | + 3.0 |
| 0.000789 | 0.000781 | − 1.0 |
| 0.00789 | 0.00799 | + 1.2 |
| 0.0158 | 0.0162 | + 2.7 |
| 0.0307 | 0.0300 | − 2.3 |
| 0.0075 | 0.0060 | − 2.2 |
| Av. rel. error | | ± 2.1 |

A four-component acid mixture of DL-citric acid, D-isocitric acid, L-lactic acid and L-glutamic acid was analyzed, and the results are given in Table IV. The sample was divided into 4 equal parts, and analysis of each acid performed with a different dehydrogenase: β-hydroxybutyrate dehydrogenase for DL-citric acid, isocitric dehydrogenase for D-isocitric acid, lactate dehydrogenase Type II for L-lactic acid and glutamate dehydrogenase for L-glutamic acid. A phosphate buffer, pH 6.5, was used for ICDH, a glycine–hydrazine buffer (pH 9.5) for β-OH-BuDH and tris buffer (pH 9.5) for LDH and GDH. Essentially the same precisions and accuracies were obtained for the analysis of the mixture (Table IV) as were obtained in the analysis of the acid alone (Table III).

Finally a fluorimetric method was developed for the sensitive analysis of isocitrate dehydrogenase (Table V). As little as $10^{-4}$ units to 0.0675 unit of enzyme can be assayed by an initial reaction-rate method with a precision and accuracy of about 2%.

*Effect of pH, buffer and other reagents*

The lactate II, malate, glutamate and β-hydroxybutyrate dehydrogenase systems all work in the pH range 6–10. At low pH the α-keto acid is reduced to the

...oxy acid; at high pH the equilibrium is shifted in favor of formation of the keto

$$\text{hydroxy acid} \xrightleftharpoons{\text{dehydrogenase}} \text{x-keto acid} \xrightarrow{\text{hydrazine}} \text{hydrazone}$$

In order to use these dehydrogenase systems for the determination of the various hydroxy acids a pH of 9.5 was used in all determinations with either tris or glycine–hydrazine buffer. In general, higher rates were obtained with glycine–hydrazine, due to reaction of the x-keto acid with hydrazine to form a hydrazone. Some typical results obtained in the malate dehydrogenase system with various acids are given in

TABLE VI

EFFECT OF BUFFER ON DETERMINATION OF VARIOUS ACIDS WITH MALIC DEHYDROGENASE

(Acid = 0.35 mg/ml; enzyme = 0.03 mg/ml, pH = 9.5)

| *Acid* | *Rate (ΔF/min)* | | |
|---|---|---|---|
| | *Tris* | *Glycine* | *Glycine–hydrazine* |
| L-Malic | 0.68 | 0.75 | 0.86 |
| L-Tartaric | 0.12 | 0.10 | 0.15 |
| Acetic | 0.12 | 0.14 | 0.16 |
| Oxalic | 0.20 | 0.22 | 0.26 |
| Adipic | 0.23 | 0.25 | 0.30 |

TABLE VII

VALUES OF THE MICHAELIS CONSTANTS FOR ENZYME–SUBSTRATE SYSTEMS

| *Substrate* | *Enzyme*[a] | $K_m$ *(mM)*[b] |
|---|---|---|
| D-Isocitric | ICDH | $5 \cdot 10^{-3}$ |
| L-Lactic | LDH-II | 2.1 |
| | LDH-IV | 0.30 |
| D-x-Hydroxybutyric | LDH-IV | 1.0 |
| Glycolic | LDH-II | 1.5 |
| D-Isocitric | LDH-II | 3.0 |
| L-Glutamic | GDH | 1.7 |
| D-Isocitric | GDH | 15.0 |
| Glycolic | GDH | 52.0 |
| D-β-Hydroxybutyric | β-OH-BDH | 1.5 |
| DL-Citric | β-OH-BDH | 3.0 |
| L-Malic | MDH | 0.030 |
| Formic | MDH | 25.0 |
| Chloroacetic | MDH | 50.0 |
| Butyric | MDH | 10.1 |
| Oxalic | MDH | 10.0 |
| Acetic | MDH | 6.6 |
| L-Tartaric | MDH | 20.0 |
| D-Isocitric | MDH | 7.5 |
| Glycolic | MDH | 9.0 |

[a] GDH = glutamate dehydrogenase; LDH = lactate dehydrogenase; II = type II; IV = type IV; MDH = malic dehydrogenase; ICDH = isocitrate dehydrogenase; β-OH-BDH = β-hydroxybutyrate dehydrogenase.

[b] Determined under optimum conditions as described in the text.

Table VI. With this enzyme system all acids reacted in [illegible] of glycine-hydrazine buffers, the rates being higher in the latter. With lactate and glutamate dehydrogenase only L-lactate and L-glutamate reacted in tris buffer. The rates of oxidation of these acids were higher in glycine-hydrazine, but several other acids were also oxidized in this buffer system, thus reducing the specificity.

The optimum pH values and buffer systems for the isocitrate and lactate IV dehydrogenase systems were pH 6.5 phosphate and pH 7.0 tris, respectively. Hydrazine was not needed in these two enzyme systems.

Phenazine methyl sulfate (PMS) and diaphorase are both good "coenzymes" that facilitate the transfer of a hydrogen atom in the fluorescence indicator reaction. PMS was used instead of diaphorase in all the dehydrogenase systems for the determination of all acids except benzilic which worked only with diaphorase. Comparable rates were obtained with PMS and diaphorase with all the other acids determined. PMS has two advantages over diaphorase, stability and cost. Solutions of PMS can be used for up to two weeks; diaphorase solutions must be prepared daily. Costwise, PMS is about 1/100 as expensive as diaphorase.

*Michaelis constants and reaction products*

The Michaelis constants for all the organic acids for each dehydrogenase system used were determined from standard Lineweaver–Burk plots of $1/V_0$ *vs.* $1/[S]$. Some of these constants are reported in Table VII. In all cases lower $K_m$ values were found than have been previously reported in the literature because of the greater sensitivity achieved in the fluorimetric resorufin procedure. This is undoubtedly due, in part, to the shift in the equilibrium of the primary enzyme–substrate reaction by the hydrazone formation and by the resazurin–resorufin indicator reaction. Thus a $K_m$ value of $5 \cdot 10^{-6}$ *M* for the D-isocitrate–isocitrate dehydrogenase system compares with a value of $2.5 \cdot 10^{-2}$ *M* obtained by the NADPH fluorimetric assay method. A $K_m$ value of $3.0 \cdot 10^{-5}$ *M* was obtained for L-malic with malic dehydrogenase compared with a value of $5.5 \cdot 10^{-5}$ *M* obtained in previous studies[9].

Finally, attempts were made to identify the products of the oxidation of the organic acids. Gas chromatography, gas adsorption, thin-layer chromatography and other techniques were used. In the case of the α-hydroxy acids, the α-keto acid was the main product. In the case of citric, isocitric and glycolic acids, carbon dioxide was identified as an additional product. The main oxidation product from the non-hydroxy acids, formic, acetic, butyric, oxalic, etc., was carbon dioxide.

Although some of the results described on the "non-specificity" of some enzyme systems, *i.e.* malic and glutamic, are in disagreement with other biochemical studies[9], we believe that definite evidence has been given in proof that many other organic acids are catalytically oxidized by enzymes that are believed to be highly specific. Reproducible calibration plots of rate *vs.* concentration of these acids are presented (Figs. 1–3), Michaelis constants are calculated and the reaction products were identified. It can probably be concluded that, as other extremely sensitive assay procedures, such as the fluorescence method developed in this paper, become available, more detailed and meaningful information on enzyme specificity will result.

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SUMMARY

Rapid fluorimetric methods are described which involve six enzyme systems for the determination of mixtures of 21 organic acids. Lactate (Types II and IV), malate, glutamate, isocitrate, and β-hydroxybutyrate dehydrogenases are used coupled with NAD, phenazine methosulfate and resazurin, in a fluorimetric procedure for the determination of acetic, adipic, benzilic, butyric, D-α- and D-β-hydroxybutyric, chloroacetic, DL-citric, formic, L-glutamic, glutaric, glycolic, threo-D-isocitric, L-lactic, L-malic, malonic, oxalic, phthalic, DL-succinic and L-tartaric acids in the approximate concentration range of 0.1–500 μg with an accuracy and precision of about 2%. The rate of production of the highly fluorescent resorufin is equated to the concentration of the acid.

RÉSUMÉ

On décrit des méthodes fluorimétriques avec enzymes pour le dosage de mélanges d'acides organiques. On utilise des déhydrogénases de lactate (types II et IV), malate, glutamate, isocitrate et β-hydroxybutyrate, avec NAD, phénazine métosulfate et résazurine, pour le dosage des acides acétique, adipique, benzylique, butyrique, D-α- et D-β-hydroxybutyrique, chloroacétique, DL-citrique, formique, L-glutamique, glutarique, glycolique, threo-D-isocitrique, L-lactique, L-malique, malonique, oxalique, phtalique, DL-succinique et L-tartrique, à une concentration de 0.1 à 500 μg avec une exactitude et une précision d'environ 2%. La vitesse de production de la résorufine (fluorescence) est fonction de la concentration de l'acide.

ZUSAMMENFASSUNG

Es wird eine schnelle fluorimetrische Methode beschrieben, die enzymatische Systeme zur Bestimmung von Mischungen von 21 organischen Säuren verwendet. Lactat, Malat, Glutamat, Isocitrat und β-Hydroxybutyrat-Dehydrogenase werden verwendet zusammen mit NAD, Phenazinmethosulfat und Resazurin in einem fluorimetrischen Verfahren zur Bestimmung der verschiedensten Säuren. Im Konzentrationsbereich von etwa 0.1–500 μg betragen die Reproduzierbarkeit und Richtigkeit etwa 2%. Die Bildungsrate des sehr stark fluoreszierenden Resorufins ist etwa gleich der Konzentration der Säure.

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# THE CITRIC ACID CONTENT OF BLOOD PLASMA AND TISSUES OF THE DOMESTIC FOWL

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**Abstract**—1. Changes with age in the citric acid content of the blood plasma, bone and soft tissues of the domestic fowl were studied.

2. Plasma citric acid levels were consistently lower in males than in females from hatching up to the age of puberty. At hatching the mean values for males and females were 6·01 and 6·56 mg/100 ml and at 5 months of age 7·77 and 8·19 mg/100 ml respectively. In females a marked fall in the plasma level to a mean value of 2·79 mg/100 ml occurred at sexual maturity, probably due to the secretion of oestrogen.

3. No marked changes associated with age, sex or reproductive activity occurred in the citric acid content of the liver, kidney, heart, brain, pancreas or lung. Mean values for these tissues from laying birds were, respectively, 10·8, 14·2, 14·2, 47·2, 66·7 and 50·7 mg/100 g fresh tissue.

4. Certain sections of the oviduct, notably the isthmus and shell gland, were extremely rich in both citric acid (up to 400 mg/100 g fresh tissue) and in calcium (up to 110 mg/100 g).

5. The citric content of dry fat-free cortical bone remained fairly constant from hatching to maturity at a level of approx. 1 per cent, but the calcium content of the bones increased with age from 22 to more than 26 per cent in adults.

6. Medullary bone generally contained more than 40 mg citric acid/g calcium compared with values of between 30 and 40 mg/g calcium for the corresponding cortical bone, but in laying birds of the White Leghorn breed the mean value was only 25 mg citric acid/g calcium.

## INTRODUCTION

The distribution of citric acid in the tissues of a number of mammalian species has been reviewed by Thunberg (1953), but little is known about its concentration in the tissues of the fowl. Accordingly, it was felt desirable to study the level of citric acid occurring naturally in the blood, bones and major organs of the fowl at various ages from hatching till maturity.

## EXPERIMENTAL

Most of the experimental birds were Rhode Island Red × Light Sussex (R.I.R. × L.S.) but some White Leghorns (W.L.), L.S. × R.I.R. and pure R.I.R.'s

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were also used. All birds were kept in cages except for the 1 2-year-old cocks which were on free-range. Birds of the following ages were studied: 1 day, 1, 2, 3 and 4 weeks, 2, 3, 4, 5 and 6 months and 1 2 years. Plasma of 3 4-year-old non-laying hens was also analysed. In addition, embryos of 18 days of age were investigated. The chicks were fed on commercial chick-starter crumbs from 2 days up to 8 weeks of age and thereafter on grower's pellets. The 1-day-old chicks were not fed. Two weeks or so before the pullets came into lay, they received a normal laying ration (120 g day).

Blood was collected into heparinized tubes either from the jugular vein when the birds were killed or from a wing vein when they were spared, usually in the morning. It was immediately centrifuged in a refrigerator centrifuge at 5 C for 5 min. To 2 ml of plasma in a 15 ml centrifuge tube was added 10 ml of 10% (w/v) trichloroacetic acid. The mixture was stirred well with a glass rod and left standing for approximately 30 min, after which it was centrifuged or filtered. Filtrates thus obtained provided sufficient material for duplicate determinations which were carried out by the method of Taylor (1953) with the following modifications: (a) 5 ml of 20% (w/v) ferrous ammonium sulphate in N sulphuric acid was used to destroy the excess bromine, (b) the sodium sulphide reagent was made up in a mixture of ethylene glycol (previously redistilled under reduced pressure) and water (4 : 1 v/v). This reagent produces a more stable yellow colour than the aqueous sodium sulphide, giving maximum intensity after approximately 45 minutes (Carlsson & Hollunger, 1954).

Tissues were dissected out as quickly as possible and placed in the freezing compartment of a refrigerator pending extraction. Samples (ca. 3–5 g) were homogenized with two portions of 5 ml trichloroacetic acid (10% w/v) in a Potter-Elvehjem glass homogenizer, and filtered through a Whatman No. 42 filter paper. Citric acid was determined on the combined filtrates.

Bones (tibia and femur) were prepared for analysis as described by Taylor & Moore (1954). In the age groups of 1 day, 7 and 14 days, the bones of five, three and two birds respectively were pooled to provide sufficient material for analysis. The bones of three female chicks aged 1 month were also bulked. In every other age group the analyses were carried out on separate samples from each bird. Fifty mg of dry fat-free bone powder was dissolved in 50 ml of 0·1 N hydrochloric acid. After filtration citric acid was determined in a 5 ml portion of the filtrate. Weighed samples were ashed in an electric furnace at 800° for the determination of calcium in bones and oviduct. The ash was dissolved in 0·5 N HCl and, after the removal of interfering phosphate on an ion-exchange resin (Lapidus & Mellon, 1958), calcium was determined as described by Hertelendy & Taylor (1961).

## RESULTS

### *Blood*

Plasma samples from 235 birds were analysed and the results are summarized in Table 1. Females showed consistently higher values than males up to the age of puberty. This difference was evident even at 1 day of age, and analysis of

variance (Table 2) showed that this difference was significant ($p < 0{\cdot}001$). The plasma citric acid levels of both sexes remained fairly constant up to the age of 2 months, but there was a marked rise between 2 and 3 months of age and the higher values were maintained until the birds reached the age of 5 months. The

TABLE 1—THE CITRIC ACID CONTENT OF BLOOD PLASMA OF MALE (M) AND FEMALE (F) CHICKENS OF DIFFERENT AGES. VALUES ARE MEANS ± STANDARD ERROR

| Age | Sex | No. of birds | Citric acid (mg/100 ml) |
|---|---|---|---|
| 1 day | F | 23 | 6·56 ± 0·27 |
| | M | 21 | 6·01 ± 0·60 |
| 7 days | F | 12 | 6·05 ± 0·12 |
| | M | 8 | 5·90 ± 0·56 |
| 2 weeks | F | 5 | 6·18 ± 0·43 |
| | M | 5 | 5·98 ± 0·57 |
| 3 weeks | F | 4 | 6·19 ± 0·72 |
| | M | 5 | 6·05 ± 0·47 |
| 4 weeks | F | 6 | 6·74 ± 0·56 |
| | M | 7 | 6·42 ± 0·38 |
| 2 months | F | 11 | 6·71 ± 0·56 |
| | M | 8 | 6·29 ± 0·62 |
| 3 months | F | 7 | 8·05 ± 0·44 |
| | M | 7 | 7·39 ± 0·50 |
| 4 months | F | 5 | 7·91 ± 0·88 |
| | M | 4 | 7·53 ± 0·09 |
| 5 months | F | 12 | 8·19 ± 0·33 |
| | M | 5 | 7·77 ± 0·42 |
| 6 months | F* | 14 | 2·79 ± 0·25 |
| | M | 4 | 9·45 ± 1·07 |
| 1–2 years | F* | 44 | 2·79 ± 0·29 |
| | M | 12 | 12·21 ± 0·50 |
| 3–4 years | F† | 6 | 9·10 ± 0·85 |

* Laying. † Non-laying.

observations of Taylor *et al.* (1960) on the drastic fall in plasma citric acid levels associated with reproductive activity in females were confirmed. The citrate content of the plasma of mature cocks, especially of those which had regular exercise, was considerably higher than that of immature birds. It was shown that the plasma citric acid of old, non-laying hens was similar to that of adult cocks.

*Main organs*

The results for the citric acid contents of the major organs are summarized in Table 3. The values for certain organs, particularly the pancreas, were very variable, and no sex differences were apparent. The lowest citrate concentration was found in the liver, the highest in the lungs. The citrate content of several

samples of skeletal muscle was similar to that of liver. Apart from the somewhat higher content of citric acid in the kidneys and hearts of embryos and day-old chicks as compared with older birds, there were no consistent differences associated with age. No significant changes accompanied reproductive activity, and the liver, pancreas, lungs and brain of embryos, day-old and growing chicks contained essentially the same amounts of citric acid as those of laying hens.

TABLE 2.—ANALYSIS OF VARIANCE OF THE PLASMA CITRATE LEVELS OF CHICKENS OF BOTH SEXES FROM HATCHING TO 5 MONTHS OF AGE

| Source of variation | Sums of squares | Degrees of freedom | Mean squares (variance) | F (variance ratio) |
|---|---|---|---|---|
| Sex | 5,832 | 1 | 5,832 | 36·8* |
| Age | 102,877 | 8 | 16,096 | 101·6* |
| Error | 1,267 | 8 | 158·4 | |
| Total | 109,976 | 17 | | |

* Significant at 0·1 per cent level of probability.

*Oviduct*

When the oviducts of laying hens were analysed it was found that the isthmus and, to a lesser extent, the uterus were considerably richer in citric acid than any other tissue examined, with the exception of the skeleton, and a brief report of this work has already been published (Taylor & Hertelendy, 1960). The distal sections of the isthmus contained between 300 and 400 mg/100 g fresh weight, i.e. about a hundred times as much as the brain or three to four hundred times as much as the liver. Furthermore, a close correlation was observed between the citric acid and calcium contents of the isthmus and uterus of laying hens ($r = +0{\cdot}97$). The regression line and its equation calculated from the analytical data are shown in Fig. 1. In another experiment the citric acid content of the entire isthmus of pre-laying pullets, i.e. birds which already possessed a well-developed oviduct but which had not begun to lay, was compared with those of laying hens, and the data are presented in Table 4. When citrate values were expressed on the basis of the nitrogen content of the fresh tissues, no consistent differences were found between pre-laying pullets and laying hens. The actual amounts of both citric acid and nitrogen, expressed as mg/100 g fresh tissue, were, however, lower in the pre-laying birds.

*Bone*

Mean percentage of citric acid and calcium in the dry fat-free bones of the experimental birds are presented in Table 5. The citric acid content of the cortical bone remained fairly constant at a level of approximately 1 per cent from hatching until the onset of reproduction. No difference was observed between sexes in the

TABLE 5. AVERAGE CITRIC ACID CONTENT OF THE MAIN ORGANS OF THE DOMESTIC FOWL AT VARIOUS AGES. RANGES OF VALUES OBTAINED ARE SHOWN UNDER THE MEANS. WHERE ONLY TWO DETERMINATIONS WERE MADE BOTH VALUES ARE SHOWN, SEPARATED BY A COMMA

| Age | Sex | No. of birds | Citric acid (μg/g/ wet tissue) Liver | Kidney | Heart | Brain | Pancreas | Lung |
|---|---|---|---|---|---|---|---|---|
| Embryo | F | 5* | 8·9 | 66·2 | 41·6 | 34·5 | — | 35·2 |
| 18 days | M | 5* | 7·5 | 44·7 | 43·7 | 32·3 | — | 27·5 |
| | F | 15 | 10·8 (8·4–12·1) | 83·5 (70·0–97·8) | 77·9 (68·5–84·5) | 47·3 (41·7–56·4) | 127·8 | 81·7 (67·5–98·3) |
| 1 day | M | 15 | 8·0 (7·2–9·0) | 71·2 (62·5–76·8) | 57·0 (54·6–60·8) | 45·1 (43·3–46·8) | 52·8 | 77·8 (68·2–82·9) |
| | F | 6 | 13·5 (10·2, 16·7) | 27·7 (25·8, 29·6) | 21·6 (19·5, 23·7) | 29·0 (27·0, 31·0) | 30·1 (28·7, 31·5) | 75·5 (72·9, 78·1) |
| 1 week | M | 3* | 16·9 | 25·8 | 30·1 | 37·1 | 50·5 | 83·4 |
| | F | 2 | 8·7 (6·8, 10·6) | 22·6 (20·6, 24·5) | 13·5 (12·2, 14·8) | 28·4 (23·9, 32·9) | 53·3 (52·2, 54·3) | 55·4 (47·2, 63·5) |
| 2 weeks | M | 2 | 14·7 (11·9, 17·5) | 31·6 (26·8, 36·4) | 26·3 (24·5, 28·0) | 23·3 (21·6, 24·9) | 80·4 (76·2, 84·5) | 65·2 (60·4, 70·0) |
| | F | 2 | 10·0 (9·2, 10·7) | 9·3 (8·0, 10·6) | 13·1 (10·4, 15·7) | 23·8 (21·7, 25·9) | 19·5 (17·2, 21·7) | 48·4 (53·8, 42·9) |
| 1 month | M | 2 | 12·7 (10·2, 15·1) | 12·1 (11·9, 12·3) | 14·7 (12·0, 17·3) | 25·1 (22·7, 27·4) | 26·7 (21·5, 31·8) | 57·9 (55·2, 60·6) |
| 3 months | F | 3 | 11·9 (4·7–19·3) | 15·9 (8·5, 23·3) | 13·1 (11·7–14·1) | 53·4 (23·2–98·8) | 83·4 (25·7–117·0) | 61·3 (47·4–78·0) |
| Laying hen | F | 5 | 10·8 (8·4–15·3) | 14·2 (9·0–20·1) | 14·2 (10·5–19·8) | 47·2 (33·7–64·3) | 66·7 (54·6–86·9) | [illegible] |
| Mature cock | M | 1 | 20·4 | 26·8 | 13·1 | 35·7 | 29·1 | [illegible] |

* Denotes pooled samples.

0957

early age groups. The calcium content of the bones increased steadily during this period, so that the citric acid : calcium ratio fell. No consistent change in the citric acid content of the cortical bone was observed with the onset of reproductive activity and the citric acid : calcium ratios for pre-laying birds of different breeds all fell within the range 30–40 mg citric acid/g calcium.

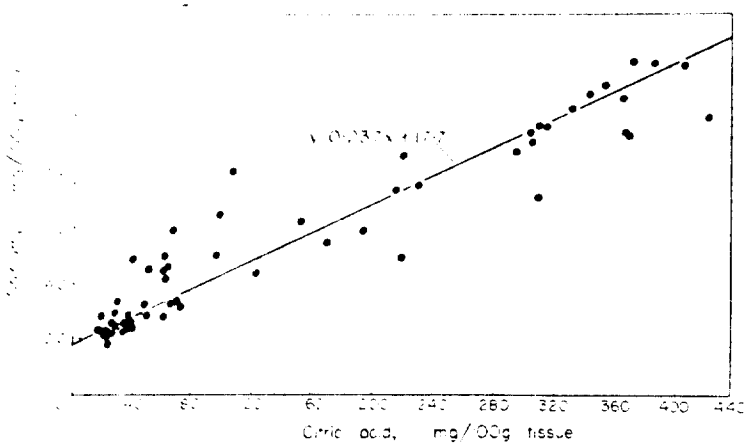


FIG. 1. The relationship between the concentrations of calcium and citric acid in the isthmus and uterus of laying hens. Each point represents data from a single segment of tissue.

In pre-laying birds the medullary bone was relatively richer in citric acid than the cortical bone from the same bird. The same was true of laying birds except in the case of the W.L. breed. The relative concentration of citric acid in the medullary bone of W.L. hens was only 25·3 mg citric acid/g calcium compared with a figure of 45·1 in pre-laying birds of the same breed and a value of a similar order for the medullary bone of laying birds of heavy breeds.

TABLE 4—CITRIC ACID AND NITROGEN CONTENT OF THE ENTIRE ISTHMUS OF PRE-LAYING AND LAYING PULLETS. VALUES ARE MEANS OF DUPLICATE DETERMINATIONS

| Bird No. | Reproductive state | Breed* | Wt. of isthmus (g) | Citric acid mg/100 g fresh tissue | Nitrogen (mg/g) fresh tissue | mg citric acid/g N |
|---|---|---|---|---|---|---|
| 2238 | Pre-laying | WL | 5·043 | 188·1 | 29·9 | 62·9 |
| 2247 | Pre-laying | WL | 4·684 | 183·9 | 27·8 | 66·2 |
| 239 | Laying | WL | 4·699 | 219·9 | 31·8 | 69·1 |
| 240 | Laying | WL | 4·265 | 195·0 | 31·9 | 61·1 |
| 6 | Laying | LS × RIR | 6·858 | 220·4 | 31·4 | 70·2 |
| 31 | Laying | LS × RIR | 7·723 | 212·4 | 31·7 | 67·0 |

* WL—White Leghorn; LS × RIR—Light Sussex × Rhode Island Red.

TABLE 5—MEAN PERCENTAGES ± STANDARD ERROR OF CALCIUM AND CITRIC ACID IN THE DRY FAT-FREE BONES (FEMUR AND/OR TIBIA) OF BIRDS OF DIFFERENT AGES AND BREEDS. CORTICAL BONE WAS TAKEN FROM THE SHAFTS. MEDULLARY BONE IS THE SECONDARY BONE FORMED UNDER THE INFLUENCE OF SEX HORMONES AND OCCUPYING THE MARROW CAVITIES OF THE BONES OF FEMALE BIRDS DURING THE PERIOD OF REPRODUCTION. ALL BIRDS WERE FEMALES UNLESS OTHERWISE STATED

| Age | Breed* | No. of birds | Calcium % | | Citric acid % | | mg citric acid/g calcium | |
|---|---|---|---|---|---|---|---|---|
| | | | Cortical | Medullary | Cortical | Medullary | Cortical | Medullary |
| 1 day† | RIR × LS | 30 | 22·15 ± 0·65 | — | 1·06 ± 0·01 | — | 47·2 ± 1·52 | — |
| 7 days† | RIR × LS | 12 | 22·41 ± 0·23 | — | 1·00 ± 0·01 | — | 44·1 ± 0·91 | — |
| 14 days† | RIR × LS | 4 | 22·98 ± 0·26 | — | 0·99 ± 0·04 | — | 43·2 ± 0·52 | — |
| 1 month‡ | RIR × LS | 3 | 23·75 | — | 1·05 | — | 44·2 | — |
| 3 months | RIR × LS | 4 | 23·35 ± 0·35 | — | 0·97 ± 0·01 | — | 37·4 ± 1·03 | — |
| 4 months | RIR × LS | 4 | 26·01 ± 0·40 | — | 1·02 ± 0·01 | — | 39·1 ± 0·79 | — |
| 5–6 months (immature) | WL | 2 | 26·46 ± 0·27 | — | 0·99 ± 0·03 | — | 37·4 ± 0·71 | — |
| | LS × RIR | 6 | 25·60 ± 0·59 | — | 0·89 ± 0·18 | — | 34·6 ± 2·00 | — |
| 5–7 months (pre-laying) | WL | 4 | 26·89 ± 0·27 | 26·22 ± 0·68 | 0·83 ± 0·02 | 1·18 ± 0·04 | 31·0 ± 0·84 | 45·1 ± 1·46 |
| | LS × RIR | 12 | 25·73 ± 0·11 | 23·36 ± 0·02 | 0·95 ± 0·04 | 0·98 ± 0·00 | 36·9 ± 1·50 | 41·9 ± 1·02 |
| 6–12 months (laying) | WL | 4 | 26·83 ± 0·70 | 24·02 ± 1·34 | 0·84 ± 0·04 | 0·62 ± 0·09 | 31·3 ± 0·97 | 25·3 ± 2·50 |
| | LS × RIR | 5 | 26·22 ± 0·33 | 24·60 ± 0·85 | 0·95 ± 0·14 | 1·01 ± 0·01 | 36·1 ± 2·50 | 41·1 ± 1·80 |

* RIR—Rhode Island Red; LS—Light Sussex; WL—White Leghorn.
† Chicks of both sexes in equal number.
‡ One pooled sample.

0957

## DISCUSSION

The level of citric acid in the plasma was shown to be consistently higher in female birds than in male birds until the age of puberty. Freeman *et al.* (1961) observed slightly but consistently higher plasma citrate levels in female rats than in males of similar age groups. These authors have pointed out that factors which control citric acid concentration in the circulation are not clearly established.

The average citric acid level for mature breeding cocks was markedly higher than that observed in caged birds. This may have been due to the fact that the former birds took regular exercise in pens, for it is known that physical work tends to raise the citrate level in serum (Thunberg, 1953).

The observation of Taylor *et al.* (1960) that a substantial drop in the concentration of plasma citrate of female birds occurs at the time of the onset of reproduction was confirmed, and it was shown in addition that this relatively low level of citric acid is maintained throughout the period of active reproduction. Old birds that had ceased to lay showed plasma citrate levels similar to those observed in mature cocks. It is not possible to offer, at this stage, any satisfactory explanation at a biochemical level for the striking fall in the concentration of citric acid coincident with reproduction in female birds. It is known that administration of female sex hormones lowers the serum citrate level (Common *et al.*, 1948; Hertelendy & Taylor, 1962) and the fall with the onset of laying is probably related to endogenous oestrogen activity. Plasma citrate values of immature birds in the present experiment agreed well with those reported by Bell & Campbell (1961) for their control groups, but the values for adult cocks were lower than those observed by Urist *et al.* (1958). Various workers (Hill *et al.*, 1960; Hill *et al.*, 1961; Hill, 1961) have studied the citrate levels in the whole blood of young chicks under normal and various experimental conditions. However, since neither haematocrit values nor blood cell citrate concentrations were reported by these authors, their values for normal controls cannot be compared directly with the values obtained in the present investigation.

The results for the citric acid contents of the various soft tissues showed no marked differences that could be associated with age, sex or reproductive activity. Variations in the plasma levels, therefore, are not reflections of variations in the tissue concentrations of the various organs. Great differences (from about 2 to 100 p.p.m.) between the citrate contents of different organs (brain, muscle, liver, kidney) of mammalian species have also been reported in the literature (Thunberg, 1953).

The tissue citrate levels observed in the present experiment are in good agreement with the values published by Buffa & Peters (1949) for pigeon tissues. Hill *et al.* (1960) found considerably higher citrate concentrations in chicken liver than those reported in this communication, viz. 42·2 $\mu$g/g in fed and 131 $\mu$g/g in starved birds. The effect of starvation was not investigated in the present work.

The physiological significance of the exceptionally high level of citric acid in the isthmus is not clear. The shell membranes are laid down in this section of the oviduct, and unpublished observations from this Department suggest that the

0957



formation of the mammillary knobs may be initiated in the distal part of this region. The main process of shell deposition, however, takes place in the uterus, which contains very much less calcium and citric acid. It is possible that the high concentrations of calcium and citric acid in the isthmus play a role in the primary calcification of the mammillae, which then act as nuclei for the subsequent calcification of the shell in the shell gland.

Levels of citric acid in the cortical bone remained virtually constant from hatching up to the age of 5 months in spite of the fact that the level of plasma citrate increased during this period. The percentage of calcium in the bone increased with age so that the citric acid : calcium ratio decreased. Citric ions are thought to occur on the surface of the apatite crystals (Hendricks & Hill, 1950; Taylor, 1960), and these observations are consistent therefore with the view that there was an increase in the size of the individual bone crystals with age.

With the exception of laying hens of the White Leghorn breed, the medullary bone of sexually mature birds was richer in citric acid than cortical bone. Since the plasma citric acid level is falling in the pre-laying pullet during the time that the medullary bone is being laid down it is fairly certain that the high citric acid content of this bone is associated with local factors rather than with the level of citric acid in the circulation. Vaes & Nichols (1961) observed that oestradiol increased the concentration of citric acid in the bones of mice by 35 per cent over the control animals, and it is possible that the higher citrate content of the medullary bone, laid down under the influence of gonadal hormones (Gardner & Pfeiffer, 1942), may be due to the increased production of citric acid by the bone cells under the influence of oestrogen. Whether the additional citric acid was associated with the mineral phase of the bone or in the form of a citrate-peptide complex (Hartles & Leaver, 1960; Leaver *et al.*, 1960) was not determined.

Another possible explanation for the differences in the citric acid content of medullary and cortical bone is that the amounts of citric acid present are related to the size of the bone crystals, high citrate concentrations being associated with small crystals. On this hypothesis the apatite crystals in the medullary bone of laying White Leghorns would be expected to be larger than in pre-laying birds of the same breed or in birds of other breeds at any time.

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0957



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# THE REGULATION OF GASTRIC EMPTYING OF MEALS CONTAINING CITRIC ACID AND SALTS OF CITRIC ACID

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It is known that when test meals containing hydrochloric acid are given, the greater the concentration of acid in the meal the slower is gastric emptying (Shay & Gershon-Cohen, 1934; Van Liere & Sleeth, 1940; Pathak, 1959; Hunt & Pathak, 1960). This slowing of gastric emptying is believed to depend upon the stimulation of duodenal receptors by the duodenal contents recently transferred from the stomach. The present studies are concerned with the rates of emptying of test meals containing citric acid and the various sodium salts of this trivalent acid. These were chosen for study because they allow the preparation of solutions with a variety of pH values and with various degrees of buffering power which can be used to decide on the relative importance of these two variables in exciting the receptors concerned.

## METHODS

The subjects were four volunteer students and HT. Test meals were given down a tube at 37° C to the fasting subjects after their stomachs had been washed out with 250 ml. of tap water, which was recovered as completely as possible before the meal was given. All meals contained about 60 mg phenol red/l. to act as a marker. Phenol red is not absorbed or secreted by the stomach (for references see Hunt, 1947). After an interval, constant for each subject but varying between 9 and 19 min, the meal was withdrawn through the tube; when this recovery appeared to be complete 250 ml. tap water was given and this portion was aspirated separately. The amount of phenol red in this second aspirate allowed the volume recorded for the first recovery to be corrected for incompleteness. When a recovery began 9 min after giving the meal, the meal was recorded as of 10 min duration to allow for the time required to recover the gastric contents. Other times were similarly adjusted by 1 min.

The majority of the test meals were made up from appropriate amounts of citric acid and sodium citrate. Test meals containing ammonium citrate or ammonium chloride were made up with the corresponding salts.

*Estimation of phenol red.* Ten millilitres of the test-meal solution or of the recovered gastric contents and 50 ml. of a solution of $Na_3PO_4$ (27·5 g/l.) were pipetted into a 250 ml. flask which was then filled up to the mark with distilled water. The mixture had a pH of about 11–12. The optical density of the purple solution so produced was estimated in a single cell photo-electric colorimeter with a green filter (Ilford 404). The concentration of phenol red was then determined from a curve relating concentration of phenol red to optical density. The green filter used minimized the effect of bile.



*Expression of results.* In each instance the volume of the original meal recovered from the stomach was calculated from the expression

$$\text{Volume gastric contents} \times \frac{\text{concentration phenol red in gastric contents}}{\text{concentration phenol red in original meal}}.$$

For each subject and each solute a plot was made of volumes of meal remaining in the stomach after a fixed interval against the concentration of the solute in the given meal. A line drawn through these points was usually straight to the eye over part of the range of concentrations of each solute: for this part of the findings a straight line was fitted by the method of least squares. Data for the regression equations of these lines are given in Table 1. The lines relating the volume of meal remaining, on the ordinate, to concentration of citric acid and salts of citric acid over the ranges studied, are shown in Fig. 1. The curved parts of the lines were drawn by eye through the mean values for several observations. When the volume of the meal recovered was in excess of about 550 ml. further increase in concentration of solute did not always proportionately increase the volume of meal recovered.

TABLE 1. The effect of citric acid and salts of citric acid and ammonium chloride in a test meal on the volume of a 750 ml. meal remaining in the stomach after a fixed interval

| | JS | JN | SW | BY | HT |
|---|---|---|---|---|---|
| Age | 22 | 19 | 22 | 21 | 44 |
| Weight (kg) | 72 | 59 | 63 | 70 | 59 |
| Height (cm) | 175 | 168 | 180 | 175 | 175 |
| Total no. of tests | 170 | 100 | 138 | 113 | 151 |
| Time (min) | 10 | 10 | 15 | 15 | 20 |
| Citric acid | | | | | |
| $a$ | 368 | 413 | 291 | 277 | 268 |
| $b \pm$ S.E. | 1·31 ± 0·16 | 1·45 ± 0·098 | 2·20 ± 0·55 | 2·79 ± 0·42 | 1·59 ± 0·25 |
| $R$ (no.) | 10–200 (9) | 10–150 (8) | 10–150 (9) | 25–120 (7) | 10–200 (14) |
| *Dihydrogen sodium citrate* | | | | | |
| $a$ | 368 | 358 | 315 | 301 | 191 |
| $b \pm$ S.E. | 0·68 ± 0·19 | 1·28 ± 0·33 | 1·01 ± 0·13 | 1·15 ± 0·13 | 1·41 ± 0·14 |
| $R$ (no.) | 45–300 (17) | 30–150 (13) | 15–300 (36) | 30–300 (16) | 30–270 (11) |
| *Hydrogen disodium citrate* | | | | | |
| $a$ | 322 | 179 | 124 | 248 | 66 |
| $b \pm$ S.E. | 0·24 ± 0·15 | 1·34 ± 0·18 | 1·17 ± 0·17 | 0·98 ± 0·15 | 1·01 ± 0·14 |
| $R$ (no.) | 45–300 (23) | 75–300 (20) | 75–300 (20) | 45–300 (23) | 100–400 (23) |
| *Trisodium citrate* | | | | | |
| $a$ | 169 | 275 | 110 | 251 | 83 |
| $b \pm$ S.E. | 0·31 ± 0·12 | 0·25 ± 0·13 | 0·50 ± 0·07 | 0·23 ± 0·12 | 0·52 ± 0·12 |
| $R$ (no.) | 100–500 (29) | 100–500 (12) | 100–500 (23) | 100–500 (28) | 100–500 (26) |
| *Ammonium citrate* | | | | | |
| $a$ | 354 | 376 | — | — | 262 |
| $b \pm$ S.E. | 0·36 ± 0·50 | 0·014 ± 0·036 | — | — | 1·22 ± 0·60 |
| $R$ (no.) | 0–100 (14) | 0–75 (15) | — | — | 0–50 (15) |
| *Ammonium chloride* | | | | | |
| $a$ | 323 | 357 | — | — | 237 |
| $b \pm$ S.E. | 0·019 ± 0·29 | 0·047 ± 0·035 | — | — | 0·41 ± 0·45 |
| $R$ (no.) | 0–70 (23) | 0–90 (8) | — | — | 0–100 (13) |

Volume of meal remaining ($y$ ml.) plotted against concentration of solute ($x$ m-equiv./l.) $y = a + bx$, where $a$ corresponds to the volume calculated to remain with zero concentration of solute, that is, the intercept of the regression line and the ordinate. $R$ (no.) = Range of concentrations (number of experiments).



1017

For this reason the calculated regression lines were extended at high concentrations to cover only the linear part of the relationship between concentration of solute and volume of meal recovered.

## RESULTS

The data for the five subjects taking meals of citric acid and its sodium salts are shown in Fig. 1. The durations of the tests, which were constant for each subject, were adjusted to give about 300–400 ml. of recovered meal when it was pure water. This is of importance in between-subject comparisons of the effects of the solutes on gastric emptying. Had there been, say, only 100 ml. of test meal leaving the stomach with no solute in the meal it would have been difficult to assess quantitatively any decrease in the rate of emptying.

There are some features common to all five subjects:

(1) Initially as the concentration of trisodium citrate in the meal was raised the volume of meal remaining after a fixed interval fell until a concentration of between 50 and 100 millinormal (mN) was reached. Thereafter at higher concentrations of trisodium citrate the volume recovered increased.

(2) A similar pattern of response was seen with hydrogen disodium citrate, but the concentration of citrate ion giving minimal volume of meal recovered was less than with the trisodium salt.

(3) In general the increase in volume of meal recovered per increase in concentration of citrate ion was greatest for citric acid, followed by dihydrogen sodium citrate, hydrogen disodium citrate and trisodium citrate, in that order.

(4) For reasons which will appear later, some similar studies were made with ammonium citrate and ammonium chloride. It may be seen from Table 1 that over the range of concentrations studied, 50–100 mN, there was no significant increase in the volume of the meal recovered as the concentration of solute in the original meal was raised.

The results for the volume of meal remaining in HT with concentrations of hydrogen disodium citrate less than 100 mN present a special feature, namely a hump in the curve at about 50 mN. The mean volume remaining with 30 mN solutions was 171 ml. (S.E. ± 12, 7 observations), whilst at 45 mN the mean volume was 239 ml. (S.E. ± 16, 16 observations), the difference being 68 ml. (S.E. ± 20), which is statistically highly significant. When all the results for volume of meal remaining with concentrations between 60 and 100 mN hydrogen disodium citrate were plotted, the slope of the line was −1·59 (S.E. ± 0·22; 22 observations). Taking these two statistical tests together, it is very unlikely that the hump in the curve for hydrogen disodium citrate for HT is a chance affair. None of the other irregularities in the curves in Fig. 1 was statistically significant in spite of many repetitions of the tests.

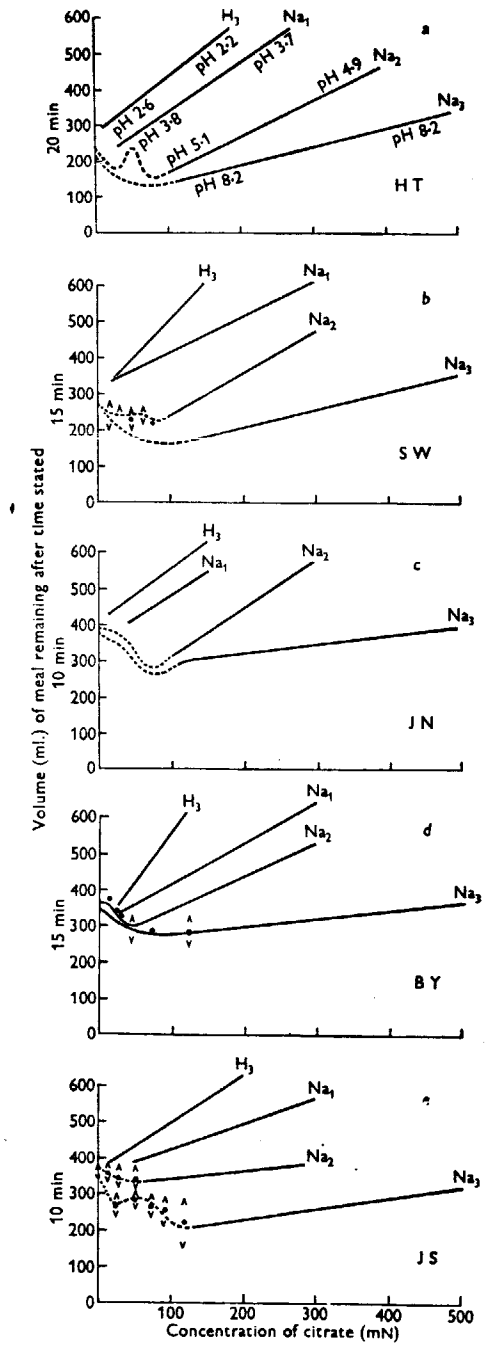


**Fig. 1.** Volumes of meal remaining in the stomach after a fixed interval plotted against concentration of solute in meal. $H_3$, citric acid; $Na_1$, dihydrogen monosodium citrate; $Na_2$, monohydrogen disodium citrate; $Na_3$, trisodium citrate. Ordinates, volume (ml.) of meal remaining at time stated; abscissae, concentration of solute (mN).

1017

## DISCUSSION

As a result of a previous study of gastric emptying with test meals containing various electrolyte and non-electrolyte solutes (Hunt & Pathak, 1960) it was suggested that gastric emptying was slowed by a duodenal osmoreceptor. The osmotic pressure of the duodenal contents, recently transferred from the stomach, was regarded as reducing the flux of water from the duodenum into a sensitive vesicle which, being thus affected, gave rise to a signal which slowed gastric emptying by reducing the vigour of gastric peristalsis. The response to the majority of solutes was a degree of slowing directly proportional to the concentration in the test meal, but with low concentrations of sodium chloride or sodium bicarbonate the rate of gastric emptying was increased relative to that of water. However, at concentrations above about 200 mN these salts slowed gastric emptying in proportion to their concentration. To explain these facts it was suggested that sodium ions moved into the sensitive vesicle by some special mechanism susceptible of saturation, chloride and bicarbonate and water following in the interest of electrical and osmotic equilibrium: these solutes in concentrations up to the saturation level therefore caused the sensitive vesicle to swell, thus decreasing the inhibitory signal which was postulated to slow emptying even with meals of water.

There was some experimental evidence consistent with the view that chloride ions did passively enter the postulated osmoreceptive vesicle. Over the range of concentration from 0–70 to 100 mN the rate of emptying of test meals containing ammonium chloride was independent of the concentration of solute. As ammonium ion and chloride ion are known to penetrate the red-cell membrane, the ammonium in the form ammonia, it seemed possible that it and the chloride ion both penetrated the sensitive vesicle of the receptor and thus had no osmotic action. On the other hand, ammonium sulphate in test meals, unlike ammonium chloride, slowed gastric emptying. This distinction was ascribed to the non-penetration of the sulphate ion into the receptor, with a corresponding limitation of movement of ammonium. Consistent with this, sodium sulphate was effective at lower concentrations than sodium chloride in slowing gastric emptying, presumably because of the postulated non-penetration of the sulphate ion into the sensitive vesicle of the osmoreceptor.

The present series of results shows a consistent difference between the effects of these four solutions containing citrate ion upon the emptying of the stomachs of the five subjects. The differences might be caused by

(1) the variations of the concentration of the three ions of citrate in the meals;

(2) the variations of the sodium concentration in the meals;



(3) the variations of the pH of the meals; or

(4) the variations of the concentration of titratable acid in the meals.

In the results from the present experiments the response to trisodium citrate was similar to that previously found for sodium chloride. The response to ammonium citrate was similar to that for ammonium chloride, in that the volume of meal recovered was independent of concentration of solute. It is therefore inferred that the citrate ion with three negative charges penetrates the osmoreceptor in a manner similar to the chloride ion in this context. That is to say, it passes freely and exerts no osmotic effect on the duodenal receptor. Thus the variation in concentration of citrate ion cannot be considered as directly responsible for the effect upon gastric emptying. With solutions of disodium citrate, monosodium citrate and citric acid the citrate ions will on an average be carrying less than three negative charges, with the possible implication that these ions will penetrate at least as readily as those carrying three charges. If this were so, the different actions of the various salts could not be attributed to the simple osmotic actions of the ions.

The results for test meals containing ammonium chloride or ammonium citrate have a limitation. There is a concentration, which varies from subject to subject, above which the meals are nauseating. When nausea occurs in these subjects gastric emptying is slowed. Such results and those for higher concentrations have been excluded from consideration because they have caused nausea, but the decision as to the occurrence of nausea is a difficult one to make. For this reason the experiments may possibly be considered biased in terms of producing results consistent with the view that ammonium chloride and citrate do not slow gastric emptying. The conclusion about meals containing ammonium chloride and ammonium citrate has, therefore, to be that they do not influence gastric emptying over a rather narrow range of concentration.

Concerning the nausea, it might be suggested that the sensation arises as a result of the slowing of gastric emptying or that the sensation arises and the slowing of gastric emptying occurs as a product of the sensation. This second hypothesis is probably preferable, since fats and sugars can cause considerable slowing of gastric emptying without co-existent nausea, whereas much less slowing of gastric emptying by ammonium chloride or ammonium citrate is associated with nausea. This does not, of course, necessarily exclude the first hypothesis from consideration.

It is clear from Fig. 1 that an increase in the concentration of citrate ion as the dihydrogen sodium salt is more effective in slowing gastric emptying than the same increase in concentration of citrate ion as the trisodium salt. This cannot be attributed simply to the differences in concentration of

1017

sodium ion, since the concentrations of sodium ion are, of course, less for equivalent concentrations of the monohydrogen and dihydrogen citrates than they are for trisodium citrate. With sodium citrate the maximal rate of emptying is at about 50–100 mN sodium ion; with dihydrogen sodium citrate the concentration of sodium is 100 mN when the total concentration of citrate is 300 mN, which can be seen from Fig. 1 to give a considerable slowing of gastric emptying relative to water. Thus the major variations in emptying cannot be ascribed to the sodium ion.

It is also unlikely that the pH of the test meal is the factor deciding the rate of emptying. With dihydrogen sodium citrate a change in concentration from 50 mN, pH 3·8, to 250 mN, pH 3·7, produces in the results of HT an increase in volume recovered from 260 to 540 ml., i.e. 280 ml. for a change of 0·1 pH unit. A change from 250 mN hydrogen disodium citrate, pH 5·0, to 250 mN dihydrogen sodium citrate, pH 3·7, a change of 1·3 pH units, is associated with an increase in volume recovered from 320 to 540 ml., that is, 220 ml., which is less than that produced for a change of 0·1 pH units with dihydrogen sodium citrate.

As an alternative to some osmoreceptor system directly sensitive to a wide range of concentrations of constituents in the duodenal lumen, there is the possibility of a system involving the action of the pancreatic and duodenal secretions in neutralizing acid. It might be suggested that when as a result of the entry of gastric contents the pH of the duodenal contents fell below, say, pH 6, effective gastric propulsive activity would cease as a result of the stimulation of a duodenal receptor. The acid should cause the release of secretin and the pancreas and Brunner's glands would secrete bicarbonate. The acid in the duodenum would be neutralized and the inhibition of gastric emptying would stop. A further portion of gastric contents would be transferred to the duodenum and the process would repeat itself.

With such a system the rate of emptying of very acid test meals would depend upon the rate of secretion of bicarbonate by the pancreas and duodenum, the amount of acid transferred from the stomach to the duodenum being equal to the rate of secretion of bicarbonate. Thus, as meals with successively higher concentrations of acid were given, a fall in the volume transferred to the duodenum in unit time should result in a constant rate of delivery of acid to the duodenum.

With such a system the rate of transfer from the stomach to the duodenum of solutions of weak acids and strong acids of equal titratable acidity should be similar and within limits independent of the pH of the solution. The data on the gastric emptying of solutions of citric acid, dihydrogen sodium citrate and of hydrogen disodium citrate are suitable for testing this hypothesis, since the respective pH values of the solutions



are about 2·4, 3·7 and 5·0, a range which should be sufficient to show up any dependence of gastric emptying on the pH of the gastric contents.

To test the view that the rate of gastric emptying is such as to deliver a fixed amount of 'titratable acid' to the duodenum during the test, it is necessary to postulate some threshold for the receptor detecting the acidity of the duodenal contents. The findings of Thomas & Crider (1940) may be relevant: their threshold for the release of secretin was not below pH 5·0.

From the present results for hydrogen disodium citrate it appears that the receptor mechanism adjusts its response to the concentration of solute at pH values above 5. As a first approach an assumption that the threshold for the inhibition of gastric emptying is effectively pH 6 has been found to explain the present results, as will be shown below.

Titration curves for various concentrations of citric acid against sodium hydroxide were made, and from these the titratable acidity up to pH 6·0 in m-equiv/l. of any of the meals containing citrate could be determined. From the data of Fig. 1 the volume of the test meal remaining for a particular concentration of solute was determined. This value, subtracted from the initial volume of the meal, 750 ml., gave the volume of the meal transferred to the duodenum in the standard time. The volume transferred multiplied by the titratable acid to pH 6·0 for the particular meal gave the amount of titratable acid transferred to the duodenum in the standard time. The results of such calculations for the meals containing citric acid, dihydrogen sodium citrate or hydrogen disodium citrate are shown in Fig. 2. For comparative purposes the results are all shown as amount of transfer in 15 min, those from meals lasting 10 min or 20 min having been corrected by multiplying by 3/2 or 3/4, respectively. It may be seen that the steady rate of transfer of titratable acid to the duodenum was between 20 and 30 m-equiv/15 min. It was substantially independent of whether titratable acid was in the form citric acid (pH about 2·4), sodium dihydrogen citrate (pH about 3·6) or hydrogen disodium citrate (pH about 5·0). These results are therefore incompatible with the direct control of gastric emptying by the pH of the gastric contents acting in the duodenum, but consistent with the concept of control somehow related to the amount of titratable acid transferred to the duodenum. If the threshold of the supposed duodenal receptors is set at pH 5 instead of pH 6, it is found that the calculated transfer to the duodenum of acid titratable to pH 5 is greater for the meals containing citric acid or monosodium dihydrogen citrate than it is for meals of disodium monohydrogen citrate. Only when the threshold of the receptor is set at about pH 6 can the results be explained on the present hypothesis. It may be seen that in the results for JS there is evidence that the steady rate of transfer of acid in the form

1017

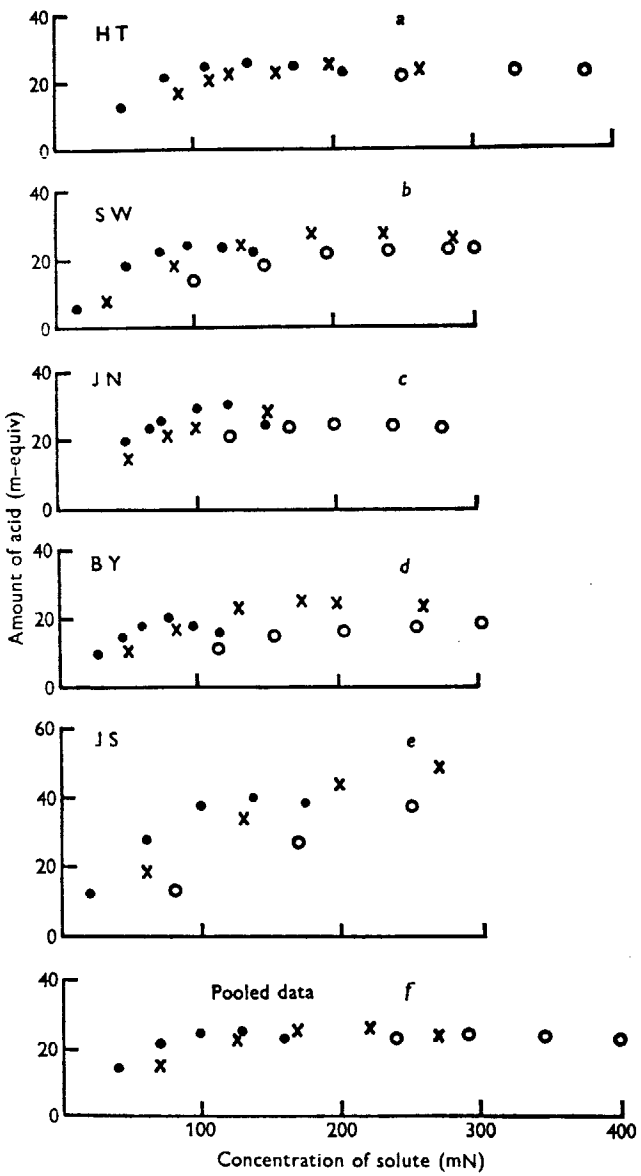


Fig. 2. Amounts of titratable acid, measured to pH 6, transferred to the duodenum in 15 min. ●, citric acid; ×, dihydrogen monosodium citrate; ○, monohydrogen disodium citrate. Ordinates, amount of titratable acid (m-equiv) leaving stomach in 15 min; abscissae, concentration of solute (mN). *f* shows pooled results.



dihydrogen sodium citrate or hydrogen disodium citrate was not reached, possibly because the duration of tests in this subject was only 10 min.

The gastric contents transferred to the duodenum contain not only the acid given by mouth, but the acid secreted by the stomach. Results on this point were obtained for these meals. The maximal amount secreted in any subject was about 5 m-equiv in the period of the test. About a quarter or less of this acid was transferred to the duodenum during the test. Thus even in the subject who secreted the most acid an error of only about 1·25 m-equiv is introduced into the calculation of the amount of acid transferred to the duodenum by neglecting the acid secreted by the stomach. In the other subjects the error is less.

Because the rates of emptying of solutions containing ammonium chloride or ammonium citrate were independent of the concentration over a limited range of these salts, it was concluded tentatively that the citrate ion and the chloride ion passed freely into the hypothetical osmoreceptor. However, this conclusion must raise a reservation about the similarity between the membrane of the osmoreceptor and the red cell, since red cells are not haemolysed in solutions of ammonium citrate. It would not be expected that a relatively large hydrated ion such as the citrate ion would penetrate as freely as chloride. However, comparison of results for HT in Hunt & Pathak (1960) and in the present paper (Table 1) is consistent with the view that chloride and citrate as sodium salts have very little action on the hypothetical osmoreceptor over a wide range of concentrations. There is therefore nothing to suggest that the gastric emptying of titratable acid with the test meals of dihydrogen sodium citrate is limited by the presence of citrate ion. As may be seen from the pooled results of Fig. 2, the general statement that the transfer of an acid meal from the stomach to the duodenum with citric acid and with salts of citric acid is independent of the pH of the meal, is well founded.

The views advanced here fit into a more comprehensive scheme for the analysis of the control of gastric emptying of acid solutions proposed by Lagerlöf, Rudewald & Perman (1960). In their experiments the volume of the gastric contents was more or less constant at 250 ml. and the transfer of acid to the duodenum was about half that calculated from the present data, with pH 6 as the end-point. They were able to show that the rate of bicarbonate secretion into the duodenum was linearly related to the amount of acid entering the duodenum from the stomach. They gave grounds for thinking that the maximal concentration of bicarbonate in the duodenal secretions is about 120 mN. Our subject JS was calculated to have transferred about 40 m-equiv of acid to the duodenum in 15 min, which would require more than 1 l. of duodenal secretions for neutraliza-

1017

tion, which is just about feasible (Wilhelmj, Sachs, Slutzky & Barak, 1950). However, Lagerlöf *et al.* (1960) found evidence of diffusion of hydrogen ion through the duodenal wall which might be relevant in our context. It is also noteworthy that in JS the tests lasted only 10 min, so that at the time of recovery there may have been a quantity of acid in the duodenum not yet neutralized, which would tend to exaggerate the amount of neutralization occurring per unit time in the duodenum.

In an earlier study of SW, JS and HT (Hunt & Pathak, 1960) with solutions containing various concentrations of sodium chloride and 20 mx hydrochloric acid there was in each instance a curve similar in shape to that for HT with hydrogen disodium citrate. It is not clear why the humped curve is seen only with HT in the present study, but it presumably depends upon a combination of a particular pH and concentration of sodium ion in the lumen of the duodenum.

## SUMMARY

1. Test meals of 750 ml. of solutions of citric acid, dihydrogen sodium citrate, hydrogen disodium citrate and trisodium citrate at various concentrations were given by tube to five subjects. Three of the subjects also received meals of ammonium chloride and ammonium citrate. The meals, which contained phenol red, were recovered after an interval fixed for each subject. The volume of original meal recovered was determined by using the amount of phenol red recovered as the index of the volume of original meal remaining in the stomach.

2. The rate of transfer of acid, measured by titration to pH 6·0, reached a steady rate for each subject. The rate of emptying of the gastric contents was independent of the pH of the gastric contents.

3. It is suggested that a receptor in the duodenum is excited when the duodenal contents are more acid than pH 6·0 and inhibits gastric emptying. The rate of gastric emptying is therefore set by the pancreas and duodenum, which neutralize the acid entering from the stomach.

4. The relations between gastric emptying and the concentrations of sodium citrate in the meal were similar to those for sodium chloride, the rate of emptying with concentrations up to 100 millinormal being more rapid than the emptying of water.

5. The rate of emptying of solutions of ammonium citrate and of ammonium chloride was independent of concentration. It is concluded that the citrate ion has little influence on receptors regulating gastric emptying.

It is a pleasure to acknowledge the co-operation and persistence of the students who were subjects in these experiments. I am indebted to Miles Laboratories Inc., Elkhart, Indiana for a generous supply of citric acid and its salts.



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1017

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# STUDIES ON THE METABOLISM OF BONE MARROW
## VI. INFLUENCE OF CITRIC, FUMARIC, MALIC AND PYRUVIC ACID ON THE OXYGEN CONSUMPTION AND THE GLUCOSE METABOLISM OF THE BONE MARROW*

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In a previous paper (1) the influence of succinic acid on the oxygen consumption and glucose metabolism of bone marrow was reported. In the experiment to be reported in this paper, citric, fumaric, malic and pyruvic acid were examined in the same way as in the previous experiment.

## EXPERIMENTAL METHOD

Experimental material: Bone marrow tissue was taken from the adult rabbit treated in the same way as in the preceding experiments (2, 3).

Malic acid and pyruvic acid were neutralized before use. As citric acid preparation, sodium citrate was used.

## EXPERIMENT

*I. Citric acid* (Table I, Fig. 1)

The concentrations used were $10^{-3}$ mol, $10^{-2}$ mol and $2 \times 10^{-2}$ mol in the final concentration. $Qo_2$ was slightly increased (10 %) by $10^{-2}$ mol and decreased (10 %) by $2 \times 10^{-2}$ mol.

TABLE I. *Influence of various citrate concentrations on $Qo_2$ and Glucose metabolism of bone marrow tissue. Average of 5 exp.*

| | $Qo_2$ | | | | | | Glucose metabolism during 130 min. | | |
|---|---|---|---|---|---|---|---|---|---|
| | I 30 | II 30 | I 60 | II 30 | IV 30 | II 60 | G. C. ($\times 10^{-3}$ mg) | P. A. ($\times 10^{-3}$ mg) | L. A. ($\times 10^{-3}$ mg) |
| a) $10^{-3}$ mol Citrate | 2.10 ±0.15 | 2.72 ±0.15 | 2.91 ±0.15 | 2.54 ±0.13 | 2.37 ±0.13 | 2.46 ±0.13 | 37.06 ±1.94 | 1.81 ±0.18 | 34.00 ±1.54 |
| b) $10^{-2}$ mol Citrate | 3.24 ±0.17 | 2.96 ±0.14 | 3.15 ±0.15 | 2.56 ±0.15 | 2.20 ±0.15 | 2.38 ±0.14 | 37.77 ±2.56 | 1.95 ±0.13 | 36.49 ±1.95 |
| c) $2 \times 10^{-2}$ mol Citrate | 2.61 ±0.09 | 2.21 ±0.12 | 2.41 ±0.10 | 2.05 ±0.10 | 1.93 ±0.09 | 1.99 ±0.09 | 34.38 ±2.46 | 1.92 ±0.14 | 38.48 ±0.86 |
| d) Control | 2.88 ±0.14 | 2.66 ±0.16 | 2.78 ±0.14 | 2.38 ±0.15 | 2.27 ±0.15 | 2.32 ±0.15 | 34.16 ±2.37 | 1.60 ±0.14 | 31.52 ±0.99 |
| a/d | 1.08 ±0.03 | 1.03 ±0.03 | 1.05 ±0.02 | 1.09 ±0.06 | 1.07 0.10 | 1.08 ±0.06 | 1.11 0.06 | 1.12 0.04 | 1.08 ±0.05 |
| b/d | 1.17 ±0.06 | 1.13 ±0.06 | 1.15 ±0.06 | 1.11 0.09 | 0.98 0.02 | 1.04 ±0.06 | 1.13 0.08 | 1.24 0.06 | 1.15 ±0.04 |
| c/d | 0.92 ±0.04 | 0.81 ±0.03 | 0.88 ±0.03 | 0.88 ±0.04 | 0.88 ±0.05 | 0.88 ±0.05 | 1.01 0.05 | 1.24 ±0.10 | 1.23 ±0.04 |

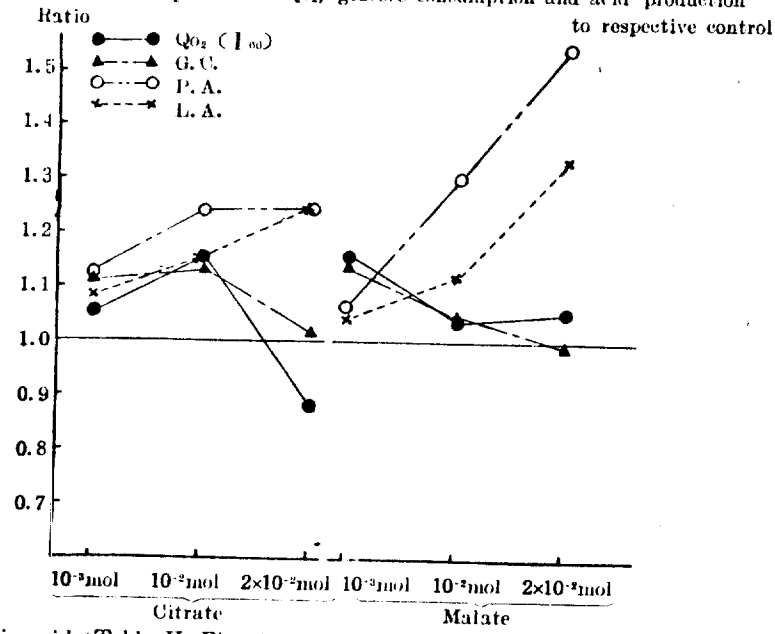
* Presented at the 32nd Meeting of the Physiological Society of Japan, 1956.

The glucose consumption (decrease of glucose in the suspension fluid) showed a tendency to increase (10 %) in the case of $10^{-3}$ and $10^{-2}$ mol.

The lactic acid showed a tendency to increase (15 %) in the case of $10^{-2}$ mol, and a distinct increase (23 %) in the case of $2 \times 10^{-2}$ mol. Production of pyruvic acid increased slightly in the case of $10^{-3}$ mol, and markedly (24 %) in the cases of $10^{-2}$ and $2 \times 10^{-2}$.

FIG. 1 Influence of various concentrations of citrate and malate on $Qo_2$ and glycolysis of bone marrow tissue

Ratio of experimental $Qo_2$, glucose consumption and acid production to respective control




## *II. Malic acid* (Table II, Fig. 2)

TABLE II. *Influence of various malate concentrations on $Qo_2$ and glucose metabolism of bone marrow tissue. Average of 5 exp.*

| | $Qo_2$ | | | | | | Glucose metabolism during 130 min. | | |
|---|---|---|---|---|---|---|---|---|---|
| | I 30 | II 30 | I 60 | III 30 | IV 30 | II 60 | G. C. ($\times 10^{-3}$mg) | P. A. ($\times 10^{-3}$mg) | L. A. ($\times 10^{-3}$mg) |
| a) $10^{-3}$mol Malate | 3.26 ± 0.10 | 3.08 ± 0.16 | 3.17 ± 0.12 | 2.64 ± 0.22 | 2.59 ± 0.17 | 2.62 ± 0.19 | 40.08 ± 2.75 | 2.24 ± 0.15 | 34.65 ± 2.28 |
| b) $10^{-2}$mol Malate | 3.12 ± 0.18 | 2.59 ± 0.01 | 2.86 ± 0.09 | 2.28 ± 0.11 | 2.14 ± 0.02 | 2.21 ± 0.06 | 37.35 ± 2.67 | 2.72 ± 0.18 | 38.83 ± 2.99 |
| c) $2 \times 10^{-2}$mol Malate | 3.22 ± 0.11 | 2.70 ± 0.13 | 2.96 ± 0.08 | 2.40 ± 0.13 | 2.23 ± 0.10 | 2.31 ± 0.08 | 35.47 ± 2.29 | 3.21 ± 0.19 | 45.69 ± 2.35 |
| d) Control | 3.10 ± 0.21 | 2.64 ± 0.18 | 2.87 ± 0.16 | 2.36 ± 0.13 | 2.06 ± 0.15 | 2.21 ± 0.13 | 36.23 ± 2.71 | 2.16 ± 0.17 | 35.35 ± 2.92 |
| a/d | 1.10 ± 0.08 | 1.21 ± 0.09 | 1.15 ± 0.09 | 1.13 ± 0.09 | 1.31 ± 0.13 | 1.21 ± 0.10 | 1.14 ± 0.09 | 1.06 ± 0.08 | 1.04 ± 0.11 |
| b/d | 1.04 ± 0.08 | 1.03 ± 0.07 | 1.03 ± 0.07 | 0.98 ± 0.06 | 1.09 ± 0.08 | 1.03 ± 0.06 | 1.04 ± 0.06 | 1.30 ± 0.10 | 1.12 ± 0.08 |
| c/d | 1.06 ± 0.04 | 1.05 ± 0.05 | 1.05 ± 0.04 | 1.03 ± 0.04 | 1.10 ± 0.04 | 1.06 ± 0.03 | 0.99 ± 0.03 | 1.54 ± 0.12 | 1.33 ± 0.07 |

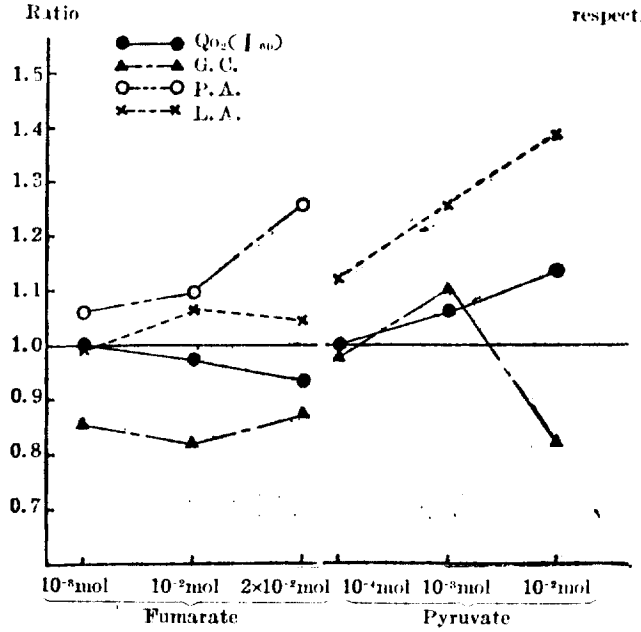
Concentrations were the same as before.

$Qo_2$ increased only slightly in the case of $10^{-3}$ mol.

The glucose consumption also increased, though slightly, in the case of $10^{-3}$ mol. Production of pyruvic acid was not influenced by $10^{-3}$ mol, while it increased in the case of $10^{-2}$ mol (30 %) and $2 \times 10^{-2}$ mol (54 %). The lactic acid was also increased slightly by $10^{-2}$ mol and markedly by $2 \times 10^{-2}$ mol (33 %).

FIG. 2 Influence of various concentrations of fumarate and pyruvate on $Qo_2$ and glycolysis of bone marrow tissue

Ratio of experimental $Qo_2$, glucose consumption and acid production to respective control




*III. Fumaric acid* (Table III, Fig. 3)

TABLE III. *Influence of various fumarate concentrations on $Qo_2$ and glucose metabolism of bone marrow tissue. Average of 5 exp.*

| | $Qo_2$ | | | | | | Glucose metabolism during 130 min. | | |
|---|---|---|---|---|---|---|---|---|---|
| | $I_{30}$ | $I_{30}$ | $I_{60}$ | $I_{30}$ | $IV_{30}$ | $I_{60}$ | G. C. ($\times 10^{-3}$mg) | P. A. ($\times 10^{-3}$mg) | L. A. ($\times 10^{-3}$mg) |
| a) $10^{-3}$mol Fumarate | 2.66 ± 0.08 | 2.51 ± 0.08 | 2.59 ± 0.08 | 2.41 ± 0.08 | 2.14 ± 0.09 | 2.28 ± 0.08 | 31.80 ± 2.89 | 2.03 ± 0.09 | 25.77 ± 2.58 |
| b) $10^{-2}$mol Fumarate | 2.61 ± 0.08 | 2.42 ± 0.10 | 2.52 ± 0.09 | 2.24 ± 0.11 | 2.11 ± 0.10 | 2.17 ± 0.10 | 30.30 ± 1.95 | 2.10 ± 0.08 | 27.15 ± 2.53 |
| c) $2\times10^{-2}$mol Fumarate | 2.46 ± 0.05 | 2.33 ± 0.09 | 2.40 ± 0.06 | 2.20 ± 0.10 | 2.09 ± 0.10 | 2.15 ± 0.10 | 32.04 ± 2.46 | 2.39 ± 0.08 | 26.66 ± 2.22 |
| d) Control | 2.61 ± 0.08 | 2.58 ± 0.07 | 2.60 ± 0.08 | 2.36 ± 0.06 | 2.26 ± 0.09 | 2.31 ± 0.07 | 36.64 ± 1.93 | 1.93 ± 0.08 | 25.61 ± 2.12 |
| a/d | 1.02 ± 0.01 | 0.97 ± 0.02 | 1.00 ± 0.01 | 1.02 ± 0.02 | 0.95 ± 0.01 | 0.99 ± 0.01 | 0.85 ± 0.04 | 1.06 ± 0.03 | 0.99 ± 0.02 |
| b/d | 1.00 ± 0.02 | 0.94 ± 0.03 | 0.97 ± 0.02 | 0.94 ± 0.03 | 0.93 ± 0.01 | 0.94 ± 0.02 | 0.82 ± 0.02 | 1.09 ± 0.04 | 1.06 ± 0.04 |
| c/d | 0.95 ± 0.02 | 0.90 ± 0.02 | 0.93 ± 0.02 | 0.93 ± 0.03 | 0.92 ± 0.02 | 0.92 ± 0.02 | 0.87 ± 0.05 | 1.25 ± 0.05 | 1.04 ± 0.04 |

$Qo_2$ was not influenced by the fumaric acid of any concentration. The glucose consumption showed a tendency to decrease in each case. Pyruvic acid increased about 25 % in the case of $2 \times 10^{-2}$ mol, but showed no change in the other concentrations. Lactic acid production was not influenced by the acid.

*IV. Pyruvic acid* (Table IV, Fig. 4)

The concentrations were $10^{-4}$ mol, $10^{-3}$ mol and $10^{-2}$ mol.

$Qo_2$ was slightly influenced (13 % increase) only in the case of $10^{-2}$ mol. The glucose consumption showed a slight increase in the case of $10^{-3}$ mol and a decrease in case of $10^{-2}$ mol. The lactic acid increased in each concentration (12 % in the case of $10^{-4}$, 25 % in the case of $10^{-3}$ and 39 % in the case of $10^{-2}$ mol).

The amount of pyruvic acid was found to be always smaller than the one experimentally added, except in the case of $10^{-4}$ mol.

TABLE IV. *Influence of various pyruvate concentrations on $Qo_2$ and glucose metabolism of bone marrow tissue. Average of 5 exp.*

| | $Qo_2$ | | | | | | Glucose metabolism during 130 min | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $I_{30}$ | $II_{30}$ | $I_{60}$ | $II_{30}$ | $IV_{30}$ | $II_{60}$ | G. C. ($\times 10^{-3}$mg) | L. A. ($\times 10^{-3}$mg) | P. A. ($\times 10^{-3}$mg) found | P. A. ($\times 10^{-3}$mg) added |
| a) $10^{-4}$mol Pyruvate | 2.21 ± 0.06 | 2.13 ± 0.06 | 2.22 ± 0.05 | 1.91 ± 0.07 | 1.87 ± 0.05 | 1.88 ± 0.06 | 35.68 ± 1.98 | 29.43 ± 2.82 | 1.62 ± 0.12 | 1.11 ± 0.07 |
| b) $10^{-3}$mol Pyruvate | 2.40 ± 0.06 | 2.28 ± 0.07 | 2.34 ± 0.06 | 2.01 ± 0.02 | 1.96 ± 0.03 | 1.99 ± 0.02 | 39.61 ± 0.90 | 32.58 ± 2.39 | 10.27 ± 0.67 | 11.94 ± 0.38 |
| c) $10^{-2}$mol Pyruvate | 2.54 ± 0.05 | 2.48 ± 0.06 | 2.51 ± 0.05 | 2.20 ± 0.03 | 2.07 ± 0.03 | 2.13 ± 0.03 | 29.68 ± 1.00 | 34.87 ± 1.64 | 109.04 ± 3.70 | 121.15 ± 5.41 |
| d) Control | 2.27 ± 0.04 | 2.17 ± 0.06 | 2.23 ± 0.05 | 1.98 ± 0.06 | 1.81 ± 0.05 | 1.90 ± 0.04 | 36.26 ± 0.94 | 26.47 ± 2.69 | 1.20 ± 0.10 | |
| a/d | 1.02 ± 0.02 | 0.98 ± 0.01 | 1.00 ± 0.02 | 0.97 ± 0.03 | 1.01 ± 0.03 | 0.99 ± 0.02 | 0.98 ± 0.03 | 1.12 ± 0.03 | | |
| b/d | 1.06 ± 0.02 | 1.05 ± 0.02 | 1.06 ± 0.03 | 1.02 ± 0.03 | 1.09 ± 0.03 | 1.05 ± 0.02 | 1.10 ± 0.02 | 1.25 ± 0.04 | | |
| c/d | 1.12 ± 0.03 | 1.15 ± 0.04 | 1.13 ± 0.03 | 1.12 ± 0.04 | 1.15 ± 0.03 | 1.13 ± 0.02 | 0.82 ± 0.02 | 1.39 ± 0.11 | | |

## COMMENT

All the acids experimented with in the present investigation belong to the components of the so-called TCA-cycle. And at some adequate concentrations they may increase the oxygen consumption of tissue. The increasing influence of citric, malic, fumaric and pyruvic acid on the oxygen consumption of bone marrow tissue was much smaller than that of succinic acid on which a report was made in the preceding paper (1). In the case of fumaric acid practically no influence was shown, a fact which was also demonstrated by Quastel and Wheatley (4) in the case of muscle, brain and so on, of rabbits.

Increasing influence of these acids on the oxygen consumption was dependent on the concentrations: $10^{-2}$ mol in the case of citric and pyruvic acids, and $10^{-3}$ mol in the case of malic acid were accelerating. $2 \times 10^{-2}$ mol of citric acid was rather inhibitory. Oohara (5) demonstrated also that the oxygen consumption of the liver and spleen of rabbits, and the liver of a toad were variously influenced by malic and citric acid, depending on their concentrations; showing the existence of an optimum concentration suitable for the tissue.

TABLE V. *Relations between glucose consumption on one hand and oxygen consumption and acid production on the other hand*

| | $10^{-2}$mol | $10^{-2}$mol | $2\times10^{-2}$mol | Control |
|---|---|---|---|---|
| **Citrate** | | | | |
| $Q_{O_2}$ | 5.86 | 6.06 | 4.80 | 5.57 |
| (1) Glucose actually disappeared ($\times10^{-3}$mg) | 37.06 | 37.77 | 34.38 | 34.16 |
| (2) Glucose consumption calculated as due to $Q_{O_2}$ ($\times10^{-3}$mg) | 7.85 | 8.12 | 6.43 | 7.46 |
| (3) Lactic acid produced ($\times10^{-3}$mg) | 34.00 | 36.49 | 38.48 | 31.52 |
| (4) Pyruvic acid produced ($\times10^{-3}$mg) | 1.81 | 1.95 | 1.92 | 1.60 |
| (1) − {(2)+(3)+(4)} Glucose consumed except by tissue oxidation and acid production ($\times10^{-3}$mg) | 6.60 | −8.79 | −12.45 | −6.42 |
| $\frac{\text{Pyruvic acid}}{\text{Glucose actually disappeared}}$ (%) | 4.9% | 5.2% | 5.6% | 4.7% |
| **Malate** | | | | |
| $Q_{O_2}$ | 6.32 | 5.55 | 5.76 | 5.56 |
| (1) Glucose actually disappeared ($\times10^{-3}$mg) | 40.08 | 37.35 | 35.47 | 36.23 |
| (2) Glucose consumption calculated as due to $Q_{O_2}$ ($\times10^{-3}$mg) | 8.47 | 7.44 | 7.72 | 7.45 |
| (3) Lactic acid produced ($\times10^{-3}$mg) | 34.65 | 38.83 | 45.69 | 35.35 |
| (4) Pyruvic acid produced ($\times10^{-3}$mg) | 2.24 | 2.72 | 3.21 | 2.16 |
| (1) − {(2)+(3)+(4)} Glucose consumed except by tissue oxidation and acid production ($\times10^{-3}$mg) | −5.28 | −11.64 | −21.15 | −8.73 |
| $\frac{\text{Pyruvic acid}}{\text{Glucose actually disappeared}}$ (%) | 5.6% | 7.3% | 9.0% | 5.9% |
| **Fumarate** | | | | |
| $Q_{O_2}$ | 5.30 | 5.11 | 4.95 | 5.34 |
| (1) Glucose actually disappeared ($\times10^{-3}$mg) | 31.80 | 30.30 | 32.04 | 36.64 |
| (2) Glucose consumption calculated as due to $Q_{O_2}$ ($\times10^{-3}$mg) | 7.10 | 6.85 | 5.63 | 7.16 |
| (3) Lactic acid produced ($\times10^{-3}$mg) | 25.77 | 27.15 | 26.66 | 25.61 |
| (4) Pyruvic acid produced ($\times10^{-3}$mg) | 2.03 | 2.10 | 2.39 | 1.93 |
| (1) − {(2)+(3)+(4)} Glucose consumed except by tissue oxidation and acid production ($\times10^{-3}$mg) | −3.10 | −5.80 | −2.64 | 1.94 |
| $\frac{\text{Pyruvic acid}}{\text{Glucose actually disappeared}}$ (%) | 6.4% | 6.9% | 7.5% | 5.3% |

Production of pyruvic acid was most remarkedly shown among the metabolites in the present experiment. Citric acid increased the production in each concentration. Malic acid increased the production in proportion to the concentration. Fumaric acid caused a distinct increase of the production only in the case of $2 \times 10^{-2}$ mol concentration (the highest concentration in this experiment). Though the changes of pyruvic acid productions under the influence of citric, malic or fumaric acids were much more marked than those of the oxygen consumption and lactic acid production under the same influences, the actual quantities of the pyruvic acid found in the suspension fluids were very small, showing small percentages of glucose (Table V). In the experiment of pyruvic acid influence, the production of the pyruvic acid could not be demonstrated, as the acid in the suspension fluid after the experiment was found to be smaller than that of the acid experimentally added to the fluid. The pyruvic acid is at a starting position of the TCA-cycle, originating from the triose phosphate which is an anaerobic product of the glucose. The quantity of the acid found in the suspension fluid shows a balance of its production and disappearance. It is clear that in the three groups other than the pyruvic group, the pyruvic acid found in the suspension fluid was a metabolite of the glucose contained in the tissue or existing in the fluid. However, it is not clear from the present experiment whether some part of the pyruvic acid produced during the experimental period played a role in the TCA-cycle, or became the lactic acid discovered in the fluid.

Lactic acid was found to have increased at higher concentrations ($1 \times 10^{-2}$, $2 \times 10^{-2}$ mol) of citric acid and malic acid. In the case of pyruvic acid addition, the lactic acid in the suspension fluid showed a tendency to increase in proportion to the concentration of the pyruvic acid added. The increase of the lactic acid production seemed to be accompanied generally by increase of the pyruvic acid production, demonstrating that the acids, citric and malic, accelerated the metabolism of the glucose.

The glucose consumption (diminution of the glucose in the suspension fluid) did not proceed in parallel with the oxygen consumption, and seemed to be in a reverse proportion to the pyruvic production. Especially in the case of the pyruvic application, consumption of the glucose decreased markedly, while the oxygen consumption increased. It seems to be that the pyruvic acid added to the suspension fluid spared the glucose; or some part of the acid might have been transformed to lactic acid, possibly due to oxygen insufficiency, or have been resynthesized to glucose.

Assuming that the oxygen consumption was due only to the oxidation of the glucose contained in the suspension fluid, the consumption was computed as glucose, $mm^3$ oxygen being multiplied by $1.34 \times 10^{-3}$ mg. (The oxygen consumption during 10 minutes prior to the actual measuring was taken as 1/6 of the consumption during the first experimental hour.) The amount of the glucose which actually disappeared from the suspension fluid was smaller than the sums of the oxygen consumption computed as glucose oxidation, and lactic and pyruvic acid production, in each case of citric or malic acid. Some substance corresponding to this difference had to be derived from a substance contained in the tissue, or the acids experimentally added to the suspension fluid, other than the glucose in the fluid.

In the case of fumaric acid, the difference between the glucose which actually

disappeared and the sum of the oxygen consumption and production of the acids was insignificant.

## SUMMARY

Influences of citric ($10^{-3}$, $10^{-2}$ and $2 \times 10^{-2}$ mol), malic ($10^{-3}$, $10^{-2}$ and $2 \times 10^{-2}$ mol), fumaric ($10^{-3}$, $10^{-2}$ and $2 \times 10^{-2}$ mol) and pyruvic acids ($10^{-4}$, $10^{-3}$ and $10^{-2}$ mol) on the oxygen consumption and glucose metabolism of the bone marrow were investigated.

1) The oxygen consumption was increased by these acids {citric ($10^{-2}$ mol), malic ($10^{-3}$ mol) and pyruvic ($10^{-2}$ mol)}, but not markedly. Fumaric acid influenced the oxygen consumption very slightly.

2) Glucose consumption (decrease of the glucose in the suspension fluid) was accelerated slightly by citric acid ($10^{-3}$, $10^{-2}$ mol), malic acid ($10^{-3}$ mol) and by pyruvic acid ($10^{-3}$ mol). The fumaric acid at $10^{-3}$, $10^{-2}$ and $2 \times 10^{-2}$ mol, and the pyruvic acid at $10^{-2}$ mol showed a tendency to decrease the consumption.

3) Lactic acid production increased under the influence of citric, malic and pyruvic acids in general, parallel to the increase of the concentrations of these acids, but showed no significant change in the case of fumaric acid.

4) Pyruvic acid production increased under the influence of citric, malic and fumaric acids in proportion to the concentrations of these acids. When pyruvic acid was added to the suspension fluid, the amount measured after the experiment was found to be less than that of the acid experimentally added to the suspension fluid.

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# The Non-volatile Organic Acids of Some Fresh Fruits and Vegetables

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## Abstract

The concentrations of citric, malic, and quinic acids were determined in several fresh fruits and of these acids and succinic and 2-pyrrolidone-5-carboxylic acids in a number of fresh vegetables. The peel of eggplant was found to have a very high content of quinic acid.

## Résumé

Les concentrations d'acide citrique, malique et quinique furent déterminées pour plusieurs fruits frais et ces acides plus l'acide succinique et l'acide 2-pyrrolidone-5-carboxylique sur un nombre de légumes frais. La pelure de l'aubergine indique une teneur très élevée en acide quinique.

## Introduction

The occurrence and concentrations of a number of the non-volatile acids have been summarized in such publications as the U.S.D.A. handbook on organic acids in higher plants (Buch, 1960) and the review by Thimann and Bonner (1950). A detailed study of certain berry fruits has been made by Whiting (1958) and similar studies have been reported for a few vegetables such as potato (Schwartz, Greenspun and Porter, 1962) and tomato (Davies, 1966).

In a general study on factors affecting quality, the acid contents of several fruits and vegetables were determined and are reported here. One point of interest in vegetables was the concentration of 2 pyrrolidone-5-carboxylic acid in the fresh materials. This has been reported as causing off-flavour in processed materials at fairly low concentrations (Mahdi, Rice and Wechel, 1961).

## Experimental

All materials were harvested at the normal state of maturity and extracted immediately. Vegetables were divided into sections as noted in Table I. Peel samples were approximately one mm thick. In berry fruit samples the stems and calyx were removed. In apples the stems and seeds were removed and in Lindia and Delicious (Table I) the quartered fruits were peeled.

A weighed portion of the material was ground in a Sorvall omni mixer with sufficient ethanol to give a final concentration of 80%. The mixture was filtered and the residue reground twice with 80% ethanol. During the grinding the container was kept immersed in crushed ice. The alcohol was removed *in vacuo* at 40° C. in a rotating evaporator and the aqueous solution treated with deactivated charcoal (Hulme and Wooltorton, 1958) to remove compounds such as chlorogenic acid. The charcoal was removed by centrifuging and washed several times by suspending in water and centrifuging. Amino acids and cations were removed by passing through a column of Dowex-50W x 4 resin, and washing until all acid was removed.

A portion of the extract containing about 2 milliequivalents of acid was passed down a column of Dowex-1 x 8 resin (14 x 0.7 cm.) in the acetate form and the column washed with water to remove sugars. A gradient- elution procedure (Hulme and Wooltorton, 1958) was used. The elution was carried out at 2 lbs. air pressure, maintained by a column of water. Samples of 3 ml. were collected and taken to dryness in a water-bath at 45° C, with compressed air delivered to the tubes through a manifold. The dried acids were titrated with 0.01 N or 0.0025 N sodium hydroxide, using thymol blue indicator.

The reproducibility of the separations was excellent, so that the fraction number gave a fairly good indication of the identity of the acid. However, as confirmation, the titrated fractions were combined as indicated by the titrations and taken to dryness as above. The residues were taken up in water and spotted on Whatman no. 1 filter paper for ascending chromatography with benzyl alcohol — *tert* — butyl alcohol — *iso*propyl alcohol — formic acid-water (24:8:8:1:8 v/v) (Stark, Goodban and Owens, 1951), the water containing bromphenol blue (0.015% w/v) and sodium acetate (0.05% w/v) (Hartley and Lawson, 1960). The indicator has an Rf value of 0.85 in this system and so serves to locate most of the plant acids when the papers are dried. The top of the paper was sprayed with the same indicator solution to locate acids of high Rf. The above solvent system maintains the spots in small zones, so that 20 spots may be applied to a 15-inch paper. Further confirmation was made with the system ethyl acetate-glacial acetic acid-water (2:1:1 v/v) (Hartley and Lawson, 1960), containing the above indicator.

The identities of quinic, 2 pyrrolidone-5-carboxylic, shikimic, and succinic acids were established by comparing their infrared spectra with those of known materials. The appropriate titrated fractions were passed through a small column of Dowex-50W x 4, the solutions evaporated *in vacuo* and the recovered acids prepared in potassium bromide disks for examination. The amount of thymol blue which was used in titration and was present in the final preparation did not interfere in the infrared.

## Results and Discussion

The concentrations of the acids (free plus that contained as salts) are listed in Tables I and II. The contents of the three acids reported in Table I are similar to those reported by others for these fruits. The differences in varieties preclude any close comparison. Shikimic acid was present in all samples of fruit (mostly a trace to a few milligrams) but under our conditions was mixed with one and sometimes two major contaminants and these were not further sepa-

Contribution No. 63 Food Research Institute

rated. The shikimic acid values for the gooseberry are for this reason slightly high.

The pyrrolidone carboxylic acid content of vegetables has been reported by some authors as probably partly or wholly due to their methods of preparation (Schwartz, Greenspun and Porter, 1961) and its formation from glutamine is said to be rapid above 65°C (Shallenberger, Pallesen and Moyer, 1959). In the present work, the temperature of sample preparation did not exceed 40°C. Some of the work was repeated at 25°C, with similar results, so that the analytical values apparently represent naturally occurring acid. However, the amounts present are considerably lower than the 0.05 percent said to cause off-flavour in certain products (Mahdi, Rice and Wechel, 1961).

The presence of the higher concentration of quinic acid in eggplant peel was confirmed in an imported sample. A peel of 0.5 mm thickness showed a content of 103 mg. per 100 g fresh weight, with a dry matter of 15.3 per cent.

## Acknowledgements

The authors wish to thank Dr. D.A. Shearer, Analytical Chemistry Research Service, for providing infrared identifications, and members of Ottawa Research Station for the plant materials.

Table II. Non-volatile Acids in Vegetables.

| Crop | Variety | Plant Part | Acids as mg/100g. of Fresh Weight | | | | |
|---|---|---|---|---|---|---|---|
| | | | Citric | Malic | Quinic | Succinic | Pyrrolidone Carboxylic |
| asparagus | Mary Washington | stem | 82.6 | 60.7 | 38.9 | 3.5 | 2.2 |
| | | tip (1 inch) | 228.1 | 170.0 | 47.2 | 11.1 | 3.3 |
| cabbage | Danish Ballhead | leaves | 48.8 | 63.1 | 3.5 | 1.1 | 6.1 |
| | | core | 16.9 | 39.7 | 3.1 | 1.4 | 5.3 |
| | Early Marvel | leaves | 49.8 | 78.0 | 4.8 | 7.4 | 9.6 |
| | | core | 21.2 | 68.8 | 3.8 | 10.6 | 3.6 |
| | Houston Evergreen | leaves | 67.6 | 99.6 | 4.5 | 7.9 | 16.9 |
| | | core | 40.8 | 102.0 | 4.4 | 4.7 | 16.2 |
| corn | Seneca Arrow | kernels | 20.9 | 29.0 | 3.5 | 7.7 | 6.4 |
| | | cob | 28.1 | 41.0 | 11.5 | 3.8 | 0.6 |
| eggplant | Black Beauty | flesh | 10.0 | 100.1 | 40.8 | 0.6 | 1.8 |
| | | peel | 10.8 | 100.2 | 241.5 | 0.9 | 2.3 |
| lettuce | Imperial | leaf blade | 15.7 | 81.2 | 3.9 | tr. | 1.7 |
| | | mid-rib | 10.8 | 98.5 | 4.6 | 1.4 | tr. |
| peas (green) | Perfection | — | 52.4 | 47.8 | 7.4 | 4.2 | 2.1 |
| squash (Butternut type) | Hercules | flesh | 6.5 | 199.0 | 11.5 | tr. | 7.8 |
| | | peel | 24.5 | 338.0 | 18.6 | tr. | 15.2 |
| tomato | Rideau | (whole) | 254.1 | 20.9 | 8.1 | tr. | 8.9 |
| turnip | Laurentian | flesh | 45.8 | 117.2 | 2.6 | 2.2 | 2.4 |
| | | peel | 112.8 | 89.0 | 7.6 | 1.6 | 4.9 |

Table I. Non-volatile Acids in Fruits

| Crop | Variety | Acids as mg/100g. of Fresh Weight Citric | Malic | Quinic |
|---|---|---|---|---|
| Apple | Delicious[1] | 3.0 | 275 | 73.0 |
| | Linda[1] | 3.3 | 881 | 27.3 |
| | McIntosh[2] | 8.1 | 461 | 20.2 |
| Currant black | Climax | 2740 | 253 | 40.2 |
| | Kerry | 1530 | 108 | 21.4 |
| | Magnus | 3060 | 173 | 38.2 |
| | Saunders | 2820 | 178 | 48.1 |
| Currant red | Cascade | 1295 | 301 | 10.8 |
| | Red Lake | 773 | 113 | 19.4 |
| | Stephen No. 9 | 1355 | 115 | 12.3 |
| Gooseberry[3] | Clark | 982 | 995 | 25.7 |
| | Poorman | 993 | 1125 | 35.4 |
| Raspberry | Comet | 1290 | tr. | 17.5 |
| | Trent | 1060 | tr. | 13.2 |
| Strawberry | Catskill | 817 | 138 | 11.5 |
| | Cavalier | 669 | 239 | 13.7 |
| | Ogallala | 653 | 139 | 17.0 |
| | Redcoat | 794 | 217 | 10.4 |
| | Sparkle | 660 | 209 | 12.8 |

[1] Cortex plus core.
[2] Whole apple minus seeds.
[3] Shikimic acid content — Clark-105, Poorman-75mg./100g. F.W.

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# EFFECT OF CITRIC ACID ON EXPERIMENTAL HYPERCHOLESTEREMIA AND ATHEROSCLEROSIS IN RABBITS

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UDC 616.153.922.01 + 616.13-004.6]-092.9 : 615.734.43

Citric acid in a dose of 960 mg/kg body weight facilitates a more rapid lowering of the cholesterol level in the blood, liver, adrenals, and aorta of rabbits after stopping cholesterol feeding. The activity of certain enzymes of the esterase group in the aorta is also restored to normal. A mixture of carbonates of the alkali and alkaline-earth metals gives the opposite effect.

* * * *

There are reports in the literature that in progressive atherosclerosis the intensity of oxidation is diminished and the quantity of incompletely oxidized products in the blood and urine increases. In cases of regression of atherosclerosis, the content of incompletely oxidized products becomes lower. Hence, an increase in the content of incompletely oxidized products may be an index of the activity of atherosclerosis [3]. Disturbance of the tricarboxylic acid cycle always leads to an increased accumulation of ketone bodies, and correspondingly, to disturbance of fatty acid metabolism [4, 5, 9]. The formation and accumulation of ketone bodies are easily suppressed by restoring the normal function of the tricarboxylic oxidative cycle of administration of citrates [2]. The antiketogenic action of citric acid has been demonstrated not only in ketones caused by ammonium salts and butyric acid, but also in diabetic ketoses in man and in alloxan diabetes in rabbits [2].

In the present investigation the effect of citric acid on the regression of cholesterol atherosclerosis was studied in rabbits.

## EXPERIMENTAL METHOD

Experiments were carried out on 27 male chinchilla rabbits initially weighing 2.3-2.5 kg. In the first period of the experiment (100 days) all the animals received a basic ration consisting of the following components: 50 g hay, 80 g oats, 100 g vegetables, and 200 mg cholesterol/kg body weight. Cholesterol was given to the animals as a fine suspension in starch paste mixed with the food. The cholesterol content in the blood plasma was measured every 20 days for 100 days by the method of Chiamori and Henry [8]. The rabbits developed cholesteremia to an unequal degree, and toward the end of the first period of the experiment the blood cholesterol level varied from 285 to 1169 mg %. To determine the degree of the morphological and biochemical changes in the organs the animals of group 1 were sacrificed after 100 days. Cholesterol in the organs was determined by Abell's method [7], total lipids gravimetrically, and the activity of the enzymes as follows: phosphomonoesterases 1 and 2 (PME-1, PME-2), acetylcholinesterase (ACE), butyrylcholinesterase (BCE), and tributyrinase in the aorta by A. A. Pokrovskii's method [6]. The remaining rabbits were subdivided into 2 groups, with 10 animals in each group, depending on their plasma cholesterol concentration. Cholesterol feeding was stopped after 100 days, and from the following day the animals of group 2 received a basic ration without cholesterol and a mixture of carbonates of alkali and alkaline-earth metals (260 mg/kg) for 50 days.

The rabbits of group 3 (experimental group) received a basic ration: citric acid in a dose of 960 mg/kg, and 260 mg of a mixture of carbonates of alkali and alkaline-earth metals (28 mg $NaHCO_3$, 113 mg

Laboratory of Biochemistry, Department of Chemotherapy, S. Ordzhonikidze All-Union Pharmaceutical-Chemical Research Institute, Moscow (Presented by Active Member of the Academy of Medical Sciences of the USSR P. D. Gorizontov). Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 65, No. 3, pp. 36-39, March, 1968. Original article submitted August 19, 1966.

TABLE 1. Effect of Citric Acid on Regression of Experimental Hypercholesteremia and Atherosclerosis in Rabbits

| Group of animals | Basic ration + test preparation | Number of animals | Wt. of animals (in g) | | Cholesterol concentration in blood plasma (mg%) | Change in cholesterol concentration in blood plasma after stopping cholesterol feeding (mg %) | | | Cholesterol concentration | | | Content of total lipids | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | days of experiment | | | in liver (mg %) | in adrenals (%) | in aorta (mg/g fresh tissue) | in liver (%) | in adrenals (%) | in aorta (mg/g fresh tissue) |
| | | | Initial | Final | 100 | 10th | 30th | 50th | | | | | | |
| 1st | Basic ration + cholesterol | 7 | 2477 | 2918 | 714 ± 122,3 | - | - | - | 1010 ± 233,7 | 17,9 ± 0,5 | 31,2 ± 4,1 | 7,9 ± 0,82 | 55,2 ± 7,7 | 75,4 ± 6,1 |
| 2nd | Basic ration + cholesterol (1st period of expt.) Basic ration + mixture of carbonates of alkali and alkaline-earth metals w/o cholesterol (2nd period of expt.) | 10 | 2472 | 2896 | 684 ± 106 | 393 ± 50,0 | 172,2 ± 40,4 | 76,7 ± 11,5 | 622,4 ± 50,2 | 17,3 ± 2,7 | 29,8 ± 4,2 | 8,1 ± 0,41 | 53,9 ± 1,5 | 67,3 ± 6,5 |
| 3rd | Basic ration + cholesterol (1st period of expt.) Basic ration w/o cholesterol + citric acid (960 mg/kg) + mixt. of carbonates of alkali and alkaline-metals (260 mg/kg)(2nd period of expt.) | 10 | 2382 | 3190 | 677,5 ± 78,9 | 261 ± 36,1 | 74,9 ± 6,7 | 47,5 ± 7,2 | 495,4 ± 33,3 | 8,8 ± 1,5 | 16,5 ± 2,7 | 6,5 ± 0,34 | 36,3 ± 3,7 | 47,0 ± 7,0 |
| | | P | | | | <0,05 | <0,05 | <0,05 | <0,05 | >0,01 | <0,02 | <0,01 | <0,001 | <0,05 |

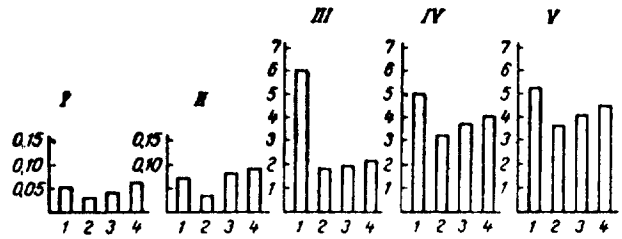


**Fig. 1.** Activity of certain enzymes in the aorta of control and experimental rabbits. I) Phosphomonoesterase-1; II) phosphomonoesterase-2; III) acetylcholinesterase; IV) butyrylcholinesterase; V) tributyrinase; 1) rabbits not receiving cholesterol with diet; 2) rabbits receiving cholesterol with diet for 100 days; 3) rabbits receiving basic ration for 50 days after stopping cholesterol feeding; 4) rabbits receiving basic diet and citric acid for 50 days after stopping cholesterol feeding. Vertical axis: enzyme activity (in μmoles substrate decomposed by 1 g fresh tissue/min).

$K_2CO_3$, 107 mg $CaCO_3$, and 20 mg $MgCO_3$). The ratio between the various elements (sodium, potassium, calcium, and magnesium) present as carbonates was determined from the content of these substances in the natural products.

## EXPERIMENTAL RESULTS

The results of biochemical investigations of the blood plasma and internal organs of the animals of group 1 are given in Table 1 and Fig. 1. In the animals of this group a high content of cholesterol and total lipids was observed in the liver, adrenals, and aorta. The atherosclerotic changes in the aorta varied in degree and were assessed from 1+ to 5+ [1]. The total lipid content in the aorta, calculated per gram fresh weight of intima and plaques, reflected the degree of involvement of the aorta. The intima of the aorta of most animals was rough throughout its extent and contained a large number of yellowish-white irregularly shaped plaques which had merged with one another over large areas. A decrease in the activity of the enzymes PME-1, PME-2, ACE, BCE, and tributyrinase was observed in the wall of the aorta. Results showing the effect of citric acid and carbonates of alkali and alkaline-earth metals on regression of the hypercholesteremia and atherosclerosis in the rabbits are given in Table 1 and Fig. 1.

Feeding with citric acid after stopping addition of cholesterol to the diet accelerated regression of the cholesteremia, and in the first 10 days a statistically significant decrease in the blood cholesterol concentration took place. After 30 days, the cholesterol level returned to the upper limit of normal, and by the end of the experiment it was within normal limits. The cholesterol content in the control animals still remained high after 30 days (92.9 to 440 mg %), and not until after 50 days did it reach a mean level of 76.7 mg %. The content of cholesterol and total lipids in the organs of the animals receiving citric acid was much lower than in the controls. The atherosclerotic changes in the aorta were more marked in the control animals (3+) than in the experimental (2+). The total content of lipids in the aorta of these animals bore a definite relationship to the macroscopic severity of the atherosclerosis.

A more rapid recovery of the activity of the enzymes PME-1 and PME-2, ACE, BCE, and tributyrinase was observed in the rabbits receiving citric acid. In the animals of group 1, the activity of these enzymes was sharply lowered, especially in rabbits with a high blood cholesterol (800-1000 mg% or more). The atherosclerotic changes in the aorta of the animals were very severe over wide areas (from the arch of the aorta to its bifurcation). In most of the experiments the correlation between a high blood cholesterol level and the severity of the atherosclerotic changes in the aorta was more or less complete.

Citric acid in a dose of 960 mg/kg accelerates the decrease in blood cholesterol level during regression of alimentary hypercholesteremia and atherosclerosis in rabbits, and also lowers the content of cholesterol and total lipids in the organs. In addition, it facilitates the more rapid recovery of activity of certain enzymes of the esterase group in the wall of the rabbits' aorta. A mixture of carbonates of alkali and alkaline-earth metals has the opposite effect.

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# Studies on Human Platelet Glycolysis. Effect of Glucose, Cyanide, Insulin, Citrate, and Agglutination and Contraction on Platelet Glycolysis *

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**Summary.** Evidence for a metabolically active plasma-free platelet system is presented. Glycogenolysis was found to be a potent pathway for lactate production. Aerobic glycolysis constituted a major fraction of glucose metabolized. Both insulin and cyanide increased lactate production in the presence of glucose. The Krebs cycle appeared to be operative for ATP synthesis when citrate was used as substrate. The first stages of gluconeogenesis were noted to be present. Glucose uptake contributed to increased lactate production.

Thrombin, epinephrine, and ADP resulted in platelet agglutination and contraction and increased platelet glycogenolysis (phosphorylase activity). Epinephrine appeared to be a more potent activator of phosphorylase, resulting in a 70% increase in glucose 6-phosphate levels. Thrombin and epinephrine both increased glucose uptake and lactate production. Glucose uptake decreased in the presence of ADP. Except for incubations with epinephrine, glucose 6-phosphate remained constant under conditions in which lactate flux increased 3-fold and glucose uptake increased 2-fold. Thrombin, epinephrine, and ADP decreased ATP levels in the presence or absence of glucose.

## Introduction

Platelets are viable cytoplasmic fragments that circulate in the bloodstream for 8 to 11 days. Their agglutination and contraction are thought to be necessary for plugging of vessel wall injury and attainment of hemostasis. Bounameaux (1) demonstrated that thrombin results in platelet agglutination and "viscous metamorphosis." Sokal (2) reported that viscous metamorphosis produced in platelet-rich plasma is accompanied by platelet contraction. Hellem (3) and Gaarder and his associates (4) demonstrated that ADP released from red blood cells causes platelet agglutination; concentrations as low as $1 \times 10^{-7}$ mole per L were effective (5). Mitchell and Sharp discovered that epinephrine, norepinephrine, and serotonin are also effective in initiating platelet agglutination (5). It has not been established whether epinephrine also results in platelet contraction. Bettex-Galland and Lüscher (6) reported the presence of thrombosthenin, a contractile protein with ATPase activity and postulated an association with platelet contraction.

Knowledge of the control of platelet energy (ATP synthesis and expenditure) during platelet agglutination and contraction is important for a proper understanding of the mechanism of hemostasis. The purpose of this series of investigations was to study the regulation of glycolysis in human platelets during basal and functioning conditions. This required metabolically active platelets, sus-

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pended in an enzyme-free medium of known composition. This communication may be divided into two sections: *1*) the biochemical evidence for the attainment of metabolically active platelets in a plasma-free system (glycolytic stimulation was initiated with glucose, insulin, citrate, and cyanide), and *2*) glycolytic changes under conditions known to result in platelet agglutination and contraction (an *in vitro* system employing thrombin, ADP, and epinephrine was utilized for this purpose).

With enzymatic techniques, lactate production, glucose consumption, adenosine triphosphate, and glucose 6-phosphate levels were measured under these conditions.

## Methods

*Preparation of platelets*

Human platelet-rich plasma,[1] collected in acid-citrate-dextrose solution, was obtained within 2 to 3 hours after phlebotomy. It was kept over ice during delivery and handled in containers not made of glass: Fenwal triple pack plastic bags, cellulose nitrate centrifuge tubes, and plastic pipettes. The platelets were separated from their plasma by differential centrifugation in an International centrifuge at 5° C. The platelet-rich plasma was centrifuged at 350 *g* to remove red blood cells. The supernatant platelet suspension was decanted and centrifuged at 2,250 *g* for 25 minutes. The platelet button obtained was suspended in 30 to 40 times its volume of a modified human Ringer solution containing 4 mM KCl, 107 mM NaCl, 20 mM $NaHCO_3$, and 2 mM $Na_2SO_4$ gassed with 5% $CO_2$-95% $O_2$ to a pH of 7.1. This suspension was accomplished by gentle aspiration and regurgitation of the Ringer solution with a plastic pipette. The suspension was resedimented for 10 minutes at 2,250 *g* and again suspended in approximately 40 times its volume of Ringer solution. This procedure was then repeated a second time. For studies with platelet agglutination and contraction, 0.1 mM EDTA was added to the Ringer solution. This eliminated to some extent the problem of platelet agglutination during the preparation of platelet suspensions. Whenever platelet agglutination occurred during the isolation and washing of platelets, the preparation was discarded. Before the 0.1 mM EDTA was introduced to the experimental design, it was first established that concentrations of EDTA in the order of 1 and 2 mmoles per L had no effect on platelet lactate production, uptake of glucose, ATP levels, or glucose 6-phosphate levels.

*Incubation procedure*

The packed cell volume of the platelet suspension was measured in a microhematocrit tube, and 3- to 5-ml samples of this suspension were employed for *in vitro* incubation. The packed cell volumes of these samples were 3% to 6% (1 ml packed platelets was equivalent to $1 \times 10^{11}$ platelets). Measurements were performed in duplicate on two different dilutions and agreed within 5 to 10% of each other and the total packed cell volume. Incubations were performed at 37° C under 5% $CO_2$-95% $O_2$ (unless otherwise noted) in tightly capped 40-ml Sorvall cellulose nitrate centrifuge tubes, in a gyratory New Brunswick shaker, model G-77, at 25 rpm. Most experiments were performed with pooled platelets from 2 to 4 U of blood. For agglutination and contraction experiments, additions consisted of the following: thrombin, 1 U per ml; ADP, $1 \times 10^{-4}$ mole per L, plus $CaCl_2$, $2.5 \times 10^{-3}$ mole per L; or epinephrine, $1 \times 10^{-4}$ mole per L, plus ascorbic acid, $1 \times 10^{-3}$ mole per L. Ascorbic acid was required to avoid oxidation of epinephrine. All additions resulted in macroscopic platelet agglutination and contraction within 5 to 8 minutes. Paired controls were devoid of clumping. Incubations were terminated by centrifuging in the same incubation tubes at 5° C at 6,000 *g* in a Sorvall RC11 centrifuge for 10 minutes.

*Preparation of samples*

The supernatant Ringer solution was treated with 10% by volume 5 N perchloric acid, followed by a neutralizing mixture containing 5 N KOH, 100 mM Tris, pH 7.5, to remove $KClO_4$. Suitably diluted samples of this neutralized solution were used to assay extracellular lactate and glucose. The platelet pellet within its centrifuge tube was treated with 3 times its volume of cold 1 N perchloric acid while immersed in an ice bucket and mixed with a glass stirring rod. The tubes were then shaken on a Vortex shaker for 1 minute and centrifuged at 6,000 *g* in the Sorvall centrifuge for 10 minutes. The supernatant obtained was treated with neutralizing mixture and again centrifuged at 6,000 *g* for 10 minutes. Undiluted samples of this neutralized tissue sample were used to assay intracellular lactic acid, ATP, and glucose 6-phosphate. It is essential that neutralizing mixture be added after removal of precipitated protein; otherwise an interfering gel is obtained.

*Enzymatic measurements*

All measurements were performed in microcuvettes, with a Gilford spectrophotometer and Honeywell recorder, employing nucleotide changes at 363 and 340 mμ for acetyl-NAD and NADP, respectively.

*Lactate.* The method employed was a minor modification of the Lowry procedure (7) and is a reproducible enzymatic method for measuring lactate provided the following precautions are observed: *1*) All glassware must be free of lactate. It is suggested that glassware be immersed for several hours in 25% NaOH, rinsed in tap water, and rinsed several times in distilled water. *2*) Final pH of at least 9.6 must be obtained. *3*) Beef heart lactic dehydrogenase must be employed. *4*) Lactate concentration should be below 0.025 mmole per L to assure adherence to Beer's law.

[1] Obtained from the American Red Cross and The New York Blood Center.

Base-line readings were obtained at 363 mμ in a final volume of 0.5 ml containing the following: 0.1 mM acetyl-NAD; 200 mM bicarbonate buffer, pH 9.6; and beef heart lactic dehydrogenase, 150 μg. The reaction was initiated with 0.015 ml of tissue sample or 0.05 ml of neutralized extracellular Ringer solution. Extracellular Ringer solution was diluted 1:10 in bicarbonate buffer. Beef heart lactic dehydrogenase was stored as a 1:10 dilution of its $(NH_4)_2SO_4$ suspension in 50 mM Tris buffer, pH 8.5, 1 mM EDTA; 0.03 ml of this dilution was used for the assay. The molar extinction coefficient for acetyl-NAD was in agreement with that obtained by Lowry, $9.3 \times 10^6$.

*Adenosine triphosphate.* ATP was measured by classical procedures coupling the hexokinase reaction to the glucose 6-phosphate dehydrogenase reaction, and employing the NADP-NADPH change at 340 mμ. The reagents employed in a final volume of 0.5 ml were these: 20 mM glucose, 0.46 mM NADP, 12.5 mM $MgCl_2$, 1 μg of crystalline hexokinase, 2 μg of glucose 6-phosphate dehydrogenase in 50 mM Tris buffer, 1 mM EDTA, pH 7.5. After base-line readings at 340 mμ, 0.02 ml of neutralized tissue extract was added directly to the microcuvettes. Tissue blanks in the absence of hexokinase were negligible.

*Glucose.* Glucose was measured with the use of hexokinase and ATP coupled to the glucose 6-phosphate dehydrogenase reaction. The reagents employed were in a final volume of 0.5 ml: 10 mM ATP, 12.5 mM $MgCl_2$, 0.46 mM NADP, 1 μg of hexokinase, and 2 μg of glucose 6-phosphate dehydrogenase in 50 mM Tris buffer, 1 mM EDTA, pH 7.5. The neutralized extracellular incubating Ringer solution was diluted 1:10 in 50 mM Tris buffer, 1 mM EDTA, pH 7.5, and 0.05 ml employed for the initiation of the reaction, after the attainment of base-line levels.

*Glucose 6-phosphate.* The reaction mixture in 0.5 ml final volume contained the following: 0.46 mM NADP in 50 mM Tris buffer, pH 7.5, 1 mM EDTA, and 0.15 ml neutralized tissue extract. The reaction was started by the addition of 2 μg of glucose 6-phosphate dehydrogenase in 0.02 ml. For these measurements it was necessary to use the expanded scale on the Honeywell recorder, employing a 2-fold amplification.

*Calculations.* Measurements were expressed per milliliter of packed platelets. In calculation of intracellular values, the platelet mass contribution to the perchloric acid volume was assumed to be 80% of its packed cell volume (8). In calculation of intracellular lactate, the contribution of entrapped lactate in the extracellular space of the platelet button was considered negligible.

*Materials.* All chemicals employed were reagent grade. Glucose 6-phosphate dehydrogenase type V,[2] crystalline hexokinase,[2] ATP,[2] ADP,[2] NADP,[2] acetyl-NAD,[2] beef heart lactic dehydrogenase,[3] and glucagon-free insulin[4] were obtained commercially. Purified human thrombin was a gift.[5] Epinephrine hydrochloride[6] was obtained commercially (intravenous injection) and was freshly diluted in 10 mM ascorbic acid.

## Results

Basal levels for ATP, intracellular lactate, and glucose 6-phosphate at 0° C before incubation were 2.7 ± 0.11, 1.95 ± 0.06, and 0.089 ± 0.004 μmoles per ml packed platelets, respectively, and may be seen from the ordinate of Figure 1. In the absence of glucose, lactate production proceeded at a linear rate of 15.6 ± 0.95 μmoles per ml per hour for at least 60 minutes, and in over one-half the experiments for 90 minutes. Thus, platelets with respect to their glycolytic end-product, lactate, remain metabolically active for at least 60 to 90 minutes. It should be noted that glucose is not required and that glycogenolysis is an active metabolic pathway. Although ATP, intracellular lactate, and glucose 6-phosphate measurements remained fairly similar in different platelet pools, total lactate production varied considerably from 7 to 22 μmoles per ml per hour. This may be a reflection of glycogen stores. Intracellular lactate rose slightly, reaching its peak level of 3.1 μmoles per ml at 90 minutes, and then declined gradually for the next 90 minutes. Glucose 6-phosphate levels remained constant over an 180-minute interval. ATP, however, declined to 38% of its initial level after 2 hours.

Platelets were responsive to increasing temperatures: 17°, 27°, and 37° C. For total lactate production $Q_{10}$ (17 to 27°) was 2.0 and $Q_{10}$ (27 to 37°) was 2.7.

In the presence of 5 mM glucose, lactate production increased 2-fold to 34.8 ± 1.6 μmoles per ml per hour (range 21 to 45) and remained constant for 90 minutes (Figure 2 and Table I). Glucose 6-phosphate levels remained constant and ATP levels declined as in the absence of glucose. Intracellular lactate levels rose to twice their basal levels. Glucose uptake was 21 μmoles per ml per hour. In the presence of 5 mM NaCN, an inhibitor of cytochrome oxidase, glucose uptake increased 2-fold, to 42.3 ± 6.9 (5 experiments); lactate production increased 1.9-fold, to 64.0 ± 15.6 (4 experiments).

In the presence of glucose and insulin, 0.4 U per

[2] Sigma Chemical Co., St. Louis, Mo.
[3] Worthington Biochemical Corp., Freehold, N. J.
[4] Eli Lilly, Indianapolis, Ind.
[5] From Dr. Kent Miller.
[6] Parke Davis, Detroit, Mich.

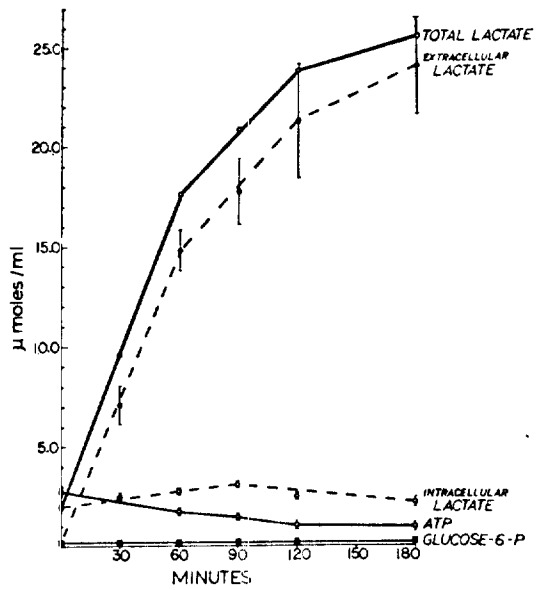


FIG. 1. LACTATE (INTRACELLULAR AND EXTRACELLULAR), ATP, AND GLUCOSE 6-PHOSPHATE LEVELS OF PLATELETS INCUBATED IN THE ABSENCE OF GLUCOSE. Each lactate point represents 6 to 24 experiments, except the 60-minute point, which represents 51 experiments. Each ATP point represents 6 to 21 experiments, except the zero time, which represents 26 experiments. Each glucose 6-phosphate point represents 6 to 30 experiments. Standard error of the mean is given.

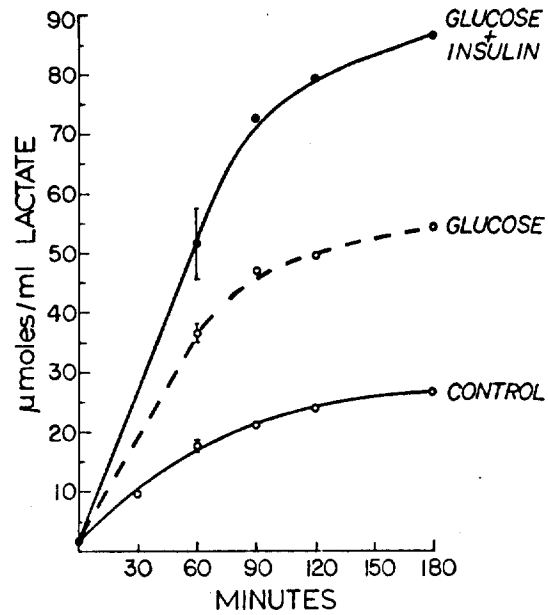


FIG. 2. TOTAL LACTATE LEVELS OF PLATELETS INCUBATED IN THE ABSENCE OF GLUCOSE, PRESENCE OF 5 mM GLUCOSE, AND PRESENCE OF GLUCOSE PLUS 0.4 U PER ML INSULIN. Standard error of the mean is given for 60-minute points, which represent 51, 40, and 5 experiments for control, glucose, and glucose plus insulin, respectively. Other points from glucose and glucose plus insulin experiments represent the average of 2 to 4 experiments.

ml (Figure 2), lactate production increased 3-fold over basal rates and remained constant for 60 to 90 minutes. Again, ATP and glucose 6-phosphate remained the same as for glucose alone (Table I), and intracellular lactate rose to 4 μmoles per ml at 1 hour. Insulin was effective at 0.004 to 0.4 U per ml in the presence of glucose. In the absence of glucose, insulin had no effect on increased lac-

TABLE I

*Comparison of lactate production and ATP and glucose 6-phosphate levels after 1-hour incubation**

| | Basal 0° C | Control | Glucose | Insulin + glucose | Citrate | Citrate + glucose |
|---|---|---|---|---|---|---|
| ATP, *μmoles/ml* | 2.70 ± 0.11 (18) | 1.74 ± 0.12 (17) | 1.75 ± 0.10 (15) | 1.72 ± 0.12 (3) | 2.19 ± 0.05 (3) | 2.19 ± 0.06 (4) |
| Glucose 6-phosphate, *μmole/ml* | 0.089 ± 0.004 (30) | 0.091 ± 0.005 (23) | 0.094 ± 0.005 (18) | 0.078 ± 0.005 (6) | 0.092 ± 0.007 (9) | 0.092 ± 0.007 (8) |
| Extracellular lactate, *μmoles/ml/hr* | 0 | 14.8 ± 0.95 (51) | 33.1 ± 1.6 (40) | 47.4 ± 6.1 (5) | 20.7 (2) | 43.4 ± 3.8 (4) |
| Intracellular lactate, *μmoles/ml/hr* | 1.95 ± 0.06 (19) | 2.77 ± 0.11 (17) | 3.61 ± 0.23 (15) | 4.0 ± 0.28 (7) | 2.6 (2) | 4.38 ± 0.43 (4) |
| Lactate production, *μmoles/ml/hr* | 0 | 15.6 | 34.8 | 49.5 | 21.4 | 45.8 |

* Basal values at 0° C are compared with values obtained after changes in incubation medium at 37° C: absence of glucose, 5 mM glucose, glucose plus 0.4 U per ml insulin, 40 mM citrate, and citrate plus glucose. Standard error of the mean is given. Number of experiments is denoted in parentheses.

tate production (control 16.5 ± 0.8 vs. insulin 16.8 ± 1.3 μmoles per ml per hour, 5 experiments).

The addition of citrate increased lactate production and prolonged the duration of linearity from 60 to 90 minutes to 120 to 180 minutes (Figure 3). Table II depicts the effect of increasing citrate concentration on lactate production and protection of ATP levels. Citrate plus glucose increased lactate production 3-fold, intracellular lactate rose to 4.4 μmoles per ml at 1 hour, and glucose 6-phosphate remained constant (Table I). Figure 4 depicts the kinetics of the effect of citrate on ATP decline. Citrate had a potent deterring action on ATP decline, whereas glucose alone had no effect. This would suggest that citrate penetrates the platelet membrane and is utilized by Krebs cycle enzymes to maintain ATP levels and increase lactate production. To substantiate this hypothesis the following experiment was employed. In the presence of 40 mM citrate, platelets were incubated with and without 2 mM, 2,4-dinitrophenol under anaerobic conditions. Both conditions were employed in order to assure cessation of ATP synthesis. (Because of technical difficulties, anaerobiosis was not always attainable.) Inhibition of the ATP-synthesizing enzymes linked to the Krebs cycle prevented the effect of citrate on increased lactate production and ATP levels (Table III). As first reported by Luganova, Seits, and Tedorovich (9), 2,4-dinitrophenol did not decrease ATP levels in platelets.

TABLE II

*Effect of citrate concentration on ATP levels and lactate production after 1 hour of incubation**

| Citrate, mM: | | 0 | 5 | 10 | 20 | 40 | 60 | 80 |
|---|---|---|---|---|---|---|---|---|
| | | % | % | % | % | % | % | % |
| Experiment 1 | ATP | 45 | 90 | 100 | 90 | 90 | | |
| | Lactate | 100 | 100 | 104 | 130 | 155 | | |
| Experiment 2 | ATP | 40 | | 60 | | 67 | 53 | 53 |
| | Lactate | 100 | | 100 | 125 | 164 | 109 | 109 |

* Per cent of initial ATP levels and per cent of 1-hour control lactate production is given.

Experiments were performed to study the effect of platelet agglutination and contraction on glycolysis.

*Thrombin experiments.* In the presence of thrombin, lactate production increased 8.0 μmoles per ml per hour or 37% (Table IV). This in-

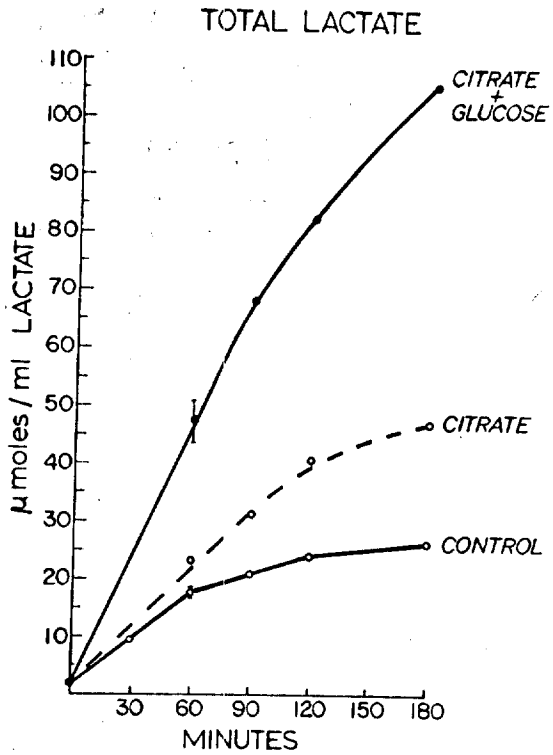


FIG. 3. TOTAL LACTATE LEVELS OF PLATELETS INCUBATED IN THE ABSENCE OF GLUCOSE, PRESENCE OF 40 mM CITRATE, AND PRESENCE OF 40 mM CITRATE PLUS 5 mM GLUCOSE. Standard error of the mean is given for 60-minute points for control and citrate plus glucose experiments, which represent 51 and 4 experiments, respectively. All other citrate points represent the average of 2 experiments.

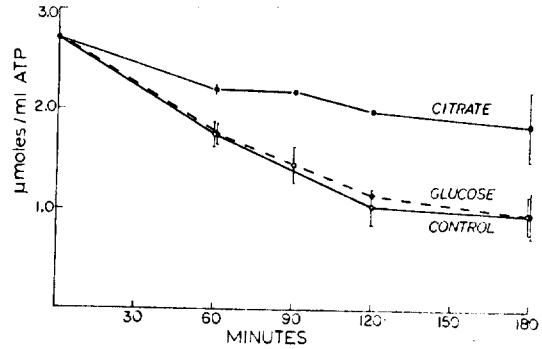


FIG. 4. ATP LEVELS FROM A 3-HOUR PERIOD OF INCUBATION IN THE ABSENCE OF GLUCOSE, PRESENCE OF 5 mM GLUCOSE, AND PRESENCE OF 40 mM CITRATE. The citrate curve combines experiments with citrate alone and citrate plus glucose (both were identical with respect to ATP decline) and represents 3 to 7 experiments. The glucose curve represents 3 to 15 experiments. Standard error of the mean is given.

TABLE III

*Reversal of citrate effect with 2 mM 2,4-dinitrophenol and anaerobiosis**

| Experiment | Glucose | | Glucose + citrate | | Glucose + citrate + NAD + An | | Glucose + NAD + An | |
|---|---|---|---|---|---|---|---|---|
| | *μmoles/ml* | (%) | *μmoles/ml* | (%) | *μmoles/ml* | (%) | *μmoles/ml* | (%) |
| 1 | 31.4 | (40) | 57.3 | (67) | 30.0 | (40) | | |
| 2 | 37.9 | (65) | 55.1 | (83) | 37.9 | (65) | | |
| 3 | 45.3 | (65) | | | | | 55.1 | (65) |

* Lactate production is after 1 hour of incubation. Per cent ATP of initial level is given in parentheses. Anaerobiosis (An) was obtained with 95% argon and 5% $CO_2$.

crease in glycolysis may be predominantly attributed to glycogenolysis, since glucose was absent from the media. ATP declined to 32% of its 60-minute control level, and glucose 6-phosphate levels remained constant. In the presence of 5 mM glucose plus thrombin, lactate production increased 51% from 37.0 to 55.7 or 18.7 μmoles per ml per hour. This was accompanied by an 82% increase in glucose uptake. ATP declined similarly to 37% of its 60-minute control level, and glucose 6-phosphate remained constant.

*Adenosine diphosphate experiments.* In the presence of ADP and $CaCl_2$, lactate production increased 15% or 3.3 μmoles per ml per hour. This increase in lactate production was noted in 10 of 11 experiments ($p < 0.01$). ATP declined to 75% of its 60-minute control value, and glucose 6-phosphate remained constant. In the presence of glucose, a significant increase in lactate production could not be demonstrated in a total of 8 experiments. Glucose uptake by the platelets declined 25%. This decline in uptake was noted in 6 of 8 experiments ($p < 0.05$). ATP declined to 73% of its 60-minute control value, and glucose 6-phosphate remained constant.

*Epinephrine experiments.* In the presence of epinephrine and ascorbic acid, lactate production increased 37% or 8.1 μmoles per ml per hour. ATP declined to 68% of its 60-minute control value, and glucose 6-phosphate remained constant. Ascorbic acid alone did not result in platelet agglutination or affect ATP levels or lactate production. In the presence of 5 mM glucose plus epinephrine, lactate production increased 43%, from 38.2 to 54.6 or 16.4 μmoles per ml per hour. This was accompanied by a 52% increase in glucose uptake. ATP declined to 61% of its 60-minute control value, and glucose 6-phosphate rose to 70% above its control value ($p < 0.05$).

## Discussion

Previous investigations of glycolytic enzymes, basal glycolytic rates, and nucleotide changes in human platelets have been published (9–13). Various methods were employed to measure ATP and lactate production, and different levels were obtained. Those for ATP were 1.61 (9), 2.5 (11), 3.8 (12), and 5.2 (13) μmoles per ml. Lac-

TABLE IV

*Effect of thrombin, ADP, and epinephrine on glycolysis**

| | Lactate production | | Glucose uptake | Adenosine triphosphate | | Glucose 6-phosphate | |
|---|---|---|---|---|---|---|---|
| | Control | Glucose | | Control | Glucose | Control | Glucose |
| | *μmoles/ml/hr* | | *μmoles/ml/hr* | *μmoles/ml* | | *μmoles/ml* | |
| Control | 21.5 ± 1.3 (8) | 37.0 ± 4.5 (7) | 19.0 ± 6.0 (7) | 2.00 ± 0.24 (8) | 1.89 ± 0.17 (8) | 0.070 ± 0.007 (5) | 0.080 ± 0.010 (5) |
| Thrombin | 29.5 ± 3.2 (8) | 55.7 ± 6.4 (7) | 34.6 ± 5.6 (7) | 0.64 ± 0.08 (5) | 0.69 ± 0.10 (5) | 0.065 ± 0.011 (3) | 0.071 ± 0.003 (5) |
| Control | 21.6 ± 1.3 (11) | 34.6 ± 2.5 (8) | 16.1 ± 1.4 (7) | 2.11 ± 0.57 (11) | 2.10 ± 0.16 (6) | 0.069 ± 0.014 (5) | 0.080 ± 0.013 (6) |
| ADP | 24.9 ± 1.4 (11) | 35.4 ± 3.1 (8) | 12.1 ± 2.1 (8) | 1.59 ± 0.15 (11) | 1.54 ± 0.12 (6) | 0.076 ± 0.009 (6) | 0.069 ± 0.006 (6) |
| Control | 22.1 ± 0.36 (15) | 38.2 ± 3.0 (9) | 17.8 ± 2.9 (9) | 1.77 ± 0.17 (9) | 1.82 ± 0.16 (7) | 0.068 ± 0.010 (5) | 0.077 ± 0.004 (5) |
| Epinephrine | 30.2 ± 2.6 (15) | 54.6 ± 5.0 (9) | 27.1 ± 4.5 (9) | 1.21 ± 0.12 (9) | 1.12 ± 0.10 (7) | 0.095 ± 0.017 (5) | 0.131 ± 0.025 (5) |

* Platelets were incubated for 1 hour at 37° C at 25 rpm in the presence and absence of 5 mM glucose. Additions consisted of thrombin, 1 U per ml; ADP, $1 \times 10^{-4}$ mole per L, plus $CaCl_2$, $2.5 \times 10^{-3}$ mole per L; epinephrine, $1 \times 10^{-4}$ mole per L, plus ascorbic acid, $1 \times 10^{-3}$ mole per L. ATP and glucose 6-phosphate levels are given at 1 hour. Standard error of the mean is given along with number of experiments in parentheses.

tate production in the absence of glucose was 27 (9) and in the presence of glucose was 70 (9) and 76.4 (10) μmoles per ml per hour at 37° C. The ATP levels of the present investigation correspond to the middle range of these values. Lactate production is approximately one-half. Waller, Löhr, Grignani, and Gross (10) reported the presence of glycolytic, Krebs cycle, and hexosemonophosphate shunt enzymes in human platelets. Platelets converted 50% of glucose uptake to lactate (glycogenolysis was not measured). Both Waller and associates (10) and Luganova and associates (9) indicated that the principal source of ATP in the platelet is the glycolytic pathway. Chernyak and Totskaya (14) also reported that the oxidative pathway is not so important as glycolysis in the generation of high energy phosphate. Bettex-Galland and Lüscher (11) first reported that thrombin increases lactate production and glucose consumption. Born (12), Bettex-Galland and Lüscher (11), and Zucker and Borrelli (13) have described ATP decline in the presence of thrombin. Little information is available on the regulation of glycolysis in a plasma-free platelet system in which glycolysis has been activated by known stimulators or platelets have been subjected to agglutination and contraction.

The results of this study confirm the importance of aerobic glycolysis in platelets but also emphasize the significance of glycogenolysis and Krebs cycle enzymes. Thus glycogen in freshly collected platelets is sufficient to maintain lactate production for at least 60 to 90 minutes. This is not to imply that the decrease in lactate production is due to decline of metabolic activity. Control mechanisms or changes in intracellular pH may be regulating the rate of lactate production (extracellular pH did not change).

In the rat (15) and anaerobic turtle (16) heart the rate of maximal glycogenolysis is dependent on the initial glycogen level. Reeves (16), employing elegant isotopic studies, demonstrated that maximal glycogenolysis proceeds at a constant rate with increasing work loads and is independent of glucose uptake. Under anoxic conditions, control dog hearts demonstrated similar 1-hour glycogen disappearance rates as did hearts from animals previously transfused with glucose and insulin (17). The assumption is made that platelet glycogenolysis similarly proceeds at a constant rate in the presence and absence of glucose. The contribution of glucose conversion to lactate can then be calculated by deducting lactate production in the absence of glucose and assuming that one glucose may be converted to two lactates. The value obtained was 46%, similar to that reported in skeletal muscle of the rhesus monkey (18). This suggests that under basal conditions 54% of glucose is metabolized by some pathway other than the Embden–Meyerhof. Waller and associates (10) reported that 20% of metabolized glucose is recovered as $CO_2$ and water. This leaves 34% of glucose utilization unaccounted for. Conversion to glycogen, amino acids, or lipids might explain this.

Thrombin– and epinephrine–induced platelet agglutination and contraction are associated with an increase in glycogen breakdown (phosphorylase activity), lactate production, and glucose uptake. It is recognized that increased glucose transport may be related to disintegration of the platelet membrane secondary to agglutination and contraction. In the presence of glucose, a further increase of lactate production was noted and can be attributed to the increase in glucose uptake. If glycogenolysis can be assumed to be independent of glucose penetration, this becomes more evident. Thus for thrombin, the theoretical lactate increase for an increase in glucose uptake of 1.8–fold should be 46% of 3.6–fold, or 1.7–fold. The actual value obtained was 1.7–fold. Similarly, the same applies for epinephrine agglutination and contraction, where glucose uptake increased 1.5–fold, the theoretical increase in lactate production being 1.4–fold. The actual increase obtained was 1.5–fold. Thus both epinephrine and thrombin can further increase glycolysis (hexokinase activity) by increasing glucose uptake.

It has been reported that a plasma factor and calcium are required for ADP agglutination of pig platelets (19). This factor does not appear to be required for human platelets although its presence increases their sensitivity to ADP (20). ADP–induced platelet agglutination resulted in a minimal but definite increase in lactate production. In the presence of glucose this effect could not be demonstrated. Surprisingly, glucose uptake decreased by 25%. Perhaps the ADP–mediated agglutination in some way impairs the sites for glucose entry.

Enhanced glucose uptake by the platelet contributed to increased lactate production. Addition of glucose to the media increased lactate production 2-fold. Cyanide increased glucose uptake 2-fold and was associated with a parallel increase in lactate production, 1.8-fold. Since free glucose was not found intracellularly, glucose disappearance from the media is a reflection of glucose utilization. This appears to be the first report of the effect of insulin on human platelets. Since insulin was not operative in the absence of glucose, the effect of insulin was probably on glucose transport. The effects of cyanide, insulin, thrombin, and epinephrine would indicate that hexokinase is not working at maximal capacity and that transport of glucose contributes to the increase of lactate production.

Skeletal muscle contraction results in increased glucose transport (21–23) and activation of the glycolytic chain (21, 22, 24, 25). The latter can be shown to be a result of the apparent activation of phosphorylase, hexokinase, and phosphofructokinase. Danforth, Helmreich, and Cori (25) have demonstrated an increase in the conversion of phosphorylase *b* to its active phosphorylase *a* form after muscle contraction. Platelet glucose 6-phosphate levels, except for incubations with epinephrine, remained constant under conditions in which lactate flux increased 3-fold and glucose uptake 2-fold. This would indicate that phosphorylase and hexokinase, key enzymes involved in the production of glucose 6-phosphate, remain closely geared to phosphofructokinase and glucose 6-phosphate dehydrogenase, the regulator enzymes controlling its removal. The assumption is made that glucose 6-phosphate is in equilibrium with fructose 6-phosphate (24, 26). Similar findings have been reported in skeletal muscle (24) and red blood cells (27). With epinephrine plus glucose, glucose 6-phosphate rose 70% above its control level. A similar finding has been reported in frog skeletal muscle (24), in which it was postulated that epinephrine activated the phosphorylase *b* to *a* conversion. With platelet phosphorylase, epinephrine appears to be a more potent activator than ADP or thrombin. As with skeletal muscle, key enzymes removing glucose 6-phosphate become rate limiting in the case of epinephrine (24).

Synthesis of ATP is considerably more efficient via the Krebs cycle than the Embden–Meyerhof pathway. This could explain the significant protection of ATP levels by a pathway contributing no more than 20% of glucose utilization. Aster and Jandl (28) reported that collection of blood in acid–citrate–dextrose solution containing increased citrate, 22 mM, enhanced platelet recovery and viability after platelet transfusion. This was explained on the basis of lowered pH (6.5), which led to reduction of platelet clumping. Perhaps maintenance of ATP levels can also explain their results. These data also suggest that enzymes capable of converting citrate to lactate are present in platelets. It appears that citrate is able to penetrate mitochondria, contribute to ATP production, and be converted to lactate via the gluconeogenic enzymes (possibly phosphoenolpyruvate carboxykinase). Thus platelets are capable of at least the first stages of gluconeogenesis.

The observation of ATP decline during incubation has also been made by Zucker and Borrelli (13). This phenomenon may result from decreased ATP synthesis or increased ATP utilization. Glucose alone or glucose plus insulin were incapable of preventing decline of ATP levels. Furthermore, lactate production did not fall during a significant span of the ATP decline. This would suggest that ATP synthesis did not decrease, but utilization increased. ATP declined further with all 3 agents: thrombin > epinephrine > ADP. These agents were capable of increasing ATP expenditure above that of control platelets. Platelet agglutination and contraction were associated with this ATP decline. It is conceivable that activation of thrombosthenin, the contractile protein ATPase, is required for platelet agglutination and contraction.

Platelet agglutination and contraction whether mediated by ADP, epinephrine, or thrombin result in increased glycolytic flux and expenditure of ATP. This universality of response was noted despite the variety of agents employed to induce agglutination and contraction. It would be interesting to speculate that this activation of the glycolytic chain and associated expenditure of ATP are a result of the cellular reorganization inherent in platelet agglutination and contraction, rather than specific agents activating specific enzymes.

## Acknowledgments

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Proc. Soc. Exp. Biol. Med.
224
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# Metabolism of Free Citric Acid in the Rat.*

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Studies in which pure citric acid was given by mouth to human subjects have shown that citric acid may be almost completely metabolized.[1-5] In the dog, large amounts of orally administered citric acid are likewise destroyed, less than 1% of the compound given appearing in the urine and no "extra" citric acid in the feces.[6] Similar results have been obtained with rabbits[7] and swine.[8, 9] The possibility that the citric acid administered was not absorbed was precluded by Langecker[7] who found a prompt and prolonged rise in the level of blood citrate and showed further that the enzymes and bacteria present in the intestinal tract of the rabbit do not destroy citric acid even when incubated for as long as 9 hours. The following experiments were conducted in order to determine whether or not the rat also possesses the ability to metabolize free citric acid.

A series of rats maintained in metabolism cages was fed the following diet *ad libitum:* lactalbumin (Borden No. 15-42) 15.0, dextrin 50.5, hydrogenated fat (Crisco) 27.0, cod liver oil (Mead) 5.0, and salt mixture[10] 2.5%. This diet was supplemented daily with 2 cc of a solution containing 80 mg of liver extract (Lilly 343) and 200 mg of Ryzamin-B (Burroughs Wellcome).

The cages were placed over large glass funnels containing a 3/16

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* The data herein presented are taken from a dissertation submitted by Carl A. Kuether in partial fulfillment of the requirements for the degree of Master of Science in Wayne University, 1940.

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1278



inch mesh screen which separated the feces from the urine, which was collected in a flask containing about 20 cc of 5% sulfuric acid. The funnels were rinsed down daily with distilled water. Citric acid was determined by the method of Pucher, Sherman and Vickery,[11] the final measurement being made in a photelometer using a filter with maximum transmission at 4250 Å.

After an adjustment period of 4 days, urine samples were collected for four 2-day intervals followed by 3 similar periods during which the rats received by stomach tube 400 mg per day of citric acid dissolved in distilled water to make 2 cc. Three more control samples were then collected. The urine samples were diluted to 200 cc and 2 cc aliquots taken for analysis. Later another group of animals was treated by the same procedure except that citric acid determinations were run on both the urine and the feces.

In order to show that the citric acid administered was actually absorbed and not destroyed by the bacteria in the gut, the intestinal contents of two rats were removed. One lot was mixed with citric acid and divided into 4 samples, 2 of which were analyzed immediately for citric acid and the other 2 incubated at 37° for 44 hours in glass stoppered flasks to prevent evaporation. The pH of the other sample of intestinal contents was measured, citric acid added and the pH brought back to the same point by addition of NaOH. This second sample was then treated the same as the first, divided into 4 parts, 2 analyzed immediately and 2 incubated at 37° for 44 hours. The citric acid found in the unbuffered control and incubated samples was 64.7 and 62.7 mg per g respectively and in the buffered samples 62.4 and 62.5 mg per g, showing that the intestinal contents of the rat do not destroy citric acid either at the normal pH of 6.5 or at the pH of 3.3 obtaining after the addition of citric acid.

Urinary pH was measured with a glass electrode on several animals before and after citric acid feeding, the urines being collected under toluene in large test tubes. Only samples uncontaminated with food or feces were used. The values differed by no more than 0.1 pH.

In Table I, the first line for each rat is the average intake and excretion of citric acid for three 2-day control periods immediately preceding the experimental periods. During these control periods each rat was excreting more citric acid in the urine than it was absorbing from the gut and consequently must have been synthesizing citric acid (Smith and Meyer[12]). The following 3 lines for

[11] Pucher, G. W., Sherman, C. C., and Vickery, H. B., *J. Biol. Chem.*, 1936, **11**[illegible] 235.

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TABLE I.
Disposition of Citric Acid by the Rat.

| | | Citric acid intake Mg per 2 days | | | Citric acid excretion Mg per 2 days | | | | Excretion of citric acid in % of total intake | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Rat | Wt | Food | Extra | Total | Urine | Feces | Total | C.A. Abs.% | Urine | Feces | Total |
| 8 | 256 | 17.7 | 0 | 17.7 | 25.1 | 0.84 | 25.9 | 95.5 | 148 | 4.97 | 153 |
| | | 16.3 | 800 | 816 | 20.6 | 1.03 | 21.6 | 99.9 | 2.46 | .123 | 2.58 |
| | | 12.9 | 600 | 613 | 31.8 | 0.56 | 32.4 | 99.8 | 5.19 | .091 | 5.28 |
| | | 12.2 | 800 | 812 | 39.0 | 0.62 | 39.6 | 99.8 | 4.81 | .076 | 4.88 |
| 9 | 232 | 17.0 | 0 | 17.0 | 22.3 | 1.28 | 23.6 | 92.4 | 142 | 8.15 | 150 |
| | | 15.0 | 800 | 815 | 30.3 | 1.13 | 31.4 | 99.9 | 3.72 | .139 | 3.86 |
| | | 12.2 | 800 | 812 | 32.8 | 0.95 | 33.8 | 99.9 | 4.05 | .117 | 4.17 |
| | | 12.2 | 800 | 812 | 38.0 | 0.52 | 38.5 | 99.9 | 4.68 | .064 | 4.74 |
| 10 | 196 | 17.0 | 0 | 17.0 | 28.7 | 1.31 | 30.0 | 92.4 | 183 | 8.34 | 191 |
| | | 13.6 | 600 | 614 | 19.1 | 1.20 | 20.3 | 99.8 | 3.11 | .196 | 3.30 |
| | | 5.5 | 800 | 805 | 12.2 | 0.63 | 12.8 | 99.8 | 1.52 | .078 | 1.60 |
| | | 5.5 | 800 | 805 | 13.6 | 0.56 | 14.2 | 99.9 | 1.69 | .070 | 1.76 |
| 11 | 156 | 10.2 | 0 | 10.2 | 19.3 | 0.43 | 19.7 | 96.1 | 197 | 4.39 | 201 |
| | | 4.8 | 800 | 805 | 15.7 | 3.49 | 19.2 | 99.6 | 1.96 | .435 | 2.39 |
| | | 5.5 | 800 | 805 | 18.2 | 12.35 | 30.6 | 98.5 | 2.29 | 1.56 | 3.85 |
| | | 6.8 | 400 | 407 | 24.2 | 0.23 | 24.4 | 99.9 | 5.94 | .056 | 6.00 |
| 12 | 290 | 20.4 | 0 | 20.4 | 74.7 | 1.72 | 76.4 | 91.6 | 399 | 9.20 | 408 |
| | | 17.7 | 400 | 418 | 60.1 | 1.10 | 61.2 | 99.7 | 14.4 | .264 | 14.7 |
| | | 12.9 | 800 | 813 | 52.3 | 0.97 | 53.3 | 99.9 | 6.44 | .119 | 6.56 |
| | | 12.9 | 800 | 813 | 72.1 | 1.72 | 73.8 | 99.8 | 8.86 | .212 | 9.08 |

each rat give the figures for individual periods during which the rats were receiving extra citric acid. During these periods, with one exception (Rat 11, 3rd period, when he had diarrhea) the absorption of the administered citric acid exceeded 99%, and the percentage of the absorbed citric acid which was excreted in the urine dropped to a very low value. In all 4 of the animals in which only urinary citric acid was followed, there was actually a decrease in the absolute amount of citric acid excreted following the feeding of free acid, and rats 10 and 12 in the table show the same result. In all of the animals, feeding of extra citric acid brought about a remarkable decrease in the percentage of the absorbed citric acid appearing in the urine, showing that under the experimental conditions imposed, the rat possesses the ability to metabolize free citric acid almost completely.

*Summary.* Citric acid administered to the rat is absorbed since no extra citric acid appears in the feces and intestinal contents do not destroy it. The albino rat has the ability to completely metabolize maximum non-fatal quantities of free citric acid.

Source: Atti della Accademia Medica Lombarda 18(3): 549-51, 1963

# Changes of Phosphorus Blood Level After Citric Acid Loading in Animals

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It is generally noted that the evolution (liberation) of energy in biological reactions is accompanied by a rapid recovery (regeneration) of phosphorus bonds of ATP. This process takes place with a gradual transfer, from one step to another, of the energy potential up to the final reactions, in which the energy is liberated and used for synthesis purposes.

At the level of tricarboxylic acids, the reactions in which energy is liberated are of two fundamental types:

1) Decarboxylation reactions, in which the keto acid, according to a scheme proposed by Lynen, is bound to acetyl coenzyme A. By dehydrogenation and decarboxylation, an acyl mercoplan rich in energy would be formed successively, and by a successive phosphololysis process. The substrate would be liberated (with one less carbonyl group) and also a phosphoryl mercaptan, which would release phosphorus for the synthesis of ATP (1).
2) Dehydrogenation reactions, with the transfer of electrons from the substrates to the respiratory chain of DPN and TPN, of flavoproteins and cytochromes, and final oxidation to $H_2O$. For each molecule of DPN oxidized, Lehninger (2) has calculated a variation (change) in energy sufficient for the synthesis of 3 phosphorus bonds. The points of the respiratory chain at which phosphorylation takes place are not known; Chance et al. (3) have indicated approximately such points in the passage (transfer) of electrons between DPN and flavoprotein, between cytochrome b and cytochrome c, and in the oxidation of cytochrome c under the action of cytochrome oxidases. In this type of reaction, phosphorylation is intimately associated with the consumption of $O_2$.

Cases are known in which the oxidative phosphorylation can be partly dissociated (such as by the action of thyroxine), and the energy evolved can be partially dispersed in the form of heat.

The entire metabolic process, from its first to last stages, is conditioned by the energy requirements of tissues, which spend (utilize) the energy of phosphorus bonds of ATP for the maintenance of vital processes, withdrawing new energy from the metabolic chain. The withdrawal (supply) of energy thus consists in the tissue availability of ADP (4).

In the course of previous studies, we have estimated the time and the intensity with which a load of citric acid induces an oxidative increment in the animal organism. If the close relations between phosphorylation and oxidation are taken into account, it would be interesting to find out if the induced oxidative increment is accompanied, in a similar experimental manner, by an energy recovery (regeneration) of phosphorus bonds, by determining in general the time and the intensity of this phenomenon in the organism, or if the two processes remain dissociated (separated) due to the lack of a particular energy requirement on the part of tissues.

This study can be carried out at the blood chemical level by observing the changes in the total phosphorus content of the blood. In fact, it has been noted (5) how variations of the blood phosphorus level may play a significant role in the synthesis of phosphorus bonds at the tissue level.

## Materials and Methods

Seven dogs of a weight ranging from 9 to 20 kg were used in the test. After a prior chloralose anesthesia, the femoral artery was isolated for the purpose of drawing blood. After the basic drawing of blood, a single and rapid infusion of 57 mg/kg of sodium citrate, corresponding to 41 mg/kg citric acid, was carried out. Successive drawings of blood were performed after 30, 60, 90, 120, and 150 minutes.

The phosphorus blood level was determined by the Fiske and Subbarow method.

## Results

Under basal conditions, a phosphorus blood level of $4.65 \pm 0.37$ mg% (see table) was obtained. This concentration is substantially similar to the one recorded

during observations made after 30 min (4.51 ±. 38 mg%)and after 60 min (4.49±0.23 mg%). After 90 minutes, the values of the phosphorus blood level were significantly lower (3.70±0.11 mg%), and remained lower after 120 min (3.52±0.10 mg%) and 150 min (3.50±0.04 mg%).

Table 1

| Dog | Basal | 30 min | 60 min | 90 min | 120 min | 150 min |
|---|---|---|---|---|---|---|
| 1 | 4,5 | 5,0 | 4,5 | 3,8 | 3,5 | 3,5 |
| 2 | 5,1 | 5,0 | 4,7 | 3,8 | 3,5 | 3,5 |
| 3 | 4,0 | 4,0 | 4,1 | 3,5 | 3,7 | 3,5 |
| 4 | 4,8 | 4,2 | 4,5 | 3,6 | 3,6 | 3,5 |
| 5 | 5,0 | 4,6 | 4,8 | 3,8 | 3,5 | 3,6 |
| 6 | 4,5 | 4,3 | 4,3 | 3,7 | 3,4 | 3,5 |
| 7 | 4,7 | 4,5 | 4,5 | 3,7 | 3,5 | 3,5 |
| x | 4,65 | 4,51 | 4,49 | 3,70 | 3,52 | 3,50 |
| SD | 0,37 | 0,38 | 0,23 | 0.11 | 0.10 | 0.04 |

Phosphorus blood level, in mg%, prior and after loading with 41 mg/kg citric acid.

x = average

SD = deviation standard

## Discussion

The results show that the phosphorus blood level is reduced due to the effect of citric acid loading. It is permissible to think that the oxidative increment produced by the same experimental conditions (6) is accompanied, at least in part, by phosphorylation, and that it is therefore possible to stimulate the synthesis of ATP by supplying an energy potential in an intermediate sector of the metabolic chain, such as the one of tricarboxylic acids, and independently of every energy requirement on the part of the tissue.

Also significant is the corresponding course of the reduction of the phosphorus blood level and of the oxidative increment. In the previous study mentioned (6), the increase in the oxidation appeared to be marked up to 60 minutes after loading, and became evident and conspicuous after 90 minutes. The reduction of the phosphorus blood level is manifested in a coinciding manner. This fact makes the connection between the two phenomena more probationary (plausible) and provides a greater validity to the hypothesis that phosphorus is utilized in the energy bonds of ATP.

In addition, the delayed appearance of the phenomenon can confirm what has previously been observed, namely that the overcoming (surmounting) of the first metabolic stages in the Krebs cycle on the part of the infused citrate is somewhat delayed; in fact, only after overcoming of the isocitric acid stage, are phenomena of oxidoreduction,and thus of phosphorylation, initiated, and these phenomena appear to be modified precisely only after 90 minutes of observation.

At 150 minutes after loading, the consumption of $O_2$ tends to be reduced, while the phosphorylation values remain low: at that time, the two phenomena become dissociated. The drop (lowering) of the phosphorus blood level with a reduction of the phosphorylation energy would appear to be in contrast (divergent) with the interpretation previously given of this phenomenon; we believe that it is possible to invoke at that point the lack of intervention of the mechanisms of phosphoric homeostasis in compensating the deficit (shortage) established.

Apart from this disagreement, there exists a corresponding course of the two phenomena, which is probationary for the utilization of the energy of the infused metabolic product.

## Summary

Administration of a citric acid load in the animal organism caused a reduction of the values of blood phosphorus. It is likely that the drop in the phosphorus blood level is linked to its utilization in the synthesis of high-energy phosphoric bonds.

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# Variazioni della fosforemia dopo carico di acido citrico nell'animale

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E' generalmente noto che nelle reazioni biologiche lo svolgimento dell'energia si accompagna a un suo pronto recupero nei legami fosforici dell'ATP. Il processo avviene con trasferimento graduale, di tappa in tappa, del potenziale energetico fino alle ultime reazioni in cui l'energia viene svolta e impiegata per la sintesi.

A livello degli acidi tricarbossilici le reazioni nelle quali si libera l'energia appartengono a due tipi fondamentali:

1) reazioni di decarbossilazione, nelle quali il chetoacido, secondo uno schema prospettato da Lynen, si lega all'acetilcoenzima A. Per deidrogenazione e decarbossilazione si formerebbe successivamente un acilmercaptano ricco di energia e per un successivo processo di fosforolisi si libererebbe il substrato (con un gruppo carbonilico in meno) e un fosforilmercaptano che cederebbe il fosforo per la sintesi di ATP (1).

2) reazioni di deidrogenazione con passaggio di elettroni dai substrati alla catena respiratoria dei DPN e TPN, delle flavoproteine e dei citocromi e ossidazione finale ad $H_2O$. Per ogni molecola di DPN ossidata, Lehninger (2) ha calcolato una variazione di energia sufficiente alla sintesi di 3 legami fosforici. I punti della catena respiratoria in cui la fosforilazione si compie non sono noti; Chance e Coll. (3) li hanno indicati approssimativamente nel passaggio di elettroni tra DPN e flavoproteina, tra citocromo *b* e citocromo *c* e nell'ossidazione del citocromo *c* per opera della citocromossidasi. In questo tipo di reazioni la fosforilazione è strettamente accomunata al consumo di $O_2$.

Esistono casi in cui la fosforilazione ossidativa può parzialmente dissociarsi (come per azione della tiroxina) e l'energia sviluppata può in parte andare dispersa sotto forma di calore.

Tutto il processo metabolico, dalle prime alle ultime fasi, è condizionato dalla richiesta energetica dei tessuti; questi spendono l'energia dei legami fosforici dell'ATP per il mantenimento dei processi vitali, richiamando nuova energia dalla catena metabolica. Il richiamo è costituito quindi dalla disponibilità tessutale di ADP (4).

Nel corso di studi precedenti abbiamo valutato i tempi e l'intensità con cui un carico di acido citrico induce nell'organismo animale l'incremento ossidativo. Se si fa conto dei rapporti stretti tra fosforilazione e ossidazione, appare interessante conoscere se l'incremento ossidativo indotto si accompagni, in una simile modalità sperimentale, a un recupero energetico nei legami fosforici, definendo i tempi e l'intensità del fenomeno nell'organismo in generale, o se i due processi restino dissociati mancando un particolare richiamo energetico da parte dei tessuti.

Lo studio può essere condotto a livello ematochimico osservando le variazioni della fosforemia totale. E' stato infatti segnalato come variazioni del fosforo ematico possano essere significative di sintesi di legami fosforici a livello tessutale (5).

## *Materiale e metodi*

Sono stati adoperati n. 7 cani di peso variabile fra 9 e 20 Kg. Previa anestesia cloralosica, veniva isolata l'arteria femorale per i prelievi di sangue. Dopo il prelievo di base, si procedeva a una infusione unica e rapida di mg 57/Kg di citrato di sodio corrispondente a mg 41 Kg di acido citrico. I prelievi successivi erano effettuati dopo 30, 60, 90, 120, 150 minuti.

La determinazione della fosforemia era effettuata secondo il metodo di Fiske e Subbarow.

*Risultati*

In condizioni basali è risultata una fosforemia di mg 4,65 ± 0,37%. Tale concentrazione è sostanzialmente simile a quella registrata nelle osservazioni fatte a 30 minuti (mg 4,51 ± 0,38%) e a 60 minuti (mg 4,49 ± ± 0,23%). Al 90° minuto la fosforemia presenta valori significatamente ridotti (mg 3,70 ± ± 0,11%), che restano bassi a 120 (mg 3,52 ± ± 0,10%) e a 150 minuti (mg 3,50 ± 0,04%).

*Discussione*

I risultati dimostrano che la fosforemia si riduce per effetto del carico di acido citrico. E' lecito pensare che l'incremento ossidativo prodotto dalle stesse condizioni sperimentali (6) si accompagni almeno in parte a fosforilazione e che è quindi possibile stimolare la sintesi di ATP apportando un potenziale energetico in un settore intermedio della catena metabolica come è quello degli acidi tricarbossilici e indipendentemente da ogni esigenza energetica di tessuto.

Significativo è l'andamento corrispondente della riduzione della fosforemia e dell'incremento ossidativo. Nel precedente lavoro segnalato (6) l'aumento delle ossidazioni appariva accennato fino al 60° minuto dal carico e diveniva evidente e cospicuo al 90° minuto. La riduzione della fosforemia si rende manifesta coincidentemente. Ciò rende più probativo il collegamento fra i due fenomeni e fornisce maggiore validità all'ipotesi dell'utilizzazione del fosforo nei legami energetici dell'ATP.

Il ritardo della comparsa del fenomeno inoltre può confermare quanto precedentemente osservato e cioè che il superamento delle prime fasi metaboliche del ciclo di Krebs da parte del citrato infuso avvenga con un certo ritardo; infatti solo dopo il superamento dello stadio di acido isocitrico si iniziano i fenomeni di ossidurazione e quindi di fosforilazione e questi appaiono modificati appunto solo al 90° minuto di osservazione.

A 150 minuti dal carico, il consumo di $O_2$ tende a ridursi mentre i valori della fosforemia restano bassi: in questo tempo i due fenomeni diventano dissociati. La caduta della fosforemia col ridursi dell'energia fosforilante sembrerebbe in contrasto con l'interpretazione precedentemente data al fenomeno; noi pensiamo che si possa invocare a tal punto il mancato intervento dei meccanismi di omeostasi fosforica nel compensare il deficit che si è costituito.

A parte questa discordanza, esiste per il resto un andamento corrispondente dei due

Table

| Cane ne Dog | Basale Basal | 30' 30 min | 60' 60 min | 90' 90 min | 120' 120 min | 150' 150 min |
|---|---|---|---|---|---|---|
| 1 | 4,5 | 5,0 | 4,5 | 3,8 | 3,5 | 3,5 |
| 2 | 5,1 | 5,0 | 4,7 | 3,8 | 3,5 | 3,5 |
| 3 | 4,0 | 4,0 | 4,1 | 3,5 | 3,7 | 3,5 |
| 4 | 4,8 | 4,2 | 4,5 | 3,6 | 3,6 | 3,5 |
| 5 | 5,0 | 4,6 | 4,8 | 3,8 | 3,5 | 3,6 |
| 6 | 4,5 | 4,3 | 4,3 | 3,7 | 3,4 | 3,5 |
| 7 | 4,7 | 4,5 | 4,5 | 3,7 | 3,5 | 3,5 |
| x | 4,65 | 4,51 | 4,49 | 3,70 | 3,52 | 3,50 |
| SD | 0,37 | 0,38 | 0,23 | 0,11 | 0,10 | 0,04 |

Valori della fosforemia, espressi in mg %, prima e dopo carico di mg. 41/Kg di acido citrico.

x = media → average
SD = deviazione standard deviation

Phosphorus blood level, in mg %, prior and after loading with 41 mg/kg citric acid.

fenomeni probativo di una utilizzazione dell'energia del materiale metabolico infuso.

*Riassunto.* La somministrazione di un carico di acido citrico nell'organismo animale ha provocato la riduzione dei valori del fosforo ematico.

E' verosimile che la caduta della fosforemia sia legata a sua utilizzazione nella sintesi di legami fosforici ad alta energia.

*Summary.* The administration of the load of citric acid in the animal organism caused the reduction of the values of the hematic phosphorus.

The fall of the phosphorus is likely to depend on its utilization in the synthesis of highly energetic phosphorical bonds.

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# Effect of Antioxidant on Rancidity Development in Frozen Creamed Turkey [a]

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**The well-recognized instability of turkey fat has been an adverse factor in the preparation of frozen creamed turkey. It can be counteracted readily with appropriate concentrations of commercial antioxidants, which are added preferably while the meat is being cooked.**

Utilization of turkeys in precooked frozen foods has been limited by the susceptibility of turkey fat to development of rancidity. The relative instability of turkey fat compared to chicken fat is generally recognized throughout the industry. This difference in stability was specifically demonstrated recently for precooked frozen creamed chicken and turkey *(1)*. In that study peroxide values were higher and off-flavor (rancidity) scores were greater in the creamed turkey than in the creamed chicken, particularly when the white sauce was made with turkey fat instead of a hydrogenated shortening. The study also revealed that the fat recovered from the turkey cooking liquid had rather high peroxide values.

This observation led to tests of the value of adding antioxidants to the water in which the turkey was cooked. The experiments reported here show that a commercially formulated antioxidant decreases rancidity development during cooking and frozen storage of creamed turkey. As expected, it also retards rancidity development if added to the creamed turkey ingredients (after cooking) rather than to the cooking liquid. The antioxidant appears to be stable under the cooking conditions used, which differ markedly from deep-fat-frying conditions that Sair and Hall *(3)* found were destructive to antioxidants. The formulated antioxidant used was Tenox II, which is composed of 20% butylated hydroxyanisole, 6% propyl gallate, 4% citric acid, and 70% propylene glycol. Sustane 3 is a similar type of mixture.

Limited tests with other antioxidants are reported also. These include nordihydroguaiaretic acid (NDGA), propyl gallate, and butylated hydroxyanisole.

## METHODS

The following procedures were used in experiments with Tenox II. Frozen turkeys were sawed in quarters and thawed overnight at room temperature (70° F., 21° C.). Half birds were cooked by simmering in covered stainless-steel kettles with 1 liter of water per 2,270 g. of turkey. The antioxidant was added to the cooking water before cooking. The amount of antioxidant added was 0.005% of the uncooked weight of the turkey halves. The other half of each turkey was cooked without added antioxidant. A peroxide test was made on the fat immediately after cooking. The cooked turkey was boned and diced by hand. The broth and fat were separated for use in preparing the white sauce. The proportion of ingredients for the sauce was as follows: liquid, 89.1-90.2%; fat, 4.6%; flour 4.6-5.7%; and salt, 0.6%.

The melted fat and flour were combined in a steam-jacketed kettle and stirred to a smooth paste. The liquid, heated to 140° F. (60° C.), was added and the mixture was heated, with constant stirring, to 194° F. (90° C.). With steam pressure at 5 lbs., the sauce was then cooked with constant stirring for 5 minutes. Salt was added at the end of the cooking period. When the antioxidant was added to the sauce instead of during cooking, enough was added to the melted fat so that the final concentration in the creamed turkey would be the same as that resulting from the addition of antioxidant during cooking. The sauce and diced turkey meat were combined in a ratio of approximately 44% meat and 56% sauce, canned or otherwise packaged, and frozen in an air blast at —10° F. (—23° C.). The products were then transferred for storage at other temperatures. The experiments varied in the type of liquid and fat used in the sauce and in the type of container as follows:

| Experiment No. | Type of liquid in sauce | Type of fat in sauce | Type of container |
|---|---|---|---|
| 1a | Turkey broth | Hydrogenated vegetable oil | Plain tin can |
| 1b | Skim milk | Hydrogenated vegetable oil | Plain tin can |
| 2 | Turkey broth | Turkey fat | Plain tin can |
| 3 | Skim milk | Turkey fat | MSAT cellophane bag, locker carton, sealed waxed paper overwrap |
| 5a | Broth plus skim milk, 3:1 | Turkey fat | MSAT cellophane bag, locker carton, sealed waxed paper overwrap |
| 5b | Broth plus skim milk, 3:1 | Turkey fat | Visqueen bags, parchment overwrap |

The initial determinations of flavor differences and of peroxide value of the fat of the creamed turkey were begun as soon as the samples were frozen. Other analyses were made after the products were stored for 4 to 12 months.

The frozen products were thawed at room temperature and heated on a steam bath for 30 minutes. The heated creamed turkey was presented to a panel of 7 judges for evaluating "rancid off-flavor" and "other off-flavor". Scoring was based on a scale of 0 to 10 points, with a score of 0 indicating no off-flavor and a score of 10 indicating extreme off-flavor. Since only the score for "rancid off-flavor" showed differences, the results of scoring "other off-flavor" are not reported. At all storage periods, each sample was judged either 4 or 5 times by each judge according to a randomized block design (in which 3 or 4 treatments were judged at a time) or according to a balanced incomplete block design (in which 4 of the 5 treatments were judged at a time). Statistical significance of the differences in scores was determined by analysis of variance of daily panel means or individual judge's means.

Peroxide values of the crude fat extracted from the creamed turkey (meat plus cream sauce) were determined by a modification of Wheeler's method *(2)*. The fat was extracted by disintegrating in a blender the contents of one package (166 g.) with 200 ml. of chloroform and about 30 g. of anhydrous sodium sulfate. After filtration, the chloroform layer was separated from

[a] Presented at the Eleventh Annual Meeting of the IFT, New York, N. Y., June 19, 1951.

[b] Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Report of a study made under the Research and Marketing Act of 1946.

the aqueous layer and dried with anhydrous sodium sulfate; the peroxides were determined in an aliquot containing 1 g. of solids.

Experiments with antioxidants other than Tenox II were concerned only with preparation of creamed turkey rather than both the preparation and storage. In the experiments the procedure was the same as above with the following exceptions: The amount of propyl gallate and/or butylated hydroxyanisole used was equivalent to their concentrations in Tenox II. Thus, propyl gallate was added at 0.001% and butylated hydroxyanisole at 0.0003% of the weight of the uncooked bird. The amount of NDGA used was 0.001%. The cream sauce was prepared with turkey broth and skim milk (3:1), and turkey fat. The sauce and diced turkey meat were combined in a ratio of approximately 3:1. Peroxide values on the fat of the creamed turkey were determined as before but rancid off-flavors were not determined.

## RESULTS

**Effect of cooking turkey with an antioxidant on peroxide value of the rendered fat.** When halves of 6 turkeys held at —30° F. (—34° C.) for less than a month were cooked without an antioxidant, the peroxide values of the fat rendered during cooking ranged from 9 to 34. Halves of these same turkeys cooked with 0.005% Tenox II added to the cooking water had peroxide values of 0. Halves of two turkeys held at —30° F. for 6 months, and cooked with an antioxidant also showed no peroxide development in the rendered fat. A half of one of the same turkeys cooked with antioxidant after 12 months at —30° F. had a peroxide value of 5.

**Effect of cooking turkey with Tenox II on flavor and peroxide value of frozen creamed turkey.** Creamed turkey prepared from half turkeys cooked with Tenox II showed little or no rancid off-flavor and only low peroxide values when examined immediately after freezing or after storage under varying temperature and packaging conditions for 4 to 12 months (Table 1). In all cases the peroxide values of the experimental samples prepared with an antioxidant during cooking were the same, within experimental error, as that of the control samples (Tables 1 and 2). Flavor scores for those samples differed from the control samples only after 12 months of storage, and even then the difference was slight. Control turkeys were held at —30° F. for Experiments 2, 3, and 5 and cooked with an antioxidant at the end of all periods. Because of probable variations between lots of birds, these birds were considered to be better controls than fresh controls from another lot.

The degree of off-flavor and peroxide value in the creamed turkey prepared from the halves cooked without antioxidant were not the same in the different experiments when examined immediately after freezing. In Experiments 2 and 3, using different turkeys from the same lot, there was no detectable rancidity, although the peroxide values were higher than those of the halves cooked with an antioxidant. In Experiment 5 there was distinct rancidity in the unstored frozen creamed turkey and the peroxide values were correspondingly higher than those of Experiments 2 and 3. In Experiment 1, where hydrogenated vegetable oil was used in the sauce in place of turkey fat, a slight rancidity was detected but was not detected in the sample cooked with an antioxidant.

The creamed turkey prepared from the halves cooked without an antioxidant were distinctly rancid after storage when turkey fat was used in the sauce, even when no rancidity had been detected originally in the unstored creamed turkey (Experiments 2 and 3). Differences between the two treatments were less in Experiment 1, where the turkey fat was not used, than in the subsequent experiments. The peroxide values of the creamed turkey samples increased during storage in all cases when the turkey had been cooked without an antioxidant, if the creamed product was stored in containers other than cans (Experiments 3 and 5). The peroxide values increased very little on storage of the creamed turkey in cans (Experiment 2, Table 2), although flavor scores indicated a pronounced change had taken place. Since different turkeys were used in each experiment it is possible that the difference in peroxide development could be related to differences in turkeys. However, since turkeys for Experiments 2 and 3 were from the same lot, it is probable that the difference was due to differences in packaging conditions. Similar effects of packaging have been reported previously *(1)*.

**Relative stability of frozen creamed turkey prepared with antioxidant addition at two different stages of preparation.** The stability of samples pre-

**TABLE 1**

**Effect of cooking turkey with Tenox II on rancidity of creamed turkey before and after frozen storage**

| Experiment no.[c] | Storage temperature | Storage time | Peroxide value of fat in creamed turkey | | Off-flavor score[d] of creamed turkey | |
|---|---|---|---|---|---|---|
| | | | With antioxidant | Without antioxidant | With antioxidant | Without antioxidant |
| | °F. | *months* | *meq./kg.* | *meq./kg.* | | |
| 1a | .... | 0 | .. | | 0.7 | 1.2 |
| | 10 | 4 | .. | .... | 0.4 | 1.6[e] |
| 1b | .... | 0 | .. | .... | 0.2 | 0.7[e] |
| | 10 | 4 | . | ... | 0.5 | 1.4 |
| 2 | .... | 0 | 1 | 8 | 0.3 | 0.2 |
| | 10 | 6 | 1 | 11 | 0.4 | 3.4[f] |
| | 0 | 6 | 1 | 11 | 0.4 | 3.2[f] |
| | 0 | 12 | 1 | 18 | 0.1 | 4.3[f] |
| 3 | .... | 0 | 4 | 11 | 0.3 | 0.3 |
| | 10 | 6 | 3 | 35 | 0.2 | 5.3[f] |
| | 0 | 6 | 4 | 36 | 0.3 | 4.3[f] |
| | 0 | 12 | 5 | 43 | 0.1 | 5.3[f] |
| 5a | .... | 0 | 3 | 37 | 0.5 | 2.9[f] |
| | 0 | 6 | 3 | 53 | 0.2 | 4.4[f] |
| | ....[g] | 2 | 4 | 77 | 1.5 | 9.4[f] |
| 5b | .... | 0 | 3 | 43 | 0.5 | 2.8[f] |
| | 0 | 6 | 6 | 66 | 0.2 | 5.4[f] |
| | ....[g] | 2 | 4 | 71 | 1.5 | 8.9[f] |

[c] Creamed turkey for Experiments 1 and 2 was stored in cans; commercial packaging materials were used in other experiments. Hydrogenated vegetable oil was used in the sauce for Experiment 1; turkey fat rendered during simmering of the turkeys was used in other experiments.

[d] 0 indicates no off-flavor, 10 indicates extreme off-flavor.

[e] Difference in scores statistically significant (P equals 0.05).

[f] Difference in scores statistically highly significant (P equals 0.01).

[g] Samples stored for 6 months at 0° F., then at 10° F. for 6 months.

**TABLE 2**

**Quality of creamed turkey prepared from control birds cooked with Tenox II**

| Experiment no. | Storage time at −30° F. (Uncooked turkey) | Peroxide value of fat in creamed turkey | Off flavor-score of creamed turkey |
|---|---|---|---|
| | *Months* | *meq./kg.* | |
| 2 | 6 | 1 | 0.2, 0.1 |
| 3 | 6 | 1 | 0.1, 0.2 |
| 2, 3 | 12 | 7 | 0.4, 0.2 |
| 5 | 6 | 5 | 0.6, 0.1 |
| 5 | 12 | 5 | 0.9, 0.2 |

pared with the following variations in treatment was studied:

| Treatment No. | Antioxidant (Tenox II) addition |
|---|---|
| 1 | None added |
| 2 | In preparation of sauce only |
| 3 | To the cooking water only |
| 4 | To both cooking water and in preparation of sauce |

Meat, fat, and broth from half of each of three turkeys cooked without antioxidant were divided equally for use in Treatments 1 and 2. The meat, fat, and broth from the other halves of the three turkeys cooked with an antioxidant were similarly divided for use in Treatments 3 and 4. Whenever an antioxidant was used, the amount was 0.005%, based on the weight of the uncooked birds. The creamed turkey from Treatments 2 and 3 above, therefore, had equivalent amounts of antioxidant, but it was added at different stages of preparation, and that from Treatment 4 had double the amount found in Treatments 2 and 3. Each of the 4 treatments was packaged in 2 ways, indicated in Table 3.

The creamed turkey prepared from the turkey cooked with the antioxidant (Treatment 3, Table 3) showed no rancidity immediately after freezing, or after 6 months of storage at 0° F. (−18° C.) and only slight rancidity after another 6 months of storage at 10° F. (−12° C.), indicating that the amount of antioxidant used during cooking afforded adequate protection from rancidity development during storage. Flavor differences between the samples cooked with an antioxidant and those cooked without an antioxidant but with antioxidant added to the sauce were not statistically significant after 6 months of storage at 0° F. However, in all cases the trend of flavor scores as well as the peroxide values showed less rancidity when the turkey was cooked with the antioxidant. After 12 months of storage at 10° F. the samples prepared by addition of antioxidant during preparation of the sauce were significantly more rancid than the samples cooked with antioxidant even though the peroxide values did not increase during this time. These results illustrate the greater effectiveness of antioxidant when present during cooking (and storage) rather than when added after cooking. The peroxide values and the off-flavor scores obtained for unstored samples containing no antioxidant were the highest encountered in any experiment. The variations from experiment to experiment or from bird to bird as already indicated may be rather large.

**Effect of individual and mixtures of antioxidants on peroxide development during cooking of turkey and preparation of sauce.** All of the antioxidant treatments reported in Table 4 effectively retarded peroxide development during cooking except for the treatment with propyl gallate. However, in another experiment with propyl gallate, the fat in the half bird cooked with the antioxidant had a peroxide value of 2.4, whereas the fat from the control had a value of 12.3. Evidently the antioxidants listed in Table 4 are effective to varying

**TABLE 3**

**Relative stability of frozen creamed turkey prepared with antioxidant addition at two different stages of preparation. The stored samples were held at —18° C. (0° F.) for 6 months and then at —12° C. (10° F.) for another 6 months**

| Antioxidant addition | Packaging | Peroxide values at | | | Off-flavor scores [h] | | |
|---|---|---|---|---|---|---|---|
| | | 0 months | 6 months | 12 months | 0 months | 6 months | 12 months |
| | | *meq./kg.* | *meq./kg.* | *meq./kg.* | | | |
| 1. None (Control) | Cellophane [i] | 37 | 53 | 77 | 2.9 | 4.4 | 9.4 |
| | Visqueen [j] | 43 | 66 | 71 | 2.8 | 5.4 | 8.9 |
| 2. In preparation of sauce only | Cellophane | 19 | 21 | 19 | 1.0 | 0.7 | 3.4 |
| | Visqueen | 24 | 23 | 21 | 1.1 | 1.3 | 2.8 |
| 3. To the cooking water only | Cellophane | 3 | 3 | 4 | 0.5 | 0.2 | 1.5 |
| | Visqueen | 3 | 6 | 4 | 0.5 | 0.2 | 1.5 |
| 4. Both to cooking water and in preparation of sauce | Cellophane | 4 | 3 | 4 | 0.6 | 0.6 | 1.3 |
| | Visqueen | 4 | 5 | 4 | 0.6 | 0.3 | 1.5 |

[h] See footnote [d], Table 1.
[i] MSAT cellophane bags in cardboard carton with heat sealed waxed paper overwrap.
[j] Visqueen bags with overwrap of parchment paper.

**TABLE 4**

**Effect of individual and mixtures of antioxidants on peroxide development that occurs during cooking of turkey and during preparation of creamed turkey therefrom**

| Antioxidants added | | Peroxide values of fat | | Peroxide values of fat in creamed turkey when antioxidant was added during | | | |
|---|---|---|---|---|---|---|---|
| | | Antioxidant added during | | | | | |
| Name | Concentration when present | None added | Cooking of bird | None (control) | Cooking of bird | Preparation of sauce | Cooking and preparation |
| | % | *meq./kg.* | *meq./kg.* | *meq./kg.* | *meq./kg.* | *meq./kg.* | *meq./kg.* |
| Butylated hydroxyanisole plus Propyl gallate plus Citric acid | 0.001 / 0.0003 / 0.0002 | 6 | 0.0 | 19 | 6 | 10 | .... |
| Butylated hydroxyanisole plus Propyl gallate | 0.001 / 0.0003 | 17 | 7 | 29 | 9 | 17 | 9 |
| Butylated hydroxyanisole | 0.001 | 13 | 8 | 23 | 10 | 14 | 8 |
| Propyl gallate | 0.0003 | 4 | 4 | 16 | 13 | 12 | 8 |
| Nordihydroguaiaretic acid (NDGA) | 0.001 | 24 | 4 | 32 | 13 | 26 | 11 |

degrees in retarding peroxide development. Because of the large bird-to-bird or sample-to-sample variations, there is at present considerable uncertainty concerning relative degrees of effectiveness.

## CONCLUSIONS

Sufficient rancidity may develop during the cooking (simmering) of turkey to be readily detected after it has been creamed. The degree of rancidity development differs for different lots of turkeys and apparently also for individual turkeys in the same lot. The presence of an antioxidant during the cooking of turkey to be used in precooked frozen foods markedly retards the development of rancid flavors and peroxide during both cooking and subsequent frozen storage. Addition of the antioxidant during preparation of the cream sauce rather than during cooking of the turkey less markedly retards development of rancid flavors during storage. Thus the greatest over-all benefit is obtained by adding the antioxidant at the beginning of the cooking process.

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# ANTIKETOGENIC AND GLYCOGENIC ACTIVITY OF CITRIC ACID

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Citric acid has long been known to be readily oxidized by the mammalian organism but until recently the details of its intermediate metabolism have remained obscure. Martius and Knoop (1, 2) have demonstrated that α-ketoglutaric acid is produced by the action of liver dehydrogenase on citric acid. Krebs and Johnson (3) found both α-ketoglutaric and succinic acids to be oxidation products of citric acid in isolated muscle tissue and Krebs (4) includes both of these compounds in his "citric acid cycle," a series of reactions through which he believes carbohydrate to be oxidized in animal tissues. Breusch (5) has strongly criticized the evidence for the existence of the citric acid cycle, at least in muscle. We are not concerned here with the details of muscle cell metabolism and it would be surprising if exogenous citric acid could be thrown into such a cycle should it exist. Of more concern here is the nature of the oxidation of citric acid, which may take place in the liver. The findings of Orten and Smith (6) that the injection of all the compounds in the citric acid cycle increased citrate excretion have been taken by Krebs to support his scheme. With his coworkers Krebs (7) confirmed Orten and Smith and found that α-ketoglutaric acid as well as citric acid was excreted after the injection of the members of the "citric acid cycle." Malonate, believed to inhibit the enzymes concerned with the cycle, caused the excretion of succinic and lesser amounts of α-ketoglutaric and citric acids. Succinic acid is very definitely a glucose former in the intact mammalian organism. It yields glucose in the phlorhizinized dog (8, 9), forms liver glycogen (10, 11), and is antiketogenic (11). If the route citric acid → . . . → α-ketoglutaric acid → . . . → succinic acid is

correct, citric acid should be glycogenic, a property which is not generally accepted for it. Lusk (12) interpreted an experiment of Baer and Blum (13) as evidence that citric acid could form glucose, as there was an increase in glucose excretion when sodium citrate was administered to a phlorhizinized dog. Greenwald (14) found that the administration of sodium citrate to phlorhizinized dogs and to a patient with diabetes mellitus was followed by an increased excretion of glucose, concluding that there was a conversion of the 6 carbon atoms of citric acid to glucose. Fürth *et al.* (15) have reported that in a phlorhizinized dog citric acid exerted neither an antiketogenic nor a glycosuric effect and failed to find any increase in liver glycogen from feeding it to rats.

The question of the glycogenic action of citric acid has been examined in a number of different ways. The ability (1) to overcome the effects of insulin hypoglycemia, (2) to form liver glycogen, and (3) to exert antiketogenic action has been considered. A study of the ability of citric acid to form glucose in the phlorhizinized dog would also have been desirable and was attempted without success. The marked influence of an alkalosis on citric acid metabolism (16-19) made it necessary to feed the free acid and not the sodium salt. Citric acid solutions, administered by stomach tube, are retained very poorly by normal fasting dogs and the phlorhizinized fasting organism is remarkably sensitive. Experiments were carried out on eleven dogs and in no case was an appreciable amount of the citric acid retained, even when doses of only 2 gm. in 1 per cent solution were used.

*Relief of Insulin Hypoglycemia by Citric Acid*—It is generally agreed that only those substances which, on enzymatic hydrolysis by the tissues or action by the liver, yield glucose are capable of overcoming insulin hypoglycemia. A comparison of the effect of citric acid with glucose in this regard is presented in Table I. Regular insulin of 40 units per cc. strength was used. The glucose and citric acid solutions were administered by stomach tube. Many of the rats which recovered were prostrate or even had convulsions, during the period after the insulin was given, before they recovered.

It is obvious from the data in Table I that citric acid is capable of overcoming insulin hypoglycemia and it would seem probable



TABLE I

*Comparison of Effect of Citric Acid with That of Glucose in Relieving Insulin Hypoglycemia in Rats*

| Experiment No. | Group No. | No. of rats | Treatment | Body weight | Average body surface | Result | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | No. died | Time | No. recovered |
| | | | | *gm.* | *sq.dm.* | | *hrs.* | |
| 1 | 1 | 3 | Controls | 154–186 | 3.5 | 3 | 8, 8, 9 | 0 |
| | 2 | 3 | Glucose | 159–173 | 3.4 | 3 | 11, 12, 13 | 0 |
| | 3 | 3 | Citric acid | 154–179 | 3.4 | 0 | | 3 |
| 2 | 1 | 3 | Controls | 228–270 | 4.4 | 3 | 14, 15, 17 | 0 |
| | 2 | 3 | Glucose | 192–238 | 4.1 | 1 | 18 | 2 |
| | 3 | 3 | Citric acid | 232–300 | 4.6 | 0 | | 3 |
| 3 | 1 | 8 | Controls | 247–345 | 5.1 | 5 | 4, 5, 6, 10, 12 | 3 |
| | 2 | 8 | Citric acid | 264–324 | 5.1 | 0 | | 8 |
| 4 | 1 | 3 | Controls | 177–208 | 3.9 | 3 | 3.3, 3.5, 4.1 | 0 |
| | 2 | 3 | Glucose | 177–227 | 3.9 | 3 | 3.5, 3.6, 5.7 | 0 |
| | 3 | 3 | Citric acid | 181–215 | 3.8 | 3 | 6.7, 7.3, 11.1 | 0 |

*Experiment 1*—Female rats fasted for 48 hours; then given 2.5 units of insulin per sq.dm. of body surface intraperitoneally at the 0 hour of the experiment. The dose was repeated in 5 hours. Solutions of 0.5 M glucose and citric acid were given to the respective groups at 0.5 hour in doses of 1.0 cc. per sq.dm. of body surface. The citric acid was too concentrated and when the doses were repeated at 6.25 hours the citric acid group received only 0.5 cc. per sq.dm. of body surface.

*Experiment 2* Male rats fasted for 48 hours; then given 5 units of insulin per sq.dm. of body surface subcutaneously at 0 hour. At 3.5 hours all were very weak and were given 0.5 cc. per sq.dm. of the two solutions in concentrations of 0.5 M. At 6 hours these doses were repeated.

*Experiment 3*—Male rats fasted for 72 hours; then given 4 units of insulin per sq.dm. of body surface subcutaneously at 0 hour. Citric acid in 0.25 M solution given to one group in a dose of 1 cc. per sq.dm. of body surface immediately afterward.

*Experiment 4* Female rats fasted for 24 hours before being given 10 units of insulin per sq.dm. of body surface by the subcutaneous route at the 0 hour of the experiment. At 1.25 hours all were prostrate. At 1.5 hours 0.5 cc. per sq.dm. of body surface of the solutions was given. At 3 hours the rats were all in convulsions and at 3.25 hours the doses of glucose and citric acid were repeated.

from this that it may form glucose in the organism. On a molecular weight basis it would seem to be more active than glucose. We would hesitate to draw any conclusions from this as to the

1425

quantity of glucose which a given amount of citric acid may yield, for as we will show elsewhere the speed of formation of glucose is just as important as the amount of glucose a substance may form in overcoming insulin hypoglycemia. It is probable that citric acid forms glucose rather slowly and for this reason is more active than glucose itself in equimolecular quantities. If we follow Krebs' scheme (4) for the oxidation of citric acid by muscle and assume that the route of glucose formation from citric acid by the liver is the same, 2 moles of citric acid = 1 mole of glucose.

*Formation of Liver Glycogen by Citric Acid*—The glycogenic action of citric acid is quite marked. The experiments in which this is demonstrated are detailed in Table II. The administration of citric acid always caused a definite increase in the amount of hepatic glycogen. Since there was no increase in nitrogen excretion which might be taken as evidence that the extra glycogen arose from protein breakdown, we must assume that the glycogen was formed from the citric acid. This is further evidence that citric acid may form glucose in the organism. It may be seen from Experiment 4 that sodium citrate formed less glycogen than the free acid. This appears to be a result of the influence of an alkalosis on citric acid metabolism, which will be considered elsewhere. The method of Good *et al.* (20) was used for the glycogen determinations.

*Antiketogenic Activity of Citric Acid*—In so far as we are aware any substance which has antiketogenic activity is able to form glucose in the organism and conversely all glycogen formers show antiketogenic action. Since citric acid is a glycogen former, it should be antiketogenic, and this is the case. Following a diet low in protein, a good fasting ketosis ensues in rats (11). The data in Table III were obtained from such fasting rats by methods described elsewhere (11). After removal from the diet the rats were fasted for a day before observations were commenced.

Collection of a blood sample for acetone body determination involved sacrifice of the animal and the number of rats in a group from which urine collections were made was reduced by two whenever blood samples were taken. Urine acetone bodies were determined by Van Slyke's method (21) and blood acetone bodies by the method of Barnes and Wick (22).

The results in Table III show very clearly, both from the urine

TABLE II

*Glycogenic Action of Citric Acid in the Rat*

| Experiment No. | Group No. | No. of rats | Treatment | Body weight | | | | Urine N over entire period per sq.dm. | Liver weight | | | Liver glycogen | | | Liver glycogen per sq.dm. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Minimum | Average | Maximum | Average body surface | | Minimum | Average | Maximum | Minimum | Average | Maximum | |
| | | | | *gm.* | *gm.* | *gm.* | *sq.dm.* | *mg.* | *gm.* | *gm.* | *gm.* | *per cent* | *per cent* | *per cent* | *mg.* |
| 1 | 1 | 6 | Controls | 137 | 149 | 160 | 3.2 | 100 | | 1.42 | | 0.18 | | | 2.5 |
| 2 | 2 | 6 | Citric acid | 139 | 154 | 184 | 3.3 | 94 | | 1.46 | | 2.98 | | | 43.4 |
| 2 | 1 | 12 | Controls | 234 | 260 | 287 | 4.6 | 87 | 1.25 | 1.43 | 1.72 | 0.09 | 0.09 | 0.10 | 1.1 |
| | 2 | 12 | Citric acid | 239 | 263 | 281 | 4.7 | 80 | 1.38 | 1.58 | 1.89 | 1.10 | 1.28 | 1.33 | 20.2 |
| 3 | 1 | 12 | Controls | 162 | 184 | 261 | 3.7 | 30 | 1.41 | 1.54 | 1.62 | 0.05 | 0.14 | 0.17 | 2.3 |
| | 2 | 12 | Citric acid | 170 | 190 | 224 | 3.8 | 32 | 1.37 | 1.60 | 1.86 | 0.98 | 1.45 | 1.68 | 23.2 |
| 4 | 1 | 8 | Controls | 168 | 214 | 250 | 4.1 | | | 1.38 | | 0.09 | 0.27 | 0.59 | 3.7 |
| | 2 | 8 | Citric acid | 170 | 215 | 251 | 4.1 | | | 1.35 | | 0.49 | 0.91 | 1.48 | 124.0 |
| | 3 | 8 | Sodium bicarbonate | 176 | 217 | 251 | 4.1 | | | 1.35 | | 0.05 | 0.07 | 0.10 | 1.0 |
| | 4 | 8 | " citrate | 186 | 215 | 246 | 4.1 | | | 1.40 | | 0.10 | 0.30 | 0.57 | 42.0 |

*Experiment 1*—Male rats fasted for 24 hours preceding the administration of 0.25 M citric acid solution in doses of 1 cc. per sq.dm. of body surface at 0, 9, 17, 21, 33, 41, 56, 59, and 64 hours; killed at 70 hours.

*Experiment 2*—Male rats fasted 48 hours and one group then given doses of 5 cc. of 0.25 M citric acid at 38, 46, 60, 66, 72, and 84 hours; killed at 90 hours.

*Experiment 3*—Female rats fasted for 48 hours and one group then given 4 cc. each of 0.25 M citric acid at 0, 4.5, 7.5, 18, 21, and 23.5 hours; killed at 27 hours.

*Experiment 4*—Male rats fasted 48 hours and then given the following: Group 1, water; Group 2, 0.2 M citric acid; Group 3, 0.6 M sodium bicarbonate; and Group 4, 0.2 M sodium citrate in amounts of 1 cc. per sq.dm. of body surface at 0, 12, 27, and 56 hours; killed at 61 hours.

1425

TABLE III

*Antiketogenic Activity of Citric Acid in Fasting Rats*

| Experiment No. | Group No. | Sex | No. of rats | Average body weight | Average body surface | Time on diet before fasting | Solution fed twice daily, 1 cc. per sq.dm. body surface | Urine N per sq.dm. body surface per day | | | | Urine acetone bodies per sq.dm. body surface per day | | | | Blood acetone bodies at end of | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *gm.* | *sq. dm.* | *days* | | 1st day | 2nd day | 3rd day | 4th day | 1st day | 2nd day | 3rd day | 4th day | 1st day | 2nd day | 3rd day | 4th day |
| | | | | | | | | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg. per cent* | *mg. per cent* | *mg. per cent* | *mg. per cent* |
| 1 | 1 | ♀ | 6 | 198 | 3.9 | 10 | Water | | | | | 1.2 | 13.1 | 12.6 | 6.2 | | | | |
| | 2 | ♀ | 3 | 217 | 4.1 | 10 | 0.50 M citric acid | | | | | 0.4 | 0.5 | 0.5 | 0.0 | | | | |
| | 3 | ♀ | 3 | 179 | 3.6 | 10 | 0.25 " " | | | | | 0.6 | 1.4 | 0.6 | 0.3 | | | | |
| | 4 | ♀ | 3 | 213 | 4.0 | 10 | 0.50 " glucose | | | | | 0.2 | 0.3 | 0.0 | 0.0 | | | | |
| 2 | 1 | ♀ | 6 | 185 | 3.7 | 5 | 0.75 " $NaHCO_3$ | 19.2 | 19.3 | 20.2 | 14.0 | 12.7 | 46.7 | 26.4 | 26.7 | | | | |
| | 2 | ♀ | 6 | 185 | 3.7 | 5 | 0.75 " " + 0.50 M citric acid | 24.8 | 13.5 | 17.0 | 15.7 | 7.8 | 7.0 | 4.0 | 5.7 | | | | |
| | 3 | ♀ | 6 | 185 | 3.7 | 5 | 0.75 M $NaHCO_3$ + 0.25 M glucose | 23.2 | 15.3 | 14.0 | 13.8 | 6.8 | 0.8 | 0.6 | 0.6 | | | | |
| 3 | 1 | ♀ | 6 | 185 | 3.7 | 23 | 0.6 M $NaHCO_3$ | 18.1 | 16.5 | 10.8 | 10.0 | 35.4 | 122.8 | 68.4 | 50.1 | | 73 | 98 | 110 |
| | 2 | ♀ | 6 | 187 | 3.7 | 23 | 0.6 " " + 0.20 M citric acid | 14.6 | 15.7 | 10.3 | 5.4 | 24.3 | 61.7 | 35.7 | 23.0 | | 60 | 62 | 68 |
| 4 | 1 | ♂ | 6 | 261 | 4.6 | 8 | Water | 24.8 | 13.3 | 26.1 | 15.2 | 20.8 | 51.0 | 26.6 | 21.0 | 41 | 47 | | 42 |
| | 2 | ♂ | 6 | 265 | 4.7 | 8 | 0.50 M citric acid | 20.6 | 12.3 | 15.3 | 16.5 | 8.5 | 11.9 | 1.1 | 0.4 | 28 | 32 | 48 | 25 |
| 5 | 1 | ♂ | 6 | 238 | 4.4 | 12 | 1.0 " $NaHCO_3$ + 0.25 M glucose | 17.2 | 17.0 | 15.9 | 8.2 | 8.0 | 24.8 | 24.5 | 14.3 | | 43 | | 31 |
| | 2 | ♂ | 6 | 236 | 4.3 | 12 | 1.0 " " + 0.25 M citric acid | 17.2 | 15.3 | 12.5 | 9.5 | 0.7 | 3.5 | 0.8 | 0.8 | | 26 | 22 | 20 |
| | 3 | ♂ | 6 | 235 | 4.3 | 12 | 1.0 M $NaHCO_3$ + 0.50 M citric acid | 16.3 | 12.6 | 12.8 | 9.1 | 0.2 | 0.3 | 0.2 | 0.4 | | 28 | 18 | 18 |

excretion of acetone bodies and the blood levels, that citric acid has a marked antiketogenic action. Although the experiments were not designed to make a quantitative comparison of the activity of glucose and citric acid, the data suggest that mole for mole citric acid is about half as active as glucose, which is what theory demands.

## SUMMARY

In the albino rat citric acid is as effective as glucose in the relief of insulin hypoglycemia, active in the formation of liver glycogen, and has a marked antiketogenic action.

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1425

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# Determination of Fixed Acids in Commercial Wines by Gas–Liquid Chromatography

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The polybasic acids in wines (succinic, fumaric, malic, tartaric, and citric) were separated from other solid material by precipitating the acids as the lead salts of the respective acids. Trimethylsilyl derivatives were formed directly from the precipitates and were identified and quantitated by vapor phase chromatography. It is interesting to note that blackberry, peach, pineapple, elderberry, loganberry, honey, and cherry wines did not contain tartaric acid in detectable amounts (10 mg 100 cm³ of sample). The citric acid concentration in commercial wines in some cases accounted for approximately 90% of the total fixed acid content. Grape wine in all cases contained malic and tartaric acids and in most cases contained citric acid.

Customarily the separations of carboxylic acids by gic are preceded by the conversion of the acids to alkyl (usually methyl) esters. Since the esters are more volatile, they can be separated at lower temperatures. These esters are less strongly adsorbed than acids and give less tailing; therefore, they can be more accurately determined.

Phenols (Ismail, 1963; Langer *et al.*, 1958) were the first compounds to be separated by gas chromatography as their silyl derivatives. Silyl ester derivatives have been used for dentification of the anomers of pentoses and hexoses by as chromatography (Bentley *et al.*, 1963; Sweeley *et al.*, 963; Martin and Eib, 1968). Flavenoids (Furuya, 1964) d inositols (Lee and Ballou, 1965) have also been converted silyl derivatives for gas chromatography. The silyl ether vatives of large complex molecules such as morphine chmann-Hanssen and Svendsen, 1963; Martin and hart, 1966) and steroids (Hammond and Leach, 1965) ono- and diglycerides (Tallent *et al.*, 1966; Wood *et al.*, ave been chromatographed. The use of silyl esters is a extension of this work to gas partition chromatography gas chromatography of trimethylsilyl esters of fatty rkofer and Donike, 1967; Dalgliesh *et al.*, 1967), ids (Mason and Smith, 1966; Ruhlmann and Gie- 1), metabolic acids (Horii *et al.*, 1965), acids in wine *et al.*, 1967), acids in whiskey (Martin *et al.*, 1965), ids (Burkhard, 1957; Blakley, 1966), resin acids , 1968), and fruit acids (Fernandez-Flores *et al.*,

s reagents that are used to synthesize the alkyl react with alcohol groups such as those on 5. If, however, hydroxycarboxylic acids are trimethylchlorosilane, both the acid and al- roups react to give the trimethyl siloxy group. imethylsilyl derivatives of hydroxycarboxylic acids are more volatile and less easily adsorbed than alkyl esters of hydroxycarboxylic acids. The silyl ether esters are superior to the methyl esters for gas chromatographic separations of hydroxycarboxylic acids (Martin and Swinehart, 1968).

## METHOD

**Apparatus and Reagents.** Gas Chromatograph. A Hewlett-Packard Model 402 gas chromatograph equipped with a flame ionization detector was used for the analysis. This instrument was placed in tandem with a Hewlett-Packard Integrator Model 3370A. The glass column for the instrument was cleaned with acetone and ethanol, and then allowed to air dry. A 250-ml solution of a 5% dimethyldichlorosilane (DMCS) in benzene was passed through the column, after which it was allowed to air dry.

The glass column for the analysis was 6-ft long, 1/4-in. o.d., and was packed with a 3% OV-3 on Chromosorb W HP 80/100 mesh. The glass wool plugs were likewise treated with 5% DMCS in benzene.

The column was operated isothermally for 10 min at 118° C and then programmed at the rate of 3° min to a temperature of 220° C. The detector block and injection port were operated at 250° C. Helium was used as the carrier gas at the rate of 40 ml min.

Lead acetate buffer solution 8 g $Pb(OAc)_2 \cdot 3\ H_2O$ plus 1 ml of acetic acid, and the final solution *qs* to 100 ml with water.

Celite. Celite 545 (Fisher Chemical).

Sulfuric acid. 0.1 *N* sulfuric acid.

Glutaric acid. A 1-mg ml solution of glutaric acid (Applied Science Laboratories, Inc., 99.9% purity) in a 50/50 ethanol water solution.

Standard acid solution. A $5 \times 10^{-2}$ *M* solution each of succinic acid (Fisher Certified), fumaric acid (Eastman Organic Chemicals) 98% + purity, l malic acid (Eastman Organic Chemicals) 98% + purity, tartaric acid (Eastman Organic Chemicals) 98% + purity, and citric acid (Fisher Scientific) anhydrous certified grade were placed in a 50/50 ethanol water solution.

, Internal Revenue Service, Washington,

Table I. Total Acid Values by Titrimetric Method, Individual Acids by the Glc Procedure

| Name | Gas Liquid Chromatography | | | | | Titrimetric | | |
|---|---|---|---|---|---|---|---|---|
| | Milliequivalent | | | | Mg/100 cc. | Mg/100 cc. | | |
| | Succinic Acid | Malic Acid | Tartaric Acid | Citric Acid | Total Fixed Acid* | Fixed Acid* | Volatile Acid* | Total Acids* |
| Apple Wine | 1.253 | 6.205 | | 0.484 | 596.0 | 543.0 | 47.0 | 590.0 |
| Blackberry Wine | 0.627 | 1.193 | | 4.950 | 508.0 | 579.0 | 81.0 | 660.0 |
| Blackberry Wine | 0.881 | 2.685 | | 3.826 | 555.0 | 642.0 | 128.0 | 770.0 |
| Chianti Wine | 1.626 | 1.462 | 2.692 | 0.437 | 467.0 | 554.0 | 76.0 | 630.0 |
| Concord Grape Wine | 1.135 | 0.746 | 2.999 | | 366.0 | 480.0 | 130.0 | 610.0 |
| Danish Blackberry Wine | 0.406 | 0.985 | 0.133 | 2.077 | 270.0 | 295.0 | 232.0 | 527.0 |
| Danish Cherry Wine | 0.745 | 6.862 | 1.346 | 0.250 | 691.0 | 673.0 | 66.0 | 739.0 |
| Dry Red Wine I (Before Treatment) | 0.932 | 0.149 | 4.544 | 0.281 | 518.0 | 533.0 | 108.0 | 641.0 |
| Dry Red Wine II (Before Treatment) | 0.982 | 0.149 | 4.598 | 0.281 | 451.0 | 545.0 | 96.0 | 641.0 |
| Dry Red Wine III (Before Treatment) | 1.745 | Trace | 3.505 | 0.531 | 434.0 | 580.0 | 134.0 | 714.0 |
| Dry Red Wine IV (After Treatment) | 1.423 | Trace | 3.691 | 0.562 | 426.0 | 557.2 | 166.8 | 724.0 |
| Elderberry Wine | 0.999 | 0.567 | | 5.075 | 498.0 | 576.0 | 104.0 | 680.0 |
| Grape Base Wine | 0.999 | 0.627 | 2.559 | Trace | 314.0 | 341.0 | 71.0 | 412.0 |
| Loganberry Wine | 0.627 | 1.119 | | 9.072 | 812.0 | 612.0 | 98.0 | 710.0 |
| Muscatel Wine | 0.881 | 1.298 | 1.626 | 1.499 | 398.0 | 296.0 | 74.0 | 370.0 |
| Peach Wine | | 0.269 | | 2.171 | 183.0 | 193.0 | 186.0 | 379.0 |
| Polish Cherry Wine | 1.135 | 1.566 | | 5.434 | 610.0 | 658.0 | 150.0 | 808.0 |
| Port Wine | 0.406 | 1.477 | 2.132 | 0.312 | 325.0 | 332.0 | 108.0 | 440.0 |
| Pure Cherry | 0.745 | 3.058 | | 2.030 | 438.0 | 508.0 | 92.0 | 600.0 |
| Natural Grape Wine Base | 0.999 | 0.507 | 3.252 | 0.484 | 393.0 | 377.0 | 110.0 | 487.0 |
| Red Dry Wine | 1.507 | 2.506 | 5.735 | | 732.0 | 672.0 | 98.0 | 770.0 |
| Straight Sherry Wine | 1.762 | 0.627 | 2.132 | 0.968 | 412.0 | 304.0 | 86.0 | 390.0 |

* Expressed as Tartaric Acid

Pyridine. Pyridine (Eastman Organic Chemicals) spectrograde was dried for 48 hr over KOH granules.

Silylating reagent. Trimethylchlorosilane (TMS) and hexamethyldisilazane (HMS) were obtained from Applied Science Laboratories, Inc.

Ethanol water solution. 80% anhydrous U.S.P. and 20% distilled water.

**Sample Preparation.** A 2-ml aliquot of wine was placed in a 15-ml centrifuge tube, and 1 ml of lead acetate buffer solution, 10 mg of celite 545, and 1 ml of sulfuric acid were added in that sequence. The precipitate was shaken with 10 ml of ethanol water solution, after which it was centrifuged for 10 min. The tube was removed and the supernatant decanted. The mat was broken with a stirring rod, and a second 10-ml

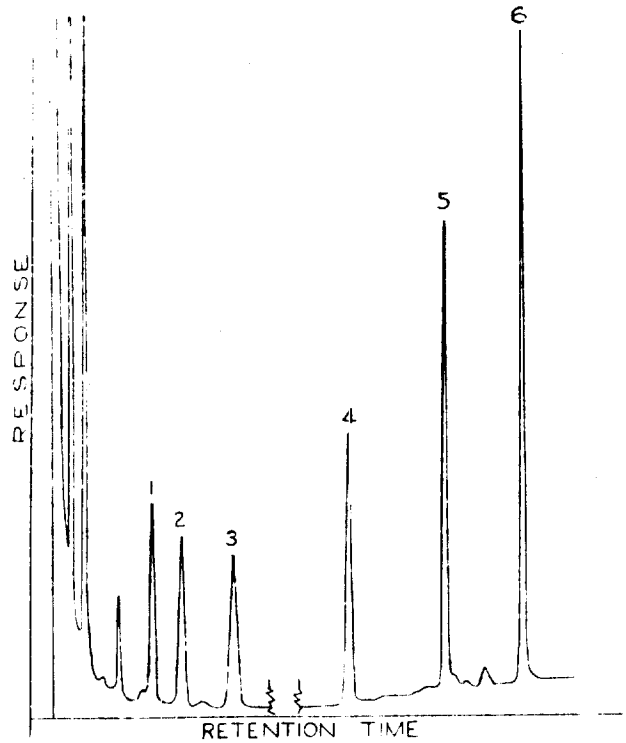


**Figure 1. Chromatogram of a standard solution of acids: 1, succinic; 2, fumaric; 3, glutaric; 4, malic; 5, tartaric; 6, citric**

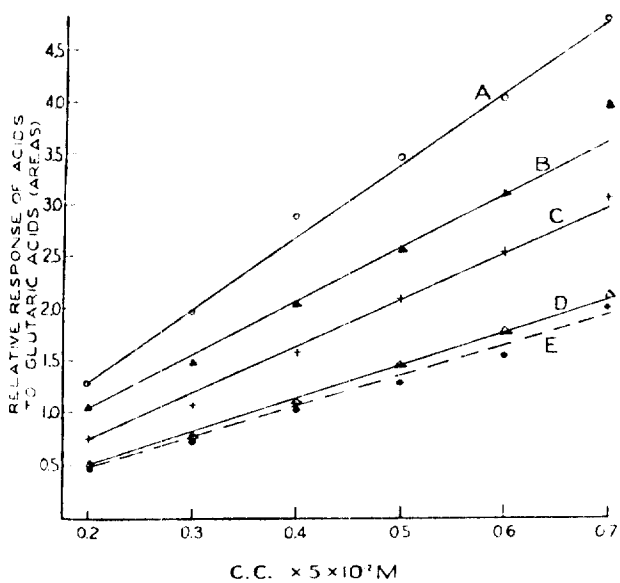


**Figure 2. This gives the relative response of A, citric, B, malic, C, tartaric, D, succinic, and E, fumaric, to glutaric acids *vs.* cm³ molar concentration**

ethanol water solution was employed to wash the precipitate. The mixture was centrifuged for another 10 min, and again the supernatant was decanted. Boiling chips were added, along with 1.5 ml of glutaric acid solution, to the precipitate, and the mixture was placed in an oven for 12 hr at 105° C. Pyridine, 1.5 ml, was added to the mixture, which immediately was capped with a rubber septum and then placed in a sand bath for 24 hr at 137° C. The sample was removed from sand bath, and 0.5 ml of TMS and 0.5 ml of HMS were added. The resulting solution was allowed to stand at room temperature for 4 hr. A 2.5 μl sample was used for the chromatographic analysis.

**Standard Solution.** 0.2, 0.3, 0.4, 0.5, 0.6, and/or 0.7 ml of a $5 \times 10^{-2}$ *M* solution of succinic, fumaric, malic, tartaric, and citric acids were placed in a 15-ml centrifuged tube, respectively. The standard was treated in the same manner as the sample solution.

**Calculation.** The amount of organic acids was calculated in the following manner:

$$\frac{Ao/Gs}{Astd/Gstd} \times Mg \times 50 = \text{mg/100 cm}^3 \text{ of sample}$$

where Ao = area of organic acid, Gs is the area of glutaric acid in sample, Astd is the area of organic acid in standard solution, and Gstd is the area of glutaric acid in standard solution. A blank sample (before introduction of internal standard) was chromatographed to ascertain possible interference from the presence of glutaric acid or other compounds with similar retention times. The recovery of fixed acids by the glc procedure was compared with recovery by titrimetric methods. The amount of fixed acids found by titrimetric methods was determined as the difference between total acids and volatile acids.

The procedural modifications employed were as follows. The end point was determined by a Fisher Automatic Titrimeter at pH 8.4 instead of the visual point of phenolphthalein.

## DISCUSSION

The di- and tricarboxylic acids used in the determination were succinic, fumaric, glutaric, malic, tartaric, and citric. The retention times of the various acids for the standard solution were noted and compared with those of the sample solution.

In prior work, an alkanoic acid ($C_{11}$) was used as an internal standard, but because of its high degree of sublimation and its monocarboxylic character, it necessitated the use of dicarboxylic or tricarboxylic acid not present in wines. Since glutaric acid was not found to occur naturally in any of the samples analyzed by glc it was selected as the internal standard of choice. The various acids were determined in wines, but further studies would be highly desirable as to varieties within a particular type of wine, *i.e.*, catawba grapes wine. Imported and domestic commercial samples were selected in order to provide a fair degree of diversification.

## RESULTS

Figure 1 shows the various acids, namely succinic, fumaric, glutaric, malic, tartaric, and citric, as sharp symmetrical peaks 1, 2, 3, 4, 5, and 6, respectively.

Figure 2 gives linearity of response of molar concentration of acid to glutaric acid by peak areas. The acids fall into a sequence which is proportional to their molecular weights.

Table I gives data for blackberry, cherry, elderberry, apple, loganberry, peach, and various classes of grape wines. In many cases, the citric acid content accounts for 90% of the fixed acid content for the commercial wines. As was expected, the malic acid is found in apples, pineapple, blackberry, loganberry, cherry, grape, elderberry, and peach wines. Tartaric acid is found in wines of grape origin, but it is not found in apple, blackberry, elderberry, loganberry, cherry, or peach wines at detectable levels (10 mg/100 ml).

In most cases, the fixed acid values found by glc procedures are lower than titrimetric values.

Samples of Dry Red Wine I and II, as well as III and IV, show before and after cation exchange treatment. For this particular treatment, there appears to be no significant change in the malic, succinic, citric, or tartaric concentration.

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# Citric and Uric Acid Excretion in the Urine of Healthy Persons and People with Uric Acid Calculi, Before and After Intake of Granules Containing Citric Acid and Citrates

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Summary

"It could be shown that the excretion of citric acid of patients with uric acid stones was not reduced in comparison to that of healthy persons. In fact, after oral application of a mixture of citric acid and citrate the excretion of citric acid in the urine even exceeded that of healthy persons. The excretion of uric acid by patients with uric acid stones was higher than by healthy persons after application of the mentioned mixture. Possible reasons for these facts are discussed."

The solubility-improving effect of alkali metal salts of weak acids on uric acid in the urine after oral application, caused by the alkalizing action of these salts on the urine, has been known for a long time. In this connection, the question arises as to whether onions also might possibly have a solubility-improving effect. Thus, within the framework of the pathogenesis of uric acid calculi, various authors have attributed a special role to citric acid excretion in the urine. Heise and Müller (1966) even describe a lower citrate excretion in all persons carrying uric acid calculi, and consider it as obvious that, as a result of a deficiency of citrates in urine, a still unknown enzyme reaction is possibly involved, which promotes the formation of uric acid calculi. Since these authors, however, provided no data on the level of citrate excretion in persons with uric acid calculi, we have investigated both the citric acid and uric acid excretion in urine, and also the uric acid blood level, in healthy test subjects and in people previously affected by uric acid calculi prior to and after administration of a citric acid-citrate mixture exerting an alkalizing effect on the urine.

## Method

Studies were carried out on 10 healthy test subjects and 15 people previously affected with uric acid calculi, in which no concrements in the urinary tract could be detected by clinical or x-ray examination at the time of the study. Formerly discharged or surgically removed concrements were identified as uric acid calculi by x-ray structural analysis.

All studies were checked prior to and after a 3-day administration of a total daily dose of 10 g of a granular preparation having an alkalizing effect on the urine. This granular preparation had the following composition: dry potassium citrate 46.3, dry sodium citrate 39.0, and dry citric acid 14.5%.

The citric acid and uric acid concentration was determined in urine collected over 24 hours, to which a thymolisopropanol mixture was added. The uric acid concentration in the blood was also checked. The citric acid was determined enzymatically, using citrate lyase. The uric acid concentration in urine and blood serum was also determined enzymatically with uricase.

## Results

The healthy test subjects excreted 513±252 mg citric acid in 24-hour urine, whereas a citric acid excretion of 496±225 mg could be detected in people previously affected by uric acid calculi. The difference between these values had no statistical significance. After administration of the granular preparation, the citric acid excretion in healthy test subjects was 710±246 mg and in people previously carrying uric acid calculi 964±336 mg. In the latter, this increase, with $P<0.001$, and in healthy test subjects, with $P<0.01$, was significant. After administration of the granules, the citric acid excretion of people previously carrying uric acid calculi was higher, with a significance of $P<0.01$, than that of healthy test subjects.

The uric acid excretion in 24-hour urine amounted to 636±249 mg in healthy test subjects and 470±183 mg in people previously affected by uric acid calculi. The difference in these values, with $P<0.05$, is close to a statistical significance. After administration of the granules, the uric acid excretion in healthy test subjects increased to 695±192 mg and in people previously affected by uric acid calculi to 709±182 mg. The increase in uric acid excretion after administration of the granules was significant, with $P<0.01$, while it was not significant in the healthy test results.

The uric acid concentration in serum amounted to 5.73±1.38 mg% in healthy test subjects and 6.49±1.80 mg% in people previously affected by uric acid calculi. The difference in the values was not statistically significant. After administration of the granules, the uric acid concentration in healthy test subjects dropped to 5.17±1.24 mg% and to 6.08±1.94 mg% in people previously carrying uric acid calculi. Whereas in healthy subjects the changes with $P<0.001$ were significant, a significance of the different values could not be detected in the people previously affected by uric acid calculi.

## Discussion of Results

The report of Heise and Müller stating that a reduced citrate excretion is present in all carriers of uric acid calculi could not be confirmed by us, since the citric acid excretion observed in the former carriers of uric acid calculi examined by us was approximately the same as that of healthy test subjects. In contrast to the nondifferent citric acid excretion prior to administration of the granules, a significantly higher citric acid excretion was found in former carriers of uric acid calculi, as compared to healthy control subjects, after intake of the granules. The uric acid excretion in former carriers of uric acid calculi, after administration of the granules, was also considerably more pronounced than in healthy test subjects, whereby the uric acid level in blood serum dropped more clearly in healthy subjects than in former carriers of uric acid calculi. If we want to assume that the increased synthesis of uric acid from glutamine, in connection with the alkalization which takes place, is the cause for the increased uric acid excretion in former carriers of uric acid calculi, then we must say that this assumption appears rather improbable already on the basis of quantitative considerations alone. The increased excretion of uric acid in former carriers of uric acid calculi without a corresponding drop of the serum uric acid level, when the synthesis of uric acid is not increased, allows us rather to assume that the increased amounts of excreted uric acid originate from deposits of uric acid crystalls in the body, whereby a reduced back diffusion of urates from the alkalized urine could possibly play a certain role in this process.

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## Citronensäure- und Harnsäureausscheidung im Urin Gesunder und Harnsäuresteinträger vor und nach Einnahme eines citronensäure-citrathaltigen Granulates*

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Seit langem ist die löslichkeitsverbessernde Wirkung der Alkalisalze schwacher Säuren auf die Harnsäure im Urin nach peroraler Applikation durch deren den Urin alkalisierende Wirkung bekannt. In diesem Zusammenhang ergibt sich die Frage, ob den Anionen möglicherweise auch eine löslichkeitsverbessernde Wirkung zukommt. So wird im Rahmen der Harnsteinpathogenese der Citronensäureausscheidung im Urin von verschiedenen Autoren eine besondere Rolle zugeschrieben. HERTLE und MÜLLER (1966) beschreiben sogar bei allen Harnsäuresteinträgern eine verminderte Citratausscheidung und sehen es als naheliegend an, daß durch das mangelnde Vorhandensein von Citraten im Urin eine zunächst noch unbekannte Fermentreaktion möglich sei, die die Harnsäuresteinbildung begünstige. Da von den Autoren aber keine Angaben über die Höhe der Citratausscheidung bei Harnsäuresteinträgern gemacht wurden, untersuchten wir neben der Citronensäureausscheidung die Harnsäureausscheidung im Urin sowie den Blutharnsäurespiegel bei gesunden Probanden und ehemaligen Harnsäuresteinträgern vor und nach Verabfolgung eines den Urin alkalisierenden Citronensäure-Citratgemisches.

### *Methodik*

Die Untersuchungen wurden an 10 gesunden Probanden und an 15 ehemaligen Harnsäuresteinträgern, bei denen zum Zeitpunkt der Untersuchung weder klinisch noch röntgenologisch ein Konkrement in den Harnwegen nachzuweisen war, durchgeführt. Früher abgegangene bzw. operativ entfernte Konkremente wurden mit Hilfe der Röntgenstrukturanalyse als Harnsäuresteine identifiziert.

Sämtliche Untersuchungen wurden vor und nach dreitägiger Verabreichung von täglich insgesamt 10 g eines den Urin alkalisierenden Granulates geprüft. Das Granulat hatte die folgende Zusammensetzung: Kalium citr. sicc. 46,3; Natrium citr. sicc. 39,0; Acidum citr. sicc. 14,5.

Im 24 Std-Sammelurin, dem ein Thymol-Isopropanol-Gemisch zugesetzt worden war, wurde die Citronensäurekonzentration und die Harnsäurekonzentration bestimmt. Ebenso wurde die Blutharnsäurekonzentration untersucht. Die Citronensäurebestimmung erfolgte enzymatisch mit Citrat-Lyase. Die Harnsäurekonzentration im Urin und Blutserum wurden ebenfalls enzymatisch mit Uricase bestimmt.

### *Ergebnisse*

Die gesunden Probanden schieden im 24 Std-Urin 513 ± 252 mg Citronensäure aus, während bei den ehemaligen Harnsäuresteinträgern eine Citronensäureausscheidung von 496 ± 225 mg nachgewiesen werden konnte. Die Differenz der Werte war ohne statistische Signifikanz. Nach Verabfolgung des Granulates betrug die Citronensäureausscheidung bei den gesunden Probanden 710 ± 246 mg und bei den ehemaligen Harnsäuresteinträgern 964 ± 336 mg. Bei den Harnsäuresteinträgern war der Anstieg mit $P < 0{,}001$ und bei den gesunden Probanden mit $P < 0{,}01$ signifikant. Nach Gabe des Granulats lag die Citronensäureausscheidung der ehemaligen Harnsäuresteinträger mit einer Signifikanz von $P < 0{,}01$ höher als bei den gesunden Probanden.

Die Harnsäureausscheidung im 24 Std-Urin betrug bei den gesunden Probanden 636 ± 249 mg und bei den ehemaligen Harnsäuresteinträgern 470 ± 183 mg. Der Unterschied der Werte liegt mit $P < 0{,}05$ nahe einer statistischen Signifikanz. Nach Verabfolgung des Granulates stieg bei den gesunden Probanden die Harnsäureausscheidung auf 695 ± 192 mg und bei den Harnsäuresteinträgern auf 709 ± 182 mg an. Der Anstieg der Harnsäureausscheidung nach Gabe des Granulats war mit $P < 0{,}01$ signifikant, bei den gesunden Probanden dagegen nicht signifikant.

Die Konzentration der Harnsäure im Serum betrug bei den gesunden Probanden 5,73 ± 1,38 mg-% und bei den Harnsäuresteinträgern 6,49 ± 1,80 mg-%. Die Differenz der Werte war statistisch nicht signifikant. Nach Verabfolgung

* Durchgeführt mit Unterstützung der Deutschen Forschungsgemeinschaft.

des Granulats sank die Konzentration der Harnsäure bei den gesunden Probanden auf 5,17 ± 1,24 mg-% und bei den ehemaligen Harnsäuresteinträgern auf 6,08 ± 1,94 mg-% ab. Während bei den Gesunden die Veränderungen mit $P < 0{,}001$ signifikant waren, war eine Signifikanz der Differenzen bei den ehemaligen Harnsäuresteinträgern nicht nachzuweisen.

*Besprechung der Ergebnisse*

Die Mitteilung von HEISE und MÜLLER, daß bei allen Harnsäuresteinträgern eine verminderte Citratausscheidung im Urin vorlage, konnte von uns nicht bestätigt werden, da die Citronensäureausscheidung der von uns untersuchten ehemaligen Harnsäuresteinträger etwa der der gesunden Probanden entsprach. Im Gegensatz zu der nicht unterschiedlichen Citronensäureausscheidung vor Gabe des Granulates ergab sich aber nach Einnahme des Granulates bei den Harnsäuresteinträgern eine signifikant höhere Citronensäureausscheidung als bei den gesunden Kontrollpersonen. Auch war die Harnsäureausscheidung nach Gabe des Granulates bei den ehemaligen Harnsäuresteinträgern wesentlich deutlicher ausgeprägt als bei den gesunden Probanden, wobei der Serumharnsäurespiegel bei den Gesunden aber deutlicher absank, als bei den ehemaligen Harnsäuresteinträgern. Als Ursache für die vermehrte Harnsäureausscheidung bei den ehemaligen Harnsäuresteinträgern eine vermehrte Synthese von Harnsäure aus Glutamin in Zusammenhang mit der erfolgten Alkalisierung annehmen zu wollen, erscheint allem schon auf Grund quantitativer Überlegungen ziemlich unwahrscheinlich. Die vermehrte Ausscheidung der Harnsäure bei den ehemaligen Harnsäuresteinträgern ohne entsprechendes Absinken des Serumharnsäurespiegels bei nicht vermehrter Harnsäuresynthese läßt eher die Vermutung zu, daß die vermehrt ausgeschiedene Harnsäure aus Ablagerungen von Harnsäurekristallen im Körper stammt, wobei eine verminderte Rückdiffusion der Urate aus dem alkalisierten Urin eine gewisse Rolle mitspielen könnte.

*Zusammenfassung.* Die Untersuchungen zeigten, daß die Citronensäureausscheidung bei Harnsäuresteinträgern gegenüber gesunden Probanden nicht vermindert ist und daß sie nach Einnahme von citronensäure-citrathaltigem Granulat gegenüber Gesunden sogar ansteigt. Auch war die Harnsäureausscheidung nach Gabe des Granulats bei Harnsäuresteinträgern wesentlich deutlicher ausgeprägt als bei gesunden Probanden. Über mögliche Ursache dieser Erscheinung wird diskutiert.

*Summary.* It could be shown that the excretion of citric acid of patients with uric acid stones was not reduced in comparison to that of healthy persons. In fact, after oral application of a mixture of citric acid and citrate the excretion of citric acid in the urine even exceeded that of healthy persons. The excretion of uric acid by patients with uric acid stones was higher than by healthy persons after application of the mentioned mixture. Possible reasons for these facts are discussed.

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# METHOD FOR REMOVING CATIONIC RADIONUCLIDES FROM MILK

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SUMMARY

A new method for the removal of radioactive strontium, barium, and cesium from milk has been developed. Fresh samples of raw whole milk with added $Sr^{85}$, $Ba^{140}$, or $Cs^{134}$ were stored at 4° C. for at least 72 hr. The samples were then adjusted to a pH of 5.4 or 5.3 with 0.1 or 0.5 *M* citric acid and passed through a column containing Dowex 50W-X8 cationic resin in the CaMgKNa cycle. The resin was previously charged with a mixed salt solution containing Ca, Mg, K, and Na chlorides in approximately the same relative proportions as in the milk, but at five times their usual concentrations, and at the same pH of acidified milk. After treatment, the milk was mixed with Dowex 2-X8 anionic resin in the OH cycle, by the batch process, and the pH was brought to 6.6. The milk was filtered through a cheese cloth to remove the spent resin. Analysis of treated milk indicated that approximately 90-95% of $Sr^{85}$, 85-95% of $Ba^{140}$, and 75% of $Cs^{134}$ were removed by this treatment from 25 resin-bed volumes of milk. The removal of radionuclides did not vary with flow rates of 5-25 ml. per minute and the cationic composition of the milk was essentially unchanged. Although the milk was not coagulable by rennin, addition of 2-3 ml. of 1 *M* $CaCl_2$ solution per liter of milk yielded coagulum comparable to that obtained with untreated milk. Further treatment of the final product with a CaMgKNa resin at pH 6.6 resulted in an additional removal of approximately 45, 30, and 90% of residual $Sr^{85}$, $Ba^{140}$, and $Cs^{134}$ remaining after the initial treatment without further change in the gross cationic composition of the milk. Results obtained at pH 5.2-5.3 with milk labeled in vivo and in vitro were similar.

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Since the advent of nuclear weapons testing, the radionuclide contamination of foods has been of concern. Surveillance studies have indicated significant amounts of biologically important radionuclides such as strontium[89] ($Sr^{89}$), strontium[90] ($Sr^{90}$), iodine[131] ($I^{131}$), barium[140] ($Ba^{140}$), and cesium[137] ($Cs^{137}$) in milk (2). Because of the health hazards associated with some of these contaminants, particularly $Sr^{90}$, methods have been sought to minimize their levels in marketed dairy products.

This problem may be approached either at the point of production on the dairy farm (6, 11) or at the processing plants before the product reaches the consumer. Control at the dairy farm may require modification of farm and animal husbandry practices. Although this approach might accomplish an over-all reduction in the concentration of radionuclides thus far observed in milk, it would not be effective in an emergency situation involving higher exposures. Under such circumstances, treatment of milk would be necessary to reduce contamination to safe levels. This treatment must be simple, economical,

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and highly effective in removing the radionuclides in question and should be compatible with the practices and equipment employed in the milk plant. The treated product would be required also to meet established criteria of quality, including those relating to mineral balance, physical stability, palatability, and sanitation.

Several laboratory studies have been made on the removal of radioactive strontium, cesium, and iodine from milks labeled in vitro and in vivo. Ion exchange techniques using commercially produced synthetic ion exchange resins or naturally occurring materials such as bone preparations and calcium phosphate have been applied.

Pulverized inorganic bone preparations were found to remove 50-70% of $Sr^{85}$ from in vitro labeled milk, but the treatment decreased the calcium content appreciably (12). Synthetic cation exchange resins in the $Na^+$, $K^+$, or $Ca^{++}$ forms have been used extensively. Evidence has been presented to show the effectiveness of these resins in the removal of radiostrontium (3-5, 7, 10) and radiocesium (7, 8) from milks labeled in vitro and in vivo. Both batch and column techniques have been employed. The removal of up to 90% of strontium and cesium was always accompanied by gross changes in the cationic composition of the milk (4, 5, 10, 12) and in some of its physical and chemical properties such as pH, titrable acidity, curd tension, and rennin coagulation time (4). Removal of radionuclides from milk labeled in vitro was always higher than from milk labeled in vivo. Milk equilibrated [2] with added radionuclides for at least 72 hr. in vitro gave results comparable to those obtained with milk labeled in vivo (8).

To maintain the cationic composition of milk unaltered, a resin charged with a synthetic mixed salt solution simulating the major cations in milk was found necessary. Although this technique preserved the cationic composition essentially unchanged, the removal of strontium was only 66% and of cesium 85%. To remove higher percentages of strontium a shift in equilibrium was accomplished in which the colloidally bound divalent cations were removed by use of pairs of CaKNa resins. In the first treatment Ca and Sr both were removed; in the second, the resin gave up Ca and removed excess Na from milk. This procedure removed approximately 85% of the strontium content (8). Although this careful manipulation of cationic composition of milk to remove Sr is possible by the batch process, its suitability for column techniques, especially to handle large volumes of milk, is questionable.

From the foregoing observations it is evident that the removal of radionuclides from milk can be achieved only if the bound ions are transformed into more readily exchangeable forms. The physico-chemical nature of milk is such that alteration of its pH from 6.6 to 5.2 will shift the equilibria so that the colloidally bound ions are released to become free ionic species. Thus, passing acidified milk through a suitable cation exchange resin charged with synthetic

[2] The term equilibrate refers to the procedure whereby milk with added tracer is allowed to stand for a given length of time to arrive at a condition of equilibrium between the tracer and other constituents of milk.

mixed salt solution under appropriate conditions should result in the removal of a high percentage of radionuclides without any significant change in cationic composition. Neutralization of acidified and resin-treated milk to pH 6.6 with an anionic resin in the hydroxyl cycle should restore some of the original physical and chemical properties, resulting in an acceptable product.

An investigation of these possibilities has resulted in the method described here.

## EXPERIMENTAL PROCEDURE

*Materials.* Milk labeled in vitro was prepared by adding an aliquot of a $Sr^{85}$, $Cs^{134}$, or $Ba^{140}$ solution of known activity to milk and allowing the mixture to equilibrate at 4° C. for at least 72 hr. before use. Milk labeled in vivo was obtained from goats dosed orally with approximately 85 $\mu c.$ of $Sr^{85}$ and 200 $\mu c.$ of $Ca^{45}$ and was obtained from the Laboratory of Radiation Biology, Department of Physical Biology, Cornell University, Ithaca.

The cation exchange resin used was Dowex 50W-X8 ($H^+$), 20-50-mesh size, with a total exchange capacity of approximately 2.0 meq/ml of resin in the wet form.

The anion exchange resin used was Dowex 2-X8 ($Cl^-$), 20-50-mesh size, and having a total exchange capacity of approximately 1.33 meq/ml of wet resin.

*Resin regeneration procedure.* Since the Dowex 50W-X8 resin, as purchased, was in the hydrogen form, it was necessary to regenerate it on a calcium, potassium, and sodium (CaKNa) or a calcium, magnesium, potassium, and sodium (CaMgKNa) cycle. The regeneration of the resin was performed as follows: First, the cationic composition of milk was determined according to the rapid technique of Murthy and Whitney (9). A mixed salt solution was prepared to contain Ca, Mg, K, and Na in the same relative proportions as in the milk but in five times the concentration, and the pH was adjusted to be the same as that of the acidified milk, with 0.1 *M* HCl. The solution was passed down-flow through a glass column (2.3 by 20 cm.) containing 30 g. of the resin, at approximately 4-5 ml. per minute, until the pH and composition of the effluent were the same as of the influent. After this equilibrium level was reached, excess salts were removed by washing the resin with distilled water.

The anion exchange resin was converted from the chloride to the hydroxyl form by treating the resin with 5% NaOH. After the resin was fully charged, excess NaOH was removed by washing the resin with distilled water.

*Procedure.* Two and one-half liters of milk equilibrated with added radioactive tracer was placed in a three-liter beaker. Citric acid solution (0.1 or 0.5 *M*) was added from a burette and the milk was stirred constantly with a mechanical stirrer until the pH reached 5.4 or 5.3. The milk was transferred to a suitable container from which it was allowed to flow through the exchange column containing 30 g. of Dowex 50W-X8 cationic resin in the CaMgKNa cycle, at 10-15 ml. per minute. The milk was collected in a beaker and 5-g. portions of Dowex 2-X8 anionic resin in the $OH^-$ cycle were added with stirring until the pH reached 6.6. The milk was filtered through a cheese cloth to remove the spent resin.

In the experiments concerning the removal of radiocesium, the neutralized milk was passed through a second column containing CaMgKNa resin at pH 6.6.

*Analytical methods.* Throughout this investigation the following analytical methods were employed:

1. pH was determined using a Beckman Model Zeromatic pH meter.
2. Calcium, magnesium, potassium, and sodium were determined by flame spectrophotometry, similar to the technique of Murthy and Whitney (9).
3. Radioactivity was determined by gross $\gamma$-counting or by gamma spectroscopy according to a method described by Branson *et al.* (1).
4. Rennin coagulation tests were performed as follows: To 50 ml. of milk maintained at 40.0 ± 0.1° C. in a water bath, was added 2 ml. of a dilute rennin solution (1 ml. of commercial rennet extract diluted to 50 ml. with distilled water) and mixed well. The time elapsing between the introduction of rennin and the first visual signs of coagulation was taken as the coagulation time.
5. Flavor score was determined after pasteurizing milk samples at 145° F. for 30 min., using a slightly modified American Dairy Science Association scoring guide (13).

## RESULTS AND DISCUSSION

*Resin regeneration characteristics.* An ideal system for charging the resin is probably the milk itself, because this would eliminate all the possible errors inherent in the preparation of synthetic salt solution. To determine what kind of results can be obtained under such conditions for the removal of radionuclides, 30 g. of Dowex 50W-X8 ($Na^+$) resin placed in a column was charged with 1.5 liters of milk at pH 6.6. Excess milk was removed by washing the resin with distilled water. Another portion of the same milk, previously equilibrated with $Sr^{85}$, was passed through the resin column. Various fractions of the effluent milk were analyzed for $Sr^{85}$ activity and cationic composition. From results presented in Table 1 it can be seen that the cationic composition of the original and the resin-treated milks are not significantly different. A constant

TABLE 1

Effect of CaMgKNa resin treatment on the cationic composition of milk and the removal of $Sr^{85}$ and $Cs^{134}$ (resin charged with milk at pH 6.6)

| Sample | Ca | Mg | K | Na | Removal of $Sr^{85}$ | Removal of $Cs^{134}$ |
|---|---|---|---|---|---|---|
| | (g/liter) | | | | (%) | |
| Untreated | 1.20 | 0.19 | 1.39 | 0.47 | ... | ... |
| Treated | | | | | | |
| 1st 100-ml. effluent | 1.17 | 0.16 | 1.41 | 0.47 | 52 | 100 |
| 4th 100-ml. effluent | 1.18 | 0.17 | 1.44 | 0.48 | 50 | 96 |
| 8th 100-ml. effluent | 1.19 | 0.16 | 1.45 | 0.49 | 47 | 71 |
| 12th 100-ml. effluent | 1.20 | 0.18 | 1.45 | 0.49 | 45 | 37 |
| 16th 100-ml. effluent | 1.21 | 0.20 | 1.41 | 0.48 | 41 | 6 |
| 20th 100-ml. effluent | 1.16 | 0.18 | 1.43 | 0.48 | 39 | 3 |
| 24th 100-ml. effluent | 1.18 | 0.18 | 1.44 | 0.50 | 36 | 0 |
| 28th 100-ml. effluent | 1.17 | 0.17 | 1.42 | 0.47 | 33 | 0 |
| Average (treated) | 1.18 | 0.17 | 1.43 | 0.49 | | |

removal of approximately 45-50% of $Sr^{85}$ was achieved from treated milk up to 30 resin-bed volumes. These experiments demonstrate the feasibility of using resin treatment for the removal of radionuclides without significant alteration in the concentration of the major cationic composition. It is clear, however, that this method of charging has no practical applicaion. Exploratory studies were conducted to determine the composition and concentration of salt solutions for charging resins. Fifty-gram portions of Dowex 50W-X8 ($H^+$) resin were charged with 5 × and 10 × the concentration of Ca, Mg, K, and Na (as chlorides) present in milk, as described under Resin Regeneration Procedure, until the pH and the cationic composition of the influent and the effluent were similar. Results indicated that approximately 1,100 ml. of 5× or 950 ml. of 10× CaMgKNa solution was required to equilibrate the resin. When 10× CaMgKNa solution was used for charging, monovalent cations were preferentially adsorbed on the resin, whereas with dilute solutions the observed preference follows as Ca > Mg > K > Na. However, with 5× CaMgKNa solution, the equilibrium condition achieved was such that passage through the column of 1× CaMgKNa solution similar to that of milk showed no significant changes in the cationic composition of the effluent solution.

To determine the effect of treating milk with resins charged as above on the cationic composition and the removal of radionuclides from milk, portions of milk equilibrated with $Sr^{85}$ were treated with the resin as described under Procedure. Results showed that while a constant removal of 90-95% of $Sr^{85}$ was obtained from both the columns up to 25 resin-bed volumes of milk, the cationic composition of milk (Table 2) from 10 ×-CaMgKNa resin column was significantly altered, whereas the cationic composition of milk from 5 ×-CaMgKNa resin column remained essentially the same. Therefore, 5 ×-CaMgKNa salt solutions were employed for charging resins in all subsequent experiments.

TABLE 2

Effect of 5 ×-CaMgKNa and 10 ×-CaMgKNa resin treatments on the cationic composition of milk (resin charged with synthetic salt solution at pH 5.4)

| Concentration of charging solution | Sample | Ca | Mg | K | Na |
|---|---|---|---|---|---|
| | | (g/liter) | | | |
| 5 × | Untreated | 1.15 | 0.13 | 1.36 | 0.50 |
| | Treated | | | | |
| | 1st 100-ml. effluent | 1.14 | 0.16 | 1.36 | 0.48 |
| | 4th 100-ml. effluent | 1.13 | 0.13 | 1.38 | 0.48 |
| | 8th 100-ml. effluent | 1.14 | 0.14 | 1.38 | 0.50 |
| | 12th 100-ml. effluent | 1.12 | 0.15 | 1.40 | 0.49 |
| | Composite | 1.14 | 0.14 | 1.40 | 0.49 |
| 10 × | Untreated | 1.14 | 0.19 | 1.46 | 0.50 |
| | Treated | | | | |
| | 1st 100-ml. effluent | 0.94 | 0.15 | 1.52 | 0.54 |
| | 4th 100-ml. effluent | 1.00 | 0.20 | 1.59 | 0.49 |
| | 8th 100-ml. effluent | 1.06 | 0.24 | 1.48 | 0.51 |
| | 12th 100-ml. effluent | 1.08 | 0.23 | 1.44 | 0.50 |
| | Composite | 1.02 | 0.20 | 1.51 | 0.52 |

*Effect of flow rates on the removal of $Sr^{85}$.* Commercial processing requires handling of large volumes of milk and, therefore, the evaluation of any strontium removal method must take this point into consideration. To obtain information on capacity or speed of the resin method, portions of milk equilibrated with $Sr^{85}$ for 24 hr. and acidified to pH 5.3 were passed through columns containing 30 g. of CaMgKNa resin at flow rates of 5, 10, 15, 20, 30, and 35 ml. per minute, respectively. When analyzed for radioactivity, the corresponding effluents indicated $Sr^{85}$ removals 97, 96, 97, 96, 95, and 91%. This suggested that increase of flow rate from 5 to 25 ml. per minute did not seriously reduce the removal of radionuclides from milk in the test columns, and that with larger columns a higher flow rate might be used.

*Effect of acidification of milk on the removal of radionuclides.* The divalent cations such as calcium, magnesium, strontium, and barium in milk are distributed between the serum and colloidal phases. That portion of salts bound with the colloids is not readily available for ion exchange. Acidification of milk ionizes colloidally bound salts which then become available for exchange. For acidifying milk, a suitable nontoxic acid is required. Since citric acid is a natural constituent of milk, and is used as a food additive, it was selected for this purpose. To determine the effect of acidification of milk on the removal of $Sr^{85}$, $Ba^{140}$, and $Cs^{134}$, 2-gal. lots of raw whole milk were equilibrated with added $Sr^{85}$, $Ba^{140}$, or $Cs^{134}$. Each lot was divided into several samples, adjusted to pH values ranging from 5.2 to 6.6 as described under Procedure, and passed through columns containing CaMgKNa resin at the same pH as the respective acidified milk sample. The 1st, 4th, 8th, 12th, etc., 100-ml. aliquots of effluents were collected and analyzed for radioactivity by gross $\gamma$-counting or by $\gamma$-spectroscopy. To determine the $Ba^{140}$ activity, the various samples were stored at 4° C. for 2 wk. to achieve transient equilibrium between $Ba^{140}$ and its daughter $La^{140}$. From the gamma spectra the concentrations of $Sr^{85}$, $Cs^{134}$, and $Ba^{140}$ were computed from counting rates at the 510, 790, and 1,600 Kev. photopeaks, respectively. Results as presented in Figures 1 and 2 show that removal of $Sr^{85}$ and $Ba^{140}$ are affected by the degree of acidification, the removal dropping from approximately 98 to 53% of $Sr^{85}$, and 94 to 23% of $Ba^{140}$, as pH was increased from 5.2 to 6.6. Removal of more $Sr^{85}$ than $Ba^{140}$ at any given pH suggests a more tenacious binding of Ba than of Sr in the colloidal-protein phases of milk. To remove a high percentage of divalent cationic radionuclides, apparently it is necessary to reduce the pH of milk to 5.3-5.4. When lowered to 5.2 the pH becomes critical to protein stability, although the ions are in the dissolved state.

Removal of $Cs^{134}$ was not affected by acidification of milk. This is to be expected, since monovalent cations are in the free ionic state in normal milk. However, the $Cs^{134}$ was not removed as effectively as $Sr^{85}$ or $Ba^{140}$, probably because of preferential adsorption of divalent rather than monovalent cationic radionuclides.

*Comparison of $Sr^{85}$ removal from milk labeled in vivo and in vitro.* Because of the differences observed by other investigators in removal of radioactive

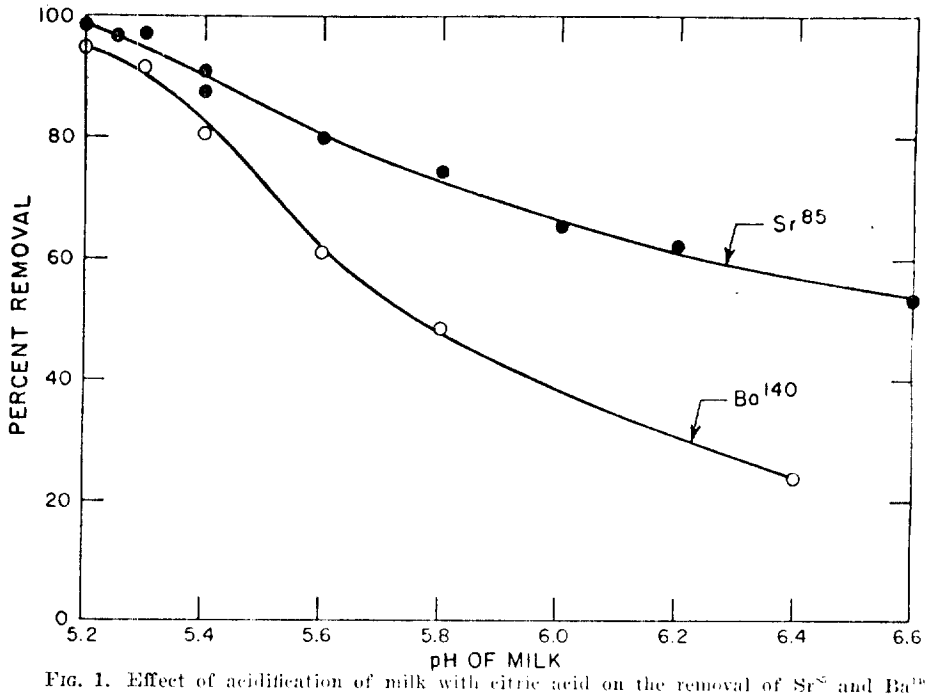


FIG. 1. Effect of acidification of milk with citric acid on the removal of $Sr^{85}$ and $Ba^{140}$ from milk.

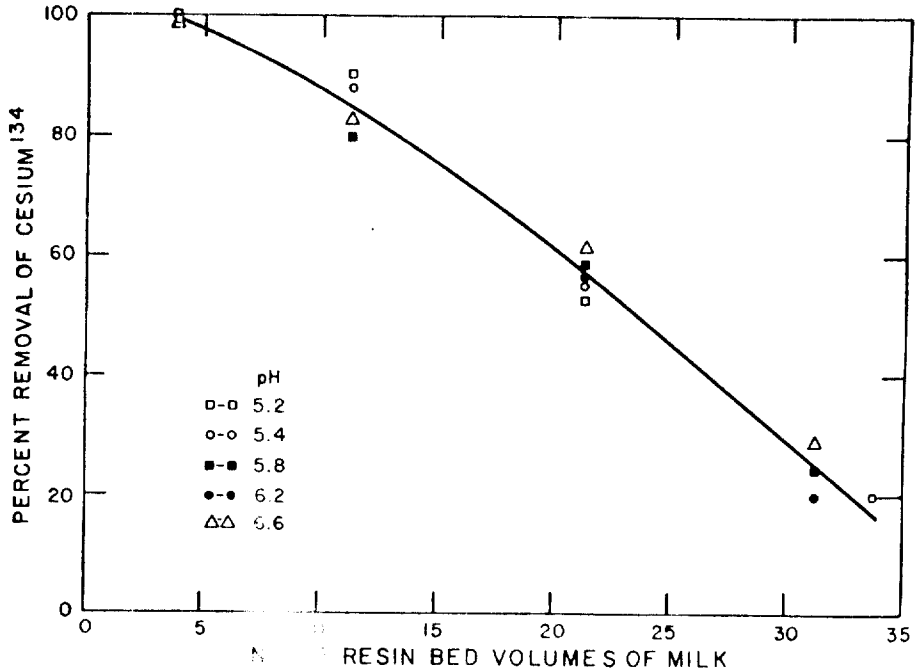


FIG. 2. Effect of acidifi[illegible] milk with citric acid on the removal of $Cs^{134}$ from milk.

strontium from milk labeled in vivo and in vitro, it was considered important to establish whether any significant differences existed between the milk samples thus labeled, especially when processed at pH 5.2-5.4. For this purpose, samples of goats milk labeled in vitro and in vivo were employed. The milk samples were divided into several portions, adjusted to pH values of 6.5, 6.2, 5.8, 5.4, and 5.25, as described before, and treated with cationic resin in the CaKNa cycle, charged at the same respective pH values. Results presented in Figure 3 show that at pH 5.2-5.3 approximately 93% of $Sr^{85}$ was removed from both types of labeled milk, whereas at higher pH values the amount of $Sr^{85}$ removed differed significantly with the labeling method. At normal pH of milk (6.65), a difference of approximately 12% was observed, which is in conformity with the published results (14). Therefore, it is reasonable to assume that the results obtained with properly equilibrated milk labeled in vitro, at pH of 5.2-5.3, will be essentially the same as those to be expected with milk labeled in vivo. These limitations of pH apply to removal of colloidally bound radionuclides. For removal of monovalent cationic radionuclides such as cesium, which occur in a free ionic state, results obtained with milk labeled in vitro and in vivo should be comparable at all the pH values studied herein.

*Effect of number of resin-bed volumes of milk passed through the column on removal of radionuclides.* To determine the volume of milk that could be treated with a given amount of resin for high percentage removal of radionuclides from milk at a constant rate, several trials were made similar to the technique de-

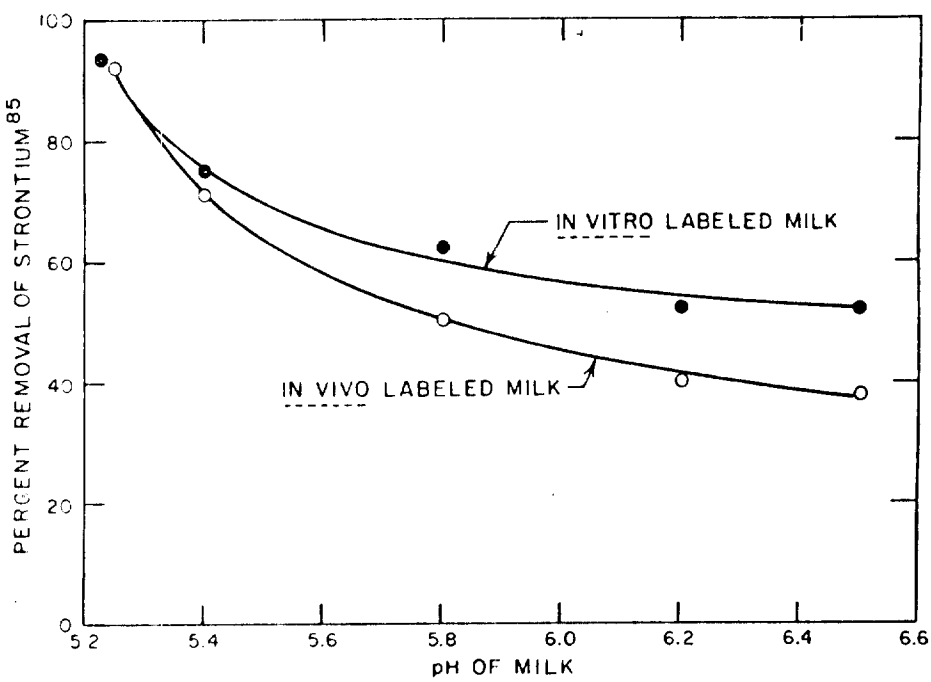


FIG. 3. Effect of pH on the removal of $Sr^{85}$ from milks labeled in vivo and in vitro.

scribed before, using $Sr^{85}$, $Ba^{140}$, and $Cs^{134}$. Results are presented in Figure 4. These data show a constant removal of 94 ± 2% of $Sr^{85}$ and 92 ± 2% of $Ba^{140}$ up to 30 resin-bed volumes of milk, beyond which a gradual decrease was observed. The behavior of $Cs^{134}$ is quite striking, in that its removal decreased from 98 to 0% with increase in the number of resin-bed volumes of milk from 5 to 50, revealing that this procedure was unsuitable for the removal of radiocesium.

*Modification of the procedure for the removal of $Cs^{134}$.* Since the previously described procedures for the removal of $Sr^{85}$ and $Ba^{140}$ were not satisfactory for the removal of radiocesium, certain modifications of the techniques were investigated. The hypothesis was advanced that when citric acid and resin treated milk is neutralized to pH 6.6 with an $OH^-$ cycle anion exchange resin, the concentrations of $Ca^{++}$, $Mg^{++}$, $Sr^{++}$, and $Ba^{++}$ in solution are reduced below that in the untreated milk by the complexing effect of these ions with the colloids and excess citrates. On the other hand, the monovalent cations such as $Na^+$, $K^+$, and $Cs^+$ do not form any such complexes and, therefore, would be present as free ions readily available to exchange reactions. To obtain information in this regard, milk equilibrated with added $Cs^{134}$ was processed as described under Procedure, then passed through a second column containing CaMgKNa resin at pH 6.6. Results are presented (Figure 5). From 100 to

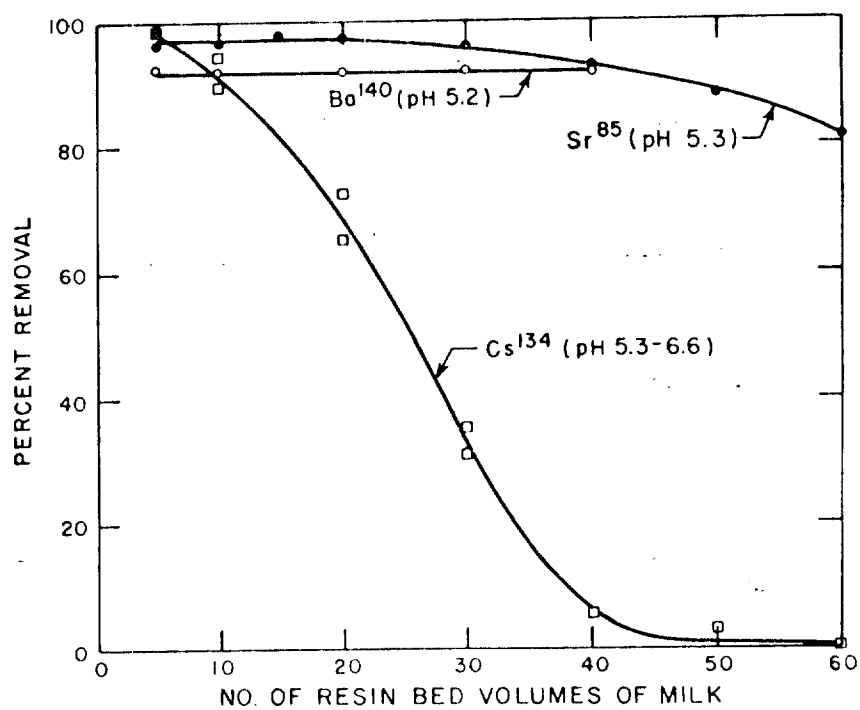


FIG. 4. Effect of number of resin bed volumes of milk passed through the column on the removal of $Sr^{85}$, $Ba^{140}$, and $Cs^{134}$ from milk.

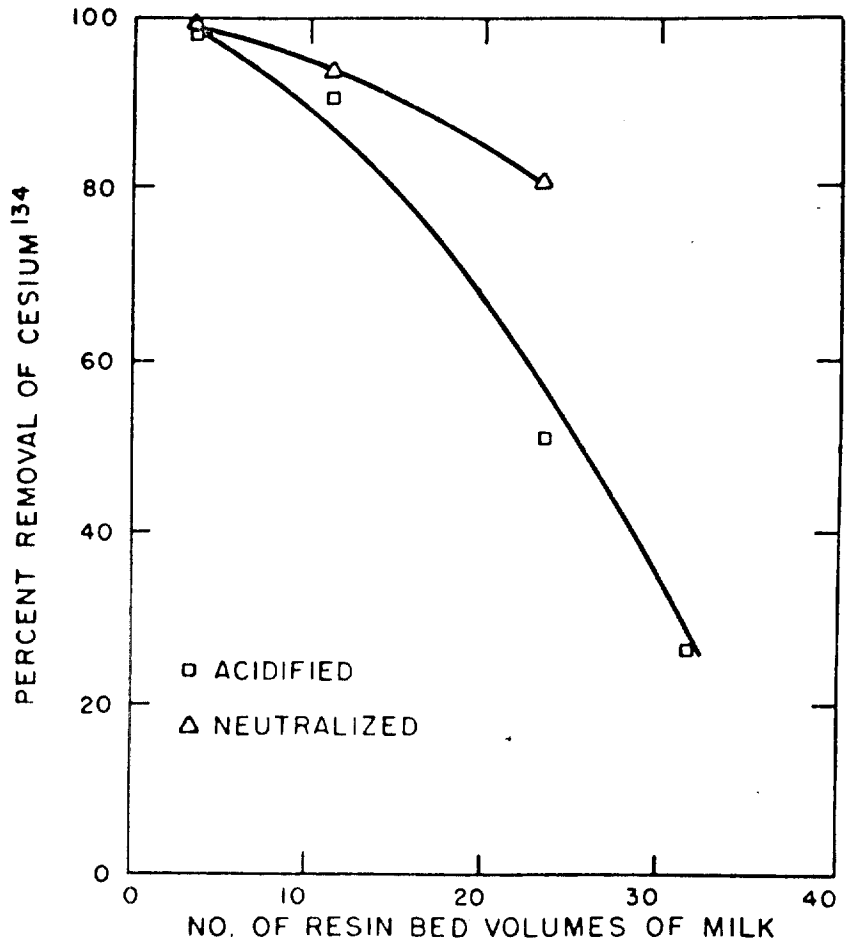


FIG. 5. Effect of acidification, CaMgKNa resin treatment at pH 5.3, and neutralization to pH 6.6, CaMgKNa resin treatment at pH 6.6 in succession on the removal of $Cs^{134}$ from milk.

80%, with an average of 90%, of the residual $Cs^{134}$ was removed by this neutralization and the second resin treatment.

As the pH of neutralization was increased above 6.6, more $Cs^{134}$ was removed (99% at pH 7.7), indicating stabilization of the divalent cation complexes with citrates and colloids. However, pH values above 6.6 were considered unsatisfactory from the point of acceptability of the final finished product. This treatment also removed approximately 45 and 30% of the residual $Sr^{85}$ and $Ba^{140}$ at pH 6.6 and, therefore, suggests that neutralization to pH 6.6 does not completely reduce the concentration of divalent cations to be unavailable for ion exchange reactions. Since more $Cs^{134}$ was removable by this neutralization and second resin treatment than from unacidified milk at pH 6.6, it makes the hypothesis valid up to a certain extent only. The over-all effect of these two-

column resin treatments resulted in the removal of 95-98% of $Sr^{85}$, 90-95% of $Ba^{140}$, and 95-100% of $Cs^{134}$.

*Cationic composition of milk.* Untreated milk and milk treated with cationic and anionic resin, as employed in some of the experiments described before, were analyzed for the major cations. The results are presented in Tables 3 and 4, which show that the Ca, Mg, K, and Na content is essentially the same in the untreated and treated milks. This indicates that the method described herein may be successfully employed for the removal of cationic radionuclides from milk.

*Rennet coagulation studies.* Several fresh samples of raw whole milk were acidified, resin-treated, and neutralized as described before. When rennet solution was added to portions of these samples, no coagulation was observed. This is to be expected because of the complexing effect of citrates with calcium, thus making calcium unavailable for the formation of coagulum. This is evident.

TABLE 3

Effect of CaMgKNa resin treatment at pH 5.4 and neutralization to pH 6.6 with $OH^-$ resin on the cationic composition of milk

| Sample | Treatment | Ca | Mg | K | Na |
|---|---|---|---|---|---|
| | | (g/liter) | | | |
| 1 | Before | 1.15 | 0.13 | 1.36 | 0.50 |
| | After | 1.14 | 0.14 | 1.38 | 0.49 |
| 2 | Before | 1.12 | 0.10 | 1.32 | 0.44 |
| | After | 1.12 | 0.10 | 1.28 | 0.44 |
| 3 | Before | 1.11 | 0.19 | 1.32 | 0.49 |
| | After | 1.12 | 0.17 | 1.36 | 0.50 |
| 4 | Before | 1.14 | 0.18 | 1.43 | 0.49 |
| | After | 1.14 | 0.15 | 1.42 | 0.48 |

TABLE 4

Effect of CaMgKNa resin treatment at pH 5.4, neutralization to pH 6.6, and CaMgKNa resin treatment at pH 6.6, in succession on the cationic composition of milk

| Treatment | Sample | Ca | Mg | K | Na |
|---|---|---|---|---|---|
| | | (g/liter) | | | |
| | Untreated | 1.15 | 0.13 | 1.36 | 0.50 |
| Column at pH 5.4 | Treated | | | | |
| | 1st 100-ml. effluent | 1.14 | 0.16 | 1.36 | 0.48 |
| | 4th 100-ml. effluent | 1.13 | 0.13 | 1.38 | 0.48 |
| | 8th 100-ml. effluent | 1.14 | 0.14 | 1.38 | 0.50 |
| | 12th 100-ml. effluent | 1.12 | 0.15 | 1.40 | 0.49 |
| | Composite | 1.14 | 0.14 | 1.40 | 0.49 |
| Column at pH 6.6 | Treated | | | | |
| | 1st 100-ml. effluent | 1.15 | 0.13 | 1.32 | 0.43 |
| | 4th 100-ml. effluent | 1.14 | 0.15 | 1.31 | 0.43 |
| | 8th 100-ml. effluent | 1.13 | 0.17 | 1.34 | 0.44 |
| | Composite | 1.14 | 0.15 | 1.32 | 0.43 |

that if the calcium is added to milk prior to rennet, near-normal coagulation will occur. Addition of 2-3 ml. of 1 *M* $CaCl_2$ per liter of milk yielded coagulum comparable to that obtained with untreated milk.

*Organoleptic quality of resin-treated milk.* Fresh raw whole milk was divided into several portions, acidified to different pH values, resin-treated, and neutralized as described before. The various samples were pasteurized at 145° F. for 30 min. and submitted to a taste panel. A portion of original milk served as a control. The judgments were evaluated by a ten-judge panel, using a slightly modified American Dairy Science Association scoring guide, and the averaged results are presented in Table 5. These limited data indicate that the

TABLE 5

Effect of resin treatment on the flavor score of milk

| Initial pH | Cation resin treatment | Method of restoring pH | Average flavor score |
|---|---|---|---|
| 5.4 | No | Anionic resin | 35.5 |
| 5.4 | Yes | Anionic resin | 36.0 |
| 5.8 | No | Anionic resin | 36.0 |
| 5.8 | Yes | Anionic resin | 36.0 |
| 6.2 | No | Anionic resin | 36.2 |
| 6.2 | Yes | Anionic resin | 35.2 |
| 6.68 | No | None | 36.6 |
| 6.68 | Yes | None | 36.1 |

organoleptic quality of milk treated by this process is comparable to that of normal pasteurized milk.

While these investigations with ion exchange resins have provided a method suitable for the removal of cationic radionuclides from milk, its applicability to commercial operations is yet to be assessed. Further work in regard to physico-chemical properties, bacteriological and nutritional qualities, and methods for sanitizing and stripping of resin columns needs to be done.

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1654

# STABILITY OF COLOR IN FRUIT JUICES[1]

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The present investigation was undertaken to study the effects of various factors on the stability of color in a variety of fruit juices during storage, and to observe the effects of such factors on the color of anthocyanin pigments isolated from some of the fresh fruits.

The agents responsible for deterioration of color in fruit products were summarized by Joslyn (1941). He pointed out that the problem of color retention in fruit products is complicated by the multiplicity of reactions which may be involved in bleaching, discoloration, and browning. The natural plant pigments may change in tint or hue during preparation, processing, or storage of the fruit products. Browning may occur through formation of pigmented substances resulting from decomposition of certain chemical constituents, or by reactions of some of the constituents of the fruit with oxygen. Browning may also result from reactions between constituents naturally present or introduced in manufacture.

Pederson, Beattie, and Stotz (1947) have indicated that changes which occur in fruit juices during storage are very complex in nature. Though enzymes are apparently inactivated, and effects of oxygen, light, and metals are reduced to a minimum, serious deteriorative changes nevertheless occur. Pederson (1947) attributed these changes to chemical reactions between various ingredients of the juice.

Studies of Tressler and Pederson (1936) and Pederson, Beattie, and Beavens (1941) showed that a progressive darkening in the color of strawberry, currant, and raspberry juices occurred during storage at higher temperatures. This was most noticeable with strawberry juice. The color changed from a bright, clear red to a dull red, and finally to brown. They also noted that color changes occurring in the juices could be partially overcome by storage at low temperatures. These observations have been more recently confirmed by Pederson (1947).

In an investigation of the color changes in fruit spreads, Kertesz (1948) observed that color loss in strawberry spread was greatly accelerated at temperatures between 18.3 and 21.1°C. (65 and 70°F.). He noted that very little loss of color occurred in these spreads during storage at 1.1°C. (34°F.) for several months, but that changes proceeded at an easily measurable rate at 15.6°C. (60°F.), and much faster at 37.8°C. (100°F.). More recent studies by Kertesz and Sondheimer (1948b) indicated that color loss in strawberry preserves due to high temperature may be lessened by greater care in cooking, immediate cooling below

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15.6°C.(60°F.), storage below 15.6°C.(60°F.), and refrigeration when product is held for long periods.

Tressler and Pederson (1936) found that pasteurized grape juice, packed in a high vacuum or in bottles containing substantially no oxygen, underwent very little deterioration, even when exposed to light at room temperature. On the other hand, juices in partially filled bottles deteriorated rapidly. Changes noted were a clouding of the juice, and a change from bright purple-red to brown. A slow deposition of brown sediment occurred, accompanied by detrimental changes in aroma and flavor. These changes occurred more rapidly at room temperature than at lower temperatures. These authors also noted that air in the headspace of bottled strawberry juice resulted in increased deterioration of color.

Powers and Esselen (1942) indicated that effects of light were of only minor importance in the deterioration of glass-packed foods in comparison with deteriorative changes caused by heat and oxygen. Studies by these workers revealed that many undesirable changes attributed to light were actually caused by heat and oxygen, and that the effects of light were negligible. Under extreme conditions of exposure, light functioned as an accelerating agent, and speeded up, slightly, reactions which occurred even in the absence of light.

Beattie, Wheeler, and Pederson (1943) observed that in certain fruit juices, changes in color occurred concurrently with progressive losses of ascorbic acid during storage. They further noted that when ascorbic acid was added as an antioxidant to strawberry, currant, and raspberry juices, deterioration of color was accelerated. Siemers (1946) indicated that ascorbic acid, although effective in retarding discoloration and development of oxidative off-flavors in some fruit products, could not be considered to be of antioxidant value in retarding such changes in all fruit and vegetable products. Products such as strawberries, citrus, and tomatoes, which normally possess a relatively high ascorbic acid content, appear to derive little benefit from added ascorbic acid.

Powers, Esselen, and Fellers (1943) and Powers and Fellers (1945) showed the addition of ascorbic acid prevented or retarded development of discoloration and associated off-flavors of certain foods such as pears, peaches, and plums.

Esselen, Powers, and Woodward (1945) reported that the addition of 30 mg. of d-isoascorbic acid per pint of tomato juice was effective in retarding flavor loss and discoloration during storage under adverse conditions. In studies on the fortification of fruit juices with ascorbic acid, Esselen, Powers, and Fellers (1946) ascertained that color and flavor of grape juice were not improved to any great extent by addition of l-ascorbic acid.

## EXPERIMENTAL PROCEDURE

This investigation was undertaken to study the effect of such factors as time and temperature of storage, and the relationship of oxygen, light, sugar, pH, and antioxidants on deteriorative changes in the color of seven representative fruit juices and strawberry fountain syrup. A second aspect dealt with the effect of these same factors on the color of the

anthocyanin pigments directly isolated from strawberries and currants.

*Preparation of Experimental Packs:* Experimental packs of blueberry, black cherry, currant, grape, red raspberry, strawberry, and tomato juices were prepared, processed, and stored under similar conditions. The grape juice was prepared as described by Chenoweth (1930). Sugar at the rate of three ounces per quart was added, except to that portion of the juice used for studies of juices prepared without the addition of sugar. For studies on the effect of pH values, the pH was adjusted by the addition of a 10 per cent solution of citric acid or sodium bicarbonate. The end points were selected to include a pH range between 0.5 and 0.75 unit above and below the original pH value of the respective juices (Table 2). This study was made to determine whether changes in pH influenced the stability of color of the different juices. For studies on the effect of oxygen, one portion of the juice was aerated by mixing in a Waring Blendor for five minutes, and another portion deaerated according to the method of Mottern and von Loesecke (1933). To one lot, ascorbic acid was added at the rate of 50 mg. per 100 ml. of juice. The various lots of juices were then filled into preheated one-half-pint fruit jars, partially sealed, and processed at 76.7°C.(170°F.) for 15 minutes. The jars were removed and completely sealed, and cooled under a fine cold-water spray.

Juices from other fruits—strawberry, red raspberry, black cherry, currant, and blueberry—were prepared by the heat extraction method. Water was added in the proportion of one cup to five pounds of fruit and the berries heated to 87.8°C.(190°F.) for two minutes. The heated berries were strained through two layers of cheesecloth, then through four layers of cheesecloth in order to further clarify the juice. Sugar was added to bring the soluble solids to approximately 15 per cent as measured by the refractometer. The finished juices were then divided into appropriate lots and treated in a manner similar to that of the grape juice.

Tomato juice was prepared by heating washed and cut tomatoes to approximately 71.1°C.(160°F.) for two minutes in a stainless steel kettle. The heated tomatoes were strained through two layers of cheesecloth, then through four layers of cheesecloth to completely remove any skin or seed particles that might have been present. The juice was heated to 82.2°C. (180°F.) and salt added at the rate of one ounce per gallon of juice. The juice was then divided into appropriate lots and filled hot into preheated one-half-pint fruit jars, partially sealed, and processed at 100°C.(212°F.) for 15 minutes. The jars were removed, completely sealed, and cooled under a fine cold-water spray.

For comparative studies on the effect of sugar, one lot from the experimental packs of each juice, with the exception of tomato juice, was put up without the addition of sugar. For similar studies on tomato juice, one lot was prepared with the addition of sugar, at the rate of four ounces per quart of juice.

Packs of strawberry fountain syrup were also studied to determine the effects of varying amounts of sugar and citric acid on the stability of color during storage at room temperature. This pack was divided into three

lots. The first lot was divided into five portions, each varying in concentration of sugar as follows:

Portion A—5 parts strawberry to 1 part sugar
Portion B—4 parts strawberry to 1 part sugar
Portion C—3 parts strawberry to 1 part sugar
Portion D—2 parts strawberry to 1 part sugar
Portion E—1 part strawberry to 1 part sugar

Lot One was prepared by mixing washed, cleaned, sliced strawberries with varying concentrations of sugar as specified above, and allowing the mixture to stand for two hours, to permit penetration of the sugar into the fruit. The strawberries were then heated to 87.8°C.(190°F.), and filled into preheated one-half-pint jars. The jars were partially sealed and processed in a hot-water bath at 100°C.(212°F.) for 15 minutes. The jars were then removed, completely sealed, and cooled under a fine cold-water spray. The second and third lots were prepared similarly, except they contained 0.5 and 0.25 per cent, respectively, by weight of citric acid which was added to the strawberries as a mixture with the sugar.

*Conditions of Storage:* Each pack of processed fruit juice was divided into groups of six jars each, representing the various experimental factors and conditions of storage. Storage studies covered a period of six months under the following conditions:

Temperature—Jars stored at 37.8°C.(100°F.); 21.1 to 26.7°C.(70 to 80°F.); 1.7°C.(35°F.); and —17.8°C.(0°F.) in the dark.

Light—Jars stored at 21.1 to 26.7°C.(70 to 80°F.) in daylight.

*Methods of Analysis:* Color changes in the juices were determined by measurement of light transmittance characteristics, using a Coleman Model 11 Spectrophotometer. Absorption maxima were determined for each juice, and subsequent measurements made at these determined wavelengths. Wavelengths used were 500 mμ. for strawberry, raspberry, grape, cherry, and blueberry juices; 450 mμ. for currant juice and strawberry fountain syrup; 475 mμ. for tomato juice (Figs. 1 and 2). The juices, with the exception of tomato juice, were analyzed as composite samples. Aliquot portions were taken, centrifuged for 10 minutes at 5,000 r.p.m. and the clear liquid decanted off. Before use the centrifuged juices were diluted to appropriate volume with distilled water so that light transmittance characteristics could be read from a sensitive portion of the galvanometer. Strawberry, raspberry, and currant juices were diluted one part in two parts of water, and blueberry, cherry, and grape juices one part in three parts of water. The Null point method of measurement was used, with water as the reference solution for the blank; high readings indicate light-colored juices, low readings darker-colored juices.

Tomato juice was prepared for spectrophotometric analysis by a modification of the method of Kramer and Smith (1946). A 10-ml. sample of the juice, to which was added 70 ml. of benzol, was transferred to a Waring Blendor, and blended for five minutes. The sample was next transferred to a 100-ml. graduated flask, and the solids allowed to settle on the bottom. A five-ml. aliquot was taken, diluted with 20 ml. of benzol.

and centrifuged for five minutes at 5,000 r.p.m. Transmission measurements were made with the centrifuged sample, using benzol as the reference solution for the blank.

The strawberry fountain syrup was first well mixed and the pieces of fruit thoroughly crushed. It was then prepared in a manner similar to the fruit juice for spectrophotometric analysis.

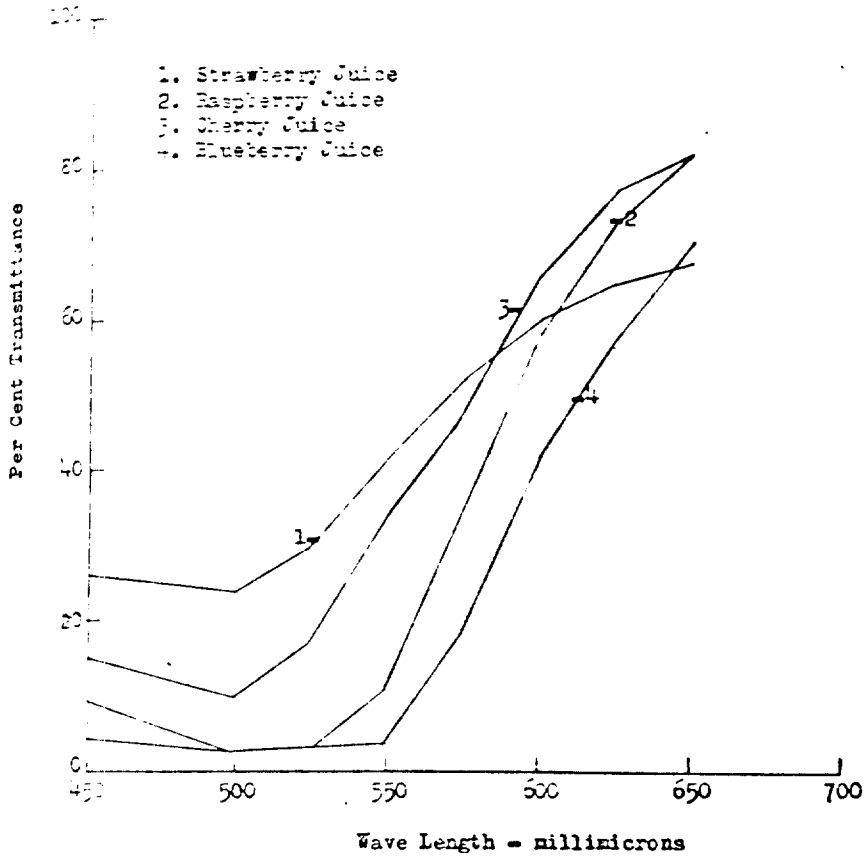


Fig. 1. Light transmittance values on control samples of strawberry, raspberry, cherry, and blueberry juices.

Visual Examination: To obtain a general estimate of acceptability of the juices in regard to color, samples were judged visually for obvious changes in appearance. Estimates of changes were based on comparison with similar samples stored at 1.7°C. 35°F.

*Anthocyanin Pigments:* Anthocyanins are known to be present in fruits mainly in combination with sugars (usually glucose, rhamnose, galactose or a combination of two sugars) as glucosides. As long as they are in this form, they are water soluble. On boiling with acid, however, the sugar is removed, leaving an anthocyanidin (a nonglucoside) insoluble in water. The intention of the present study was to obtain the antho-

cyanin pigment in relatively pure form as a glucoside to use for experimental purposes.

The red anthocyanin pigment of strawberry was recently isolated by Kertesz and Sondheimer (1948a) and identified as pelaronidin 3-monoglucoside. In the present investigation the anthocyanin pigment was isolated from strawberries and currants by chromatographic adsorption of crude

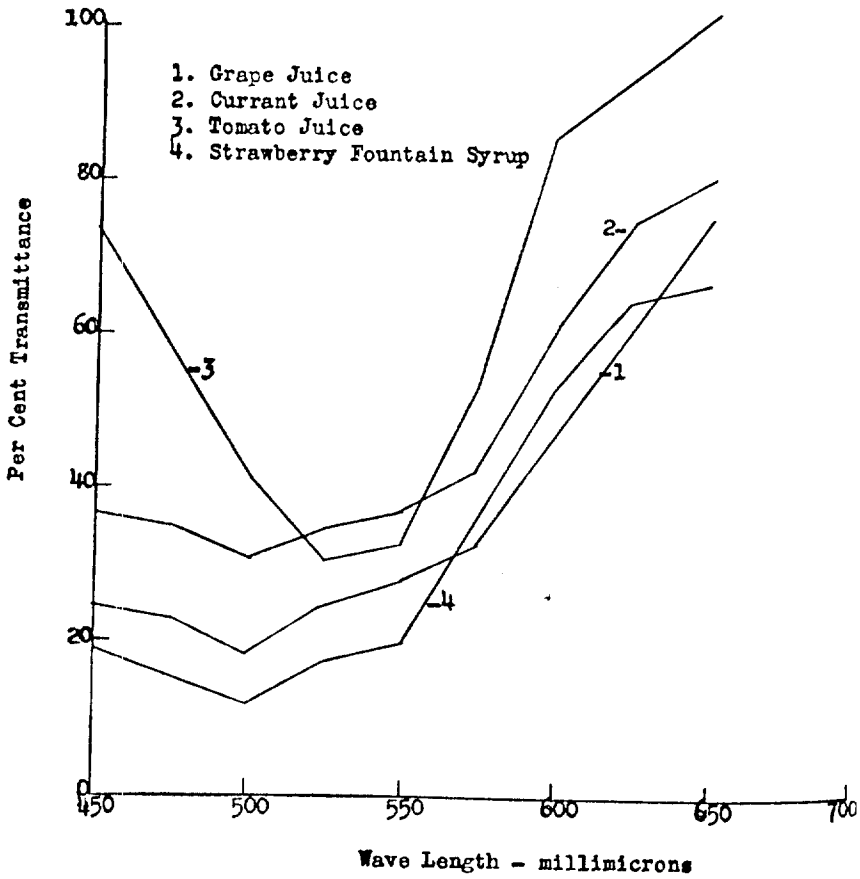


FIG. 2. Light transmittance values on control samples of currant, grape, and tomato juice and strawberry fountain syrup.

extracts of the pigment. This method consisted essentially of extraction of the pigment in crude-water extracts, purified by chromatographic adsorption. Procedures for each of the fruits are outlined in detail below.

*Procedure for Isolation of Anthocyanin From Strawberries:* 1. Solution of pigment was effected from seven and one-half pounds of frozen berries, containing four parts of strawberries to one part of sugar, by extraction with distilled water in proportion of 100 ml. of water for each pound of berries. Heat, not exceeding 48.9°C.(120°F.), was employed. Temperatures above 60.0 to 65.6°C.(140 to 150°F.) must be avoided dur-

ing the process since anthocyanin is readily converted to the anthocyanidin form at these temperatures.

2. The pigment was then separated from other substances in water solution by adding equal quantities of isobutyl alcohol. The anthocyanin was found to be more soluble in isobutyl alcohol than in water, and by thorough mixing in a Waring Blendor for approximately five minutes the greater portion of the pigment could be transferred to the alcoholic phase. Since isobutyl alcohol and water are immiscible, the alcoholic solution could then be easily decanted from the water.

3. The pigment in the crude alcoholic extract was purified by chromatographic separation, by passage of the solution through an adsorption column of alumina. Fisher chromatographic adsorption alumina especially prepared for chromatographic analysis was used in this procedure. A definite blue-purple band appeared at the top of the column. Just above this color band was a faint yellow band, believed to be a flavone.

4. The chromatogram was further developed by washing the column with fresh portions of isobutyl alcohol.

5. The pigment was eluted from the column with distilled water. The small percentage of flavone present on the column was not soluble in the water.

6. The water extract containing the pigment was then centrifuged and filtered to remove any particles of alumina or solid matter that might have come down from the column. The volume of the final pigment extract solution was made up to 750 ml. with distilled water.

*Procedure for Isolation of Anthocyanin From Currants:* A procedure similar to that employed for strawberries was followed for currants, with certain exceptions. Adsorption of the crude isobutyl alcohol solution on the alumina column disclosed similar bands, except that extracts of currants gave a chromatogram in which the flavone-like material was located just below the anthocyanin band. This material was water-soluble. This necessitated the following modification in the method. Steps One through Three were similar to procedure used with strawberries.

4. The chromatogram was further developed by washing the column with fresh portions of isobutyl alcohol followed by washing with water to remove the small percentage of flavone-like material present on the column.

5. Anthocyanin was eluted from the column with a citric acid solution at pH 2.0. A higher pH value for the eluant did not entirely remove the pigment from the column.

6. The pigment solution was centrifuged, filtered, and made up to volume as in the procedure for strawberry pigment.

*Preparation of Samples for Pigment Studies:* Samples of the pigment solutions as prepared above were arranged insofar as possible to simulate the conditions provided for study of the fruit juices. Conditions of storage were identical to those previously employed, and observations extended over a period of three months. Eleven lots of six samples each were prepared for observation of the various conditions of experimental treatment and storage. For studies on the effect of the pH of the solution, adjustments of pH values were made with a solution of citric acid (shown Table

5). For studies on the effect of oxygen, one portion of the solution was aerated by mixing in a Waring Blendor for five minutes, and another portion deaerated by bubbling nitrogen gas through the solution for five minutes. To one lot, ascorbic acid was added at the rate of 50 mg. per 100 ml. of solution. Sugar was added to another lot to bring the soluble solids to approximately 15 per cent. The prepared solutions were then filled into small glass vials in 10-ml. portions and sealed, leaving a 10 per cent headspace.

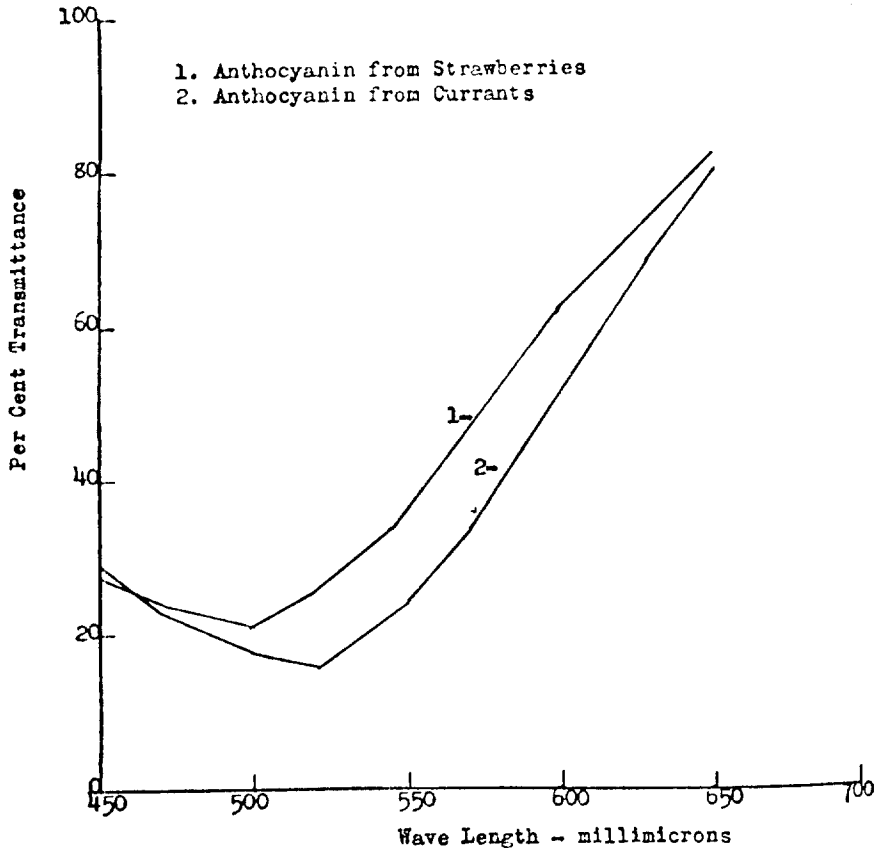


FIG. 3. Light transmittance values on control samples of anthocyanin pigment isolated from strawberries and currants.

Experimental samples of the anthocyanin pigments were removed at intervals of one, two, and three months for color analysis. Analytical procedures were the same as those employed in previous studies. Absorption maxima were determined for the anthocyanin pigment from the respective juices (Fig. 3) and subsequent measurements made at these determined wavelengths. The wavelengths used were 500 mμ. for anthocyanin from strawberry and 520 mμ. for anthocyanin from currant. Results are presented (Tables 4 and 5).

## DISCUSSION OF RESULTS

Results obtained in the first part of the investigation indicated that color changes occurring in different fruit juices during storage were not necessarily uniform, but varied with the specific fruit (Tables 1 and 2). In general, all juices showed a change in color during storage. Color changes were most marked in juices which normally possess high color. The stability of the color of blueberry, strawberry, raspberry, and currant juices decreased in descending order. Cherry, grape, and tomato juices were much more stable, and exhibited less color deterioration. Spectrophotometric tests indicated that the percentage transmittance in juices was greatest after storage for three months, and darkening was not observed until after about six months' storage. Results obtained from visual observations also indicated that the juices changed from a bright, clear red to

TABLE 1

*Effect of Storage Temperatures on Light Transmittance Characteristics of Fruit Juices*

| Juice | Period of storage | *Temperature of storage* | | | |
|---|---|---|---|---|---|
| | | −17.8°C. (0°F.) | 1.7°C. (35°F.) | 21.1 to 26.7°C. (70 to 80°F.) | 37.8°C. (100°F.) |
| | | Per cent transmittance [1] | | | |
| Blueberry | Control | 3 | 3 | 3 | 3 |
| | 6 weeks | 3 | 3 | 3 | 15 |
| | 3 months | 5 | 5 | 21 | 68 |
| | 6 months | 4 | 4 | 27 | 62 |
| Cherry | Control | 10 | 10 | 10 | 10 |
| | 6 weeks | 8 | 10 | 11 | 16 |
| | 3 months | 10 | 9 | 10 | 15 |
| | 6 months | 10 | 9 | 10 | 15 |
| Currant | Control | 32 | 32 | 32 | 32 |
| | 6 weeks | 31 | 32 | 45 | 55 |
| | 3 months | 33 | 33 | 55 | 65 |
| | 6 months | 30 | 34 | 52 | 51 |
| Grape | Control | 13 | 13 | 13 | 13 |
| | 6 weeks | 13 | 13 | 14 | 19 |
| | 3 months | 15 | 14 | 17 | 18 |
| | 6 months | 15 | 15 | 19 | 14 |
| Raspberry | Control | 3 | 3 | 3 | 3 |
| | 6 weeks | 5 | 3 | 6 | 10 |
| | 3 months | 6 | 3 | 21 | 20 |
| | 6 months | 5 | 3 | 12 | 16 |
| Strawberry | Control | 24 | 24 | 24 | 24 |
| | 6 weeks | 24 | 24 | 28 | 30 |
| | 3 months | 25 | 28 | 50 | 55 |
| | 6 months | 26 | 30 | 44 | 43 |
| Tomato | Control | 32 | 32 | 32 | 32 |
| | 6 weeks | 32 | 32 | 32 | 41 |
| | 3 months | 34 | 35 | 33 | 35 |
| | 6 months | 32 | 34 | 27 | 35 |

[1] Light transmittance values were determined at wavelengths of 500 mμ. for blueberry, cherry, grape, raspberry, and strawberry juices; 475 mμ. for tomato juice; and 450 mμ. for currant juice.

a light, dull red after storage for three months, while darkening was not observed until after six months. This observation, as previously suggested by Pederson *et al.* (1947), reveals two types of changes may have occurred: one, complete destruction of the pigment; and the other, a formation of a new pigment or development of other pigments that are present resulting from reactions between the various constituents present in the juice.

TABLE 2

***Effect of Experimental Treatment on Light Transmittance Characteristics of Fruit Juices Stored at 21.1 to 27.7°C (70 to 80°F.) in the Dark***

| Juice | Period of storage | Untreated juice | Experimental treatment: Stored in daylight | Aerated | Deaerated | Ascorbic[2] acid added | No sugar added | pH[3]: Raised | pH[3]: Lowered |
|---|---|---|---|---|---|---|---|---|---|
| | | Per cent transmittance[1] | | | | | | pH 3.5 | pH 2.5 |
| Blueberry | Control | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| | 6 weeks | 3 | 3 | 10 | 3 | 3 | 2 | 4 | 3 |
| | 3 months | 21 | 20 | 50 | 12 | 20 | 9 | 23 | 19 |
| | 6 months | 27 | 30 | 38 | 11 | 17 | 8 | 27 | 25 |
| | | | | | | | | pH 4.25 | pH 2.75 |
| Cherry | Control | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| | 6 weeks | 11 | 10 | 15 | 10 | 10 | 10 | 11 | 9 |
| | 3 months | 10 | 11 | 15 | 11 | 13 | 10 | 10 | 18 |
| | 6 months | 10 | 10 | 14 | 10 | 13 | 8 | 11 | 9 |
| | | | | | | | | pH 4.0 | pH 2.75 |
| Currant | Control | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 |
| | 6 weeks | 45 | 42 | 55 | 34 | 50 | 43 | 45 | 40 |
| | 3 months | 55 | 55 | 65 | 36 | 60 | 52 | 57 | 53 |
| | 6 months | 52 | 52 | 62 | 38 | 56 | 52 | 54 | 50 |
| | | | | | | | | pH 4.0 | pH 2.75 |
| Grape | Control | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 |
| | 6 weeks | 14 | 15 | 22 | 14 | 16 | 13 | 16 | 14 |
| | 3 months | 17 | 17 | 17 | 15 | 10 | 16 | 17 | 16 |
| | 6 months | 19 | 18 | 16 | 14 | 19 | 18.5 | 16 | 15 |
| | | | | | | | | pH 4.0 | pH 2.5 |
| Raspberry | Control | 3 | 3 | 3 | 3 | 3 | 3 | 5 | 3 |
| | 6 weeks | 6 | 6 | 8 | 7 | 22 | 6 | 9 | 6 |
| | 3 months | 21 | 21 | 22 | 9 | 23 | 19 | 23 | 19 |
| | 6 months | 11 | 11 | 16 | 7 | 28 | 14 | 21 | 16 |
| | | | | | | | | pH 4.25 | pH 2.75 |
| Strawberry | Control | 24 | 24 | 24 | 24 | 24 | 24 | 24 | 24 |
| | 6 weeks | 28 | 28 | 30 | 23 | 31.5 | 27 | 28 | 23 |
| | 3 months | 50 | 49 | 55 | 30 | 62 | 47 | 52 | 52 |
| | 6 months | 44 | 4[illegible] | 43 | 34 | 52 | 44 | 42 | 40 |
| | | | | | | | | pH 4.75 | pH [illegible] |
| Tomato | Control | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 |
| | 6 weeks | 32 | 31 | 30 | 32 | 25 | 32 | 32 | 30 |
| | 3 months | 33 | 32 | 31 | 33 | 33 | 29 | 33 | 34 |
| | 6 months | 27 | 33 | 30 | 22 | 25 | 27 | 36 | 35 |

[1] Light transmittance values were determined at wavelengths of 540 mμ. for blueberry, cherry, grape, raspberry, and strawberry juices; 475 mμ. for tomato juice; and 450 mμ. for currant juice.
[2] Ascorbic acid added at the rate of 50 mg. per 100 ml. of juice. [3] Original pH values of the juices were: Blueberry—3.20; cherry—3.50; currant—3.40; grape—3.40; raspberry—3.25; strawberry—3.50; tomato—4.25.

Heat and oxygen content were the most specific accelerating agents responsible for deterioration of color during storage. Increase in temperature of storage or increase in oxygen content resulted in more rapid deterioration of the juices. Deaerated juices stored at 21.1 to 26.7°C.(70 to 80°F.) retained their color and exhibited very little change during storage for six months. Low temperature likewise permitted storage of the juices with little deleterious effect on the color.

Addition of l-ascorbic acid to the juices, except for blueberry and grape juice, accelerated color deterioration, in the form of progressive bleaching. Bleaching was clearly evident in visual examination of stored juices. This effect has been attributed to the reducing action of the ascorbic acid. As previously pointed out by Esselen *et al.* (1946) and other workers in the case of apple juice, addition of ascorbic acid to apple or cranberry juices had a marked effect on lightening color and in retarding darkening during storage. These authors also noted that color and flavor of grape juice were not improved to any great extent by the addition of l-ascorbic acid.

As pointed out by Siemers (1946), ascorbic acid, although effective in retarding discoloration and development of oxidative off-flavors in some fruit products, could not be considered of antioxidant value in retarding color changes in all fruit products. Products such as strawberries and tomatoes, which normally possess a relatively high ascorbic acid content, appeared to derive little benefit from added ascorbic acid.

Beattie *et al.* (1943) observed that an increase in the concentration of ascorbic acid or its isomers to strawberry, currant, or raspberry juices increased the rate of color changes, the rate of change paralleling the loss of ascorbic acid. The same authors also indicated that since the red pigments are readily reducible, and the ascorbic acid readily oxidizable, interaction between the two seems likely.

Comparison of samples stored in light and darkness revealed light had little if any effect on color. Likewise sugar and pH value played only minor roles in regard to stability of color in fruit juices. Addition of sufficient concentrations of sugar (one part of sugar to one, two, or three parts of strawberries) to strawberry fountain syrup preserved the bright-red color when products were stored at from 21.1 to 26.7°C.(70 to 80°F.) for periods of six months (Table 3). Addition of citric acid to packs containing lower concentrations of sugar (one part of sugar to four and five parts strawberry) did have some preservative effect on the color during similar conditions of storage.

Results obtained in the second phase of the investigation showed that the stability of the anthocyanin pigments isolated from strawberries and currants were subject to some variation (Tables 4 and 5). Increased storage temperatures and the presence of oxygen accelerated color deterioration with both pigments, while a low storage temperature generally preserved the color. Deaerated samples of the isolated pigments stored at from 21.1 to 26.7°C.(70 to 80°F.) retained their color and exhibited very little change during storage for three months. Added l-ascorbic acid had a bleaching effect on the color of the pigment from strawberries and greater bleaching action on the pigment from currants, ultimately resulting in nearly complete decolorization of the pigment solution. Light, on the other

hand, caused rapid bleaching of the pigment from strawberries, but had only a moderate effect on the pigment from currants.

Of interest was the fact that deteriorative effects resulting from exposure to light occurred in solutions of isolated pigments but not in the juices of these same fruits. These observations suggest a number of possible explanations. A change in chemical structure of the pigments may have occurred during the process of isolation which rendered the pigment more susceptible to photolytic decomposition. Also probable is the possibility that certain constituents present in the juice may have exerted protective effects on the pigments which would be lacking in solution of isolated pigment. Another possibility is the formation or development of new pigments resulting from chemical reactions between various ingredients of the fruit. Reactions of this latter type may be the cause of alternate bleaching and darkening observed with certain of the juices.

TABLE 3

*Effect of Different Concentrations of Sugar and Citric Acid on Light Transmittance Characteristics of Strawberry Fountain Syrup Stored at 21.1 to 26.7°C. (70 to 80°F.) in the Dark*

| Sample[1] | Control | Period of storage | | |
|---|---|---|---|---|
| | | 6 weeks | 3 months | 6 months |
| | | Per cent transmittance at 450 mμ. | | |
| A | 19 | 29 | 39 | 24 |
| A1 | 19 | 25 | 27 | 16 |
| A2 | 19 | 26 | 31 | 22 |
| B | 19 | 27 | 34 | 24 |
| B1 | 19 | 23 | 25 | 16 |
| B2 | 19 | 25 | 28 | 18 |
| C | 19 | 19 | 24 | 20 |
| C1 | 19 | 17 | 23 | 19 |
| C2 | 19 | 19 | 19 | 20 |
| D | 19 | 22 | 20 | 24 |
| D1 | 19 | 18 | 24 | 19 |
| D2 | 19 | 20 | 22 | 22 |
| E | 19 | 21 | 22 | 20 |
| E1 | 19 | 19 | 19 | 19 |
| E2 | 19 | 19 | 19 | 19 |

[1]A—5 parts strawberries to one part sugar; B—4 parts strawberries to one part sugar; C—3 parts strawberries to one part sugar; D—2 parts strawberries to one part sugar; E—1 part strawberries to one part sugar; 1—0.5 per cent by weight citric acid added; 2—0.25 per cent by weight citric acid added.

Decreasing pH values appeared to intensify and protect the color during storage, whereas increasing pH values diminished the intensity of color and increased deteriorative effects during storage.

The anthocyanin pigment solution isolated from strawberries developed a faint precipitate after storage for a period of one week. No such precipitation occurred in the anthocyanin pigment solution isolated from the currants. It was also noted that the degree of precipitation increased progressively during storage for periods up to six months. This precipitation

may have occurred as a result of acid hydrolysis which converted the anthocyanin (glucoside) to the water-soluble anthocyanidin. Precipitation of the pigment from strawberries and not from currants was attributed possibly to a lower stability of the strawberry anthocyanin.

TABLE 4

*Effect of Storage Temperatures on Light Transmittance Characteristics of the Anthocyanin Pigments Isolated From Strawberries and Currants*

| Pigment | Period of storage | Temperature of storage | | | |
|---|---|---|---|---|---|
| | | −17.8°C. (0°F.) | 1.7°C. (35°F.) | 21.1 to 26.7°C. (70 to 80°F.) | 37.8°C. (100°F.) |
| | | Per cent transmittance [1] | | | |
| Anthocyanin from strawberry | Control | 20.0 | 20.0 | 20.0 | 20.0 |
| | 1 month | 22.5 | 22.5 | 28.5 | 36.5 |
| | 2 months | 23.0 | 21.0 | 26.0 | 39.0 |
| | 3 months | 23.0 | 22.0 | 27.0 | 50.0 |
| Anthocyanin from currant | Control | 16.5 | 16.5 | 16.5 | 16.5 |
| | 1 month | 17.0 | 17.0 | 19.5 | 24.0 |
| | 2 months | 16.5 | 17.0 | 20.0 | 31.0 |
| | 3 months | 16.5 | 17.0 | 24.0 | 56.0 |

[1] Light transmittance values were determined at wavelengths of 500 mμ. and 520 mμ. for strawberry and currant pigments, respectively.

TABLE 5

*Effect of Experimental Treatment on Light Transmittance Characteristics of the Anthocyanin Pigments Isolated From Strawberries and Currants Stored at 21.1 to 26.7°C.(70 to 80°F.) in the Dark*

| Pigment | Period of storage | Experimental treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Untreated pigment | Stored in daylight | Aerated | Deaerated | Ascorbic[2] acid added | Sugar added | pH [3] Raised | pH [3] Lowered |
| | | Per cent transmittance [1] | | | | | | pH 4.5 | pH 2.5 |
| Anthocyanin from strawberry | Control | 20.0 | 20.0 | 20.0 | 20.0 | 20.0 | 20.0 | 40.0 | 5.5 |
| | 1 month | 28.5 | 64.5 | 36.0 | 23.0 | 29.0 | 25.5 | 49.0 | 5.5 |
| | 2 months | 26.0 | 65.5 | 40.0 | 24.0 | 34.0 | 27.0 | 52.0 | 6.0 |
| | 3 months | 27.0 | 72.0 | 44.0 | 25.0 | 34.0 | 25.5 | 55.0 | 5.5 |
| | | | | | | | | pH 3.0 | pH 2.0 |
| Anthocyanin from currant | Control | 16.5 | 16.5 | 16.5 | 16.5 | 16.5 | 16.5 | 25.0 | 18.0 |
| | 1 month | 19.5 | 24.5 | 24.0 | 18.0 | 74.0 | 21.5 | 28.0 | 20.0 |
| | 2 months | 20.0 | 28.0 | 30.0 | 19.0 | 74.0 | 21.5 | 28.0 | 20.0 |
| | 3 months | 24.0 | 37.0 | 38.0 | 21.0 | 74.0 | ..... | 34.0 | 22.0 |

[1] Light transmittance values were determined at wave lengths of 500 mμ. and 520 mμ. for pigments from strawberry and currant, respectively. [2] Ascorbic acid added at the rate of 50 mg. per 100 ml. of sample. [3] Original pH values of the pigments were strawberry—3.5, currant—2.5.

## SUMMARY AND CONCLUSIONS

Studies were made on the effect of length and temperature of storage, and the relationship of oxygen, light, sugar, pH, and ascorbic acid to deteriorative changes in the color of seven representative fruit juices and strawberry fountain syrup. Similar studies were made on solutions of purified anthocyanin pigments isolated from strawberries and currants to observe whether deteriorative changes in juices of these fruits were directly associated with changes in these pigments.

Temperature of storage and oxygen content were the most specific agents responsible for deterioration of color during storage of both juices and isolated pigments.

Exposure to light caused little deterioration of color in juices, but exerted a bleaching effect on the isolated anthocyanin pigments.

Adjustment of pH values had little if any effect on deterioration of color in fruit juices during storage, but some effect was observed in solutions of isolated pigments. Lower pH values exerted a protective action on pigment solutions.

Sugar had little effect on stability of color in either juices or pigment solutions.

When used as an antioxidant, l-ascorbic acid exerted no marked protective properties in respect to color stability. With all the juices except blueberry and grape juice, progressive bleaching resulted from addition of ascorbic acid in concentration of 50 mg. per 100 ml. of juice. Addition of similar concentrations of solutions of strawberry anthocyanin resulted in virtually complete decolorization of the pigment.

Increasing sugar and/or citric acid concentration enhanced the stability of the color of strawberry fountain syrup during storage.

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1716

# Determination of Citric Acid in Fats

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## Abstract

The direct analysis of small amounts (2–50 ppm) of citric acid in refined glyceride oils and fats is described. Fat, containing citric acid, is titrated potentiometrically in a pyridine–acetone mixture with tetrabutylammonium hydroxide (TBAH). The method is relatively simple and rapid with an accuracy better than ±5%.

## Introduction

Owing to its complexing properties towards metals, citric acid is frequently used as an additive to oils and fats. It thus functions as a scavenger and chelates heavy metals, which will decrease their prooxidative abilities toward the oil or fat. In order to study the processing problems in connection with the use of citric acid, a quantitative analytical method with high accuracy and reproducibility was developed.

Several methods for the determination of small amounts of citric acid in biological materials have been described. Most of these methods are based on color reactions, viz., the pentabromoacetone method (1) and the so-called Fürth-Herrmann reaction (2). However there are few reports of methods for the determination of citric acid in oils and fats. With a micro method based on pentabromoacetone Buschbeck (3) found poor agreement when fatty matter was present, and he concluded that a necessary condition for the applicability of the method in such cases was to remove the oil, e.g., by extraction. Such a method has been described by Masuyama (4). Another procedure is reported by Choy et al. (5), based on the Fürth-Herrmann reaction and suitable for "oily" samples. However none of these methods has given sufficient quantitative accuracy in the 0–50 ppm range.

The direct titrimetric procedure which will be described in the following sections has been found to function satisfactorily after the problems with solvents and the electrode system were solved.

## Experimental Procedures

### Solvents

In pyridine, citric acid is protolyzed in two distinct steps: in the first, one proton is split off; and in the second, the two remaining. In the pH range of the second step, disturbances from "acidic impurities" were observed. At low citric acid levels the amounts of these disturbing compounds in proportion to the citric acid are so dominating that, in practice, only the first step can be used since this step is not influenced to any appreciable degree.

Pyridine (ACS spec.) is purified in the following way. After distillation between 114–117C, the solvent is shaken with 20 g liter of fused potassium hydroxide, filtered, and thereafter redistilled. Pyridine purified in this manner has a low blank value, and the titration curve is well developed.

Acetone (ACS spec.) was used without further purification. From these solvents a mixture was made, consisting of two parts of pyridine and one part of acetone.

### Apparatus

The measurements were performed with a Metrohm Potentiograph E 336. The volume of the burette was 5 ml. The burette tip and the storage bottle were connected by glass tubes with ball joints. The storage bottle was also provided with absorption tubes against moisture and carbon dioxide. The titration vessel was an ordinary 50-ml beaker (high form), which during the titration was covered with a lid that had the necessary holes for electrodes and burette tip.

### Electrodes

The electrode system was composed of a Beckman glass electrode No. 4263 with a Metrohm sleeve type of calomel electrode EA 409 as reference electrode. The salt solution of the electrode consisted of ethylene diamine saturated with lithium chloride, according to Gran and Althin (6), in which agar had been slurried. The porous glass of the electrode had previously been plugged with this gel-like salt solution.

After completion of the measurements, the calomel electrode was cleaned and stored in a saturated potassium chloride solution; the bridge together with the glass electrode were placed in a 0.01 M ammonium acetate solution.

### Titrant

Tetrabutylammonium hydroxide solution, 0.1 N in benzene/methanol 10:1 (BDH), was purified by passage through Amberlite IRA-400 in hydroxylic form, according to Cundiff and Markunas (8), and thereafter diluted with benzene 2-propanol 6:1 (7).

### Titration Procedure

Ten grams of oil was weighed directly in the titration vessel and dissolved in 15 ml of pyridine–acetone (2:1).[1] The sample was titrated with TBAH-solution, standardized against salicylic acid[2] in 10 g of oil.

[1] Standards of citric acid were prepared by adding aliquot amounts of citric acid (p.a., Riedel-DeHaen AG), dissolved in pyridine to 10 g of oil.
[2] BDH, Analar, mw 138.13.

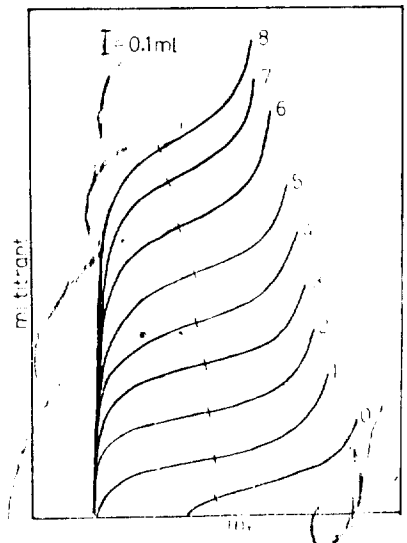


Fig. 1. Titration curve [illegible] value; 1.8, [illegible] ppm.

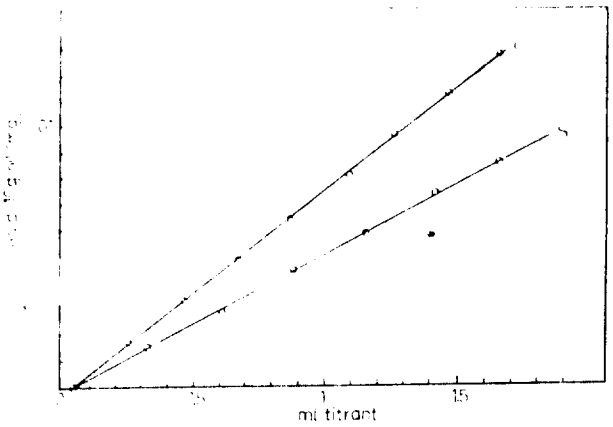


FIG. 2. The linear relationship of concentration and titrant in the 0–15 ppm range. C, citric acid; S, salicylic acid.

Normality range: $5 \cdot 10^{-4}$ – $10^{-3}$. The titration curve was automatically recorded on chart paper (Fig. 1). At the end of the titration the electrodes were thoroughly washed with trichloroethylene. Complications arising from oil films on the electrodes were thus eliminated (9).

## Results and Discussion

Table I shows the results from four series of determinations. The figures are mean values of 10 titrations. As appears from Table I, the accuracy and reproducibility are satisfactory. The difference between Samples 1 and 2 depends on different normalities of the TBAH solution. The lower normality (Sample 2) is the most accurate, which is also to be expected. The linearity of the titration curves, absolute values of citric acid at least up to 0.17 mg (Fig. 2). By lowering sample quantities, the concentration range can be extended with maintainance or even enhancement (owing to smaller proportions of fat) of accuracy, according to Table I.

TABLE I

Titration of Citric Acid in Oil

| Sample | Citric acid theor (ppm) | Citric acid found (ppm) | Standard deviation (ppm) | Relative deviation (%) |
|---|---|---|---|---|
| 1 | 2.94 | 2.94 | 0.10 | 3.5 |
| 2 | 2.97 | 2.97 | 0.03 | 0.9 |
| 3 | 5.82 | 5.82 | 0.14 | 1.4 |
| 4 | 17.42 | 17.41 | 0.08 | 0.9 |

The titration curves in Fig. 1 show that the potential jumps gradually decrease with increasing volumes of titrant. This phenomenon is a solvent effect and leads to lower accuracy since the equivalence point will be more difficult to interpret. When titrations are performed within an expanded concentration range, it is advisable to change the normality of the TBAH solution or to change the sample weight in order to achieve maximum accuracy.

The method described is not specific for citric acid since other acids will also be titrated if they are protolyzed in the same pH range. However this limitation is of minor significance since the analysis, first of all, is intended to be used in connection with model investigations for which the accuracy is of primary importance. Besides, in refined oils and fats, there are normally no acidic components in the particular pH range. As to carboxylic acids, which of course are always present, it has been found that such acids (carbon length of 2 C and longer) have no influence on the determination.

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# A Micromethod for Silica-Gel Column Chromatography of Urinary Organic Acids

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## INTRODUCTION

Ever since its introduction by Martin and Synge (5), silica-gel chromatography has become an important tool for the separation and purification of chemical compounds. One of the earlier workers, Isherwood (2), successfully used it for the separation of the organic acids of fruits. Later, Donaldson, Tulane, and Marshall (1) modified the original method by the introduction of an automatic, continuous gradient elution technique. More recently, this method, as reported here, has been improved and simplified in the authors' laboratory and has been used successfully in the separation and quantitative estimation of the levels of different urinary organic acids under various physiological as well as pathological states.

## EXPERIMENTAL METHOD

*Preparation of urine samples for extraction.* All urine specimens are collected during a 24-hour period and kept refrigerated. One g of sodium chloride is added to a 10-ml aliquot of rat urine, or to a 25-ml aliquot of urine from human subjects and the samples are lyophilized at a temperature of −60°C and a reduced pressure of 50 μ. The dried samples are stored in a freezer at −12°C and ready for extraction.

*Preparation of acid tert-amyl alcohol.* Four ml of water is pipetted into a 100-ml volumetric flask and cooled in an ice-bath. From a burette, 6 ml of concentrated sulfuric acid are added into the flask while it is still cold. The flask is kept cool, and freshly distilled *tert*-amyl alcohol is added to bring the volume to exactly the 100-ml mark. This solution is kept refrigerated until ready for use.

*Extraction procedure.* The residue obtained after lyophilization of the urine sample is made into a fine powder, to which 10 ml of acid *tert*-amyl alcohol is added and the mixture is shaken by an automatic wrist action shaker (250 oscillations per minute) for 8 hours. The [illegible] can be stored overnight in a refrigerator. Then, *tert*-amyl [illegible] is added carefully to a total volume of exactly 200 ml. The container is shaken again for 4 hours, then the contents are centrifuged at 5000 rpm. The supernatant contains the organic acids which are chromatographed by the silica gel chromatography method described below.

*Assembly of the apparatus for column chromatography.* The general assembly of the column chromatography apparatus is shown in Fig. 1. Into each of 180 clean Pyrex test tubes (18 × 150 mm) are placed exactly 2 ml of distilled water by means of a constant-volume automatic pipette, set to deliver 2 ml. The test tubes are then transferred to the rack of an automatic fraction collector, having an attached drop-counter unit. The purpose of the water is to retard the evaporation of the organic solvent as well as to facilitate the titration of the acids. Into Flask A are poured 450 ml of water-saturated chloroform. While the curved ends of Tubes I and H are held beneath the surface of the chloroform, 50 ml of water are added to Flask A. The stopcock at I is closed, while stopcock E is opened. Tube H is completely filled with chloroform by blowing on Tube L and immediately closing stopcock when Tube H is filled. Flask A is stoppered tightly and the stopper is held in position by fastening it with a string. To Flask B is added 750 ml. of 60% *tert*-amyl alcohol in chloroform. The *tert*-amyl alcohol–chloroform mixture is previously saturated by shaking it with 220 ml of water in a 1500-ml separatory funnel. After separation, the organic layer is removed and filtered on Whatman No. 1 filter paper to remove the excess water.

*Preparation of the column.* A Pyrex glass column (12 × 360 mm), fitted with a sintered-glass disk of medium porosity at the bottom, is ideal for this type of column chromatography. A disk of Whatman No. 3 filter paper, the same size as the above sintered-glass disk, is placed at the bottom of the column. The whole assembly is wetted by water-saturated chloroform. Silicic acid (100 mesh) is dried in an electric oven at 98°C for 3 days. Nine g of this dried, hot silicic acid is weighed in a 150-ml beaker, and 5.5 ml of 0.05 *M* sulfuric acid are



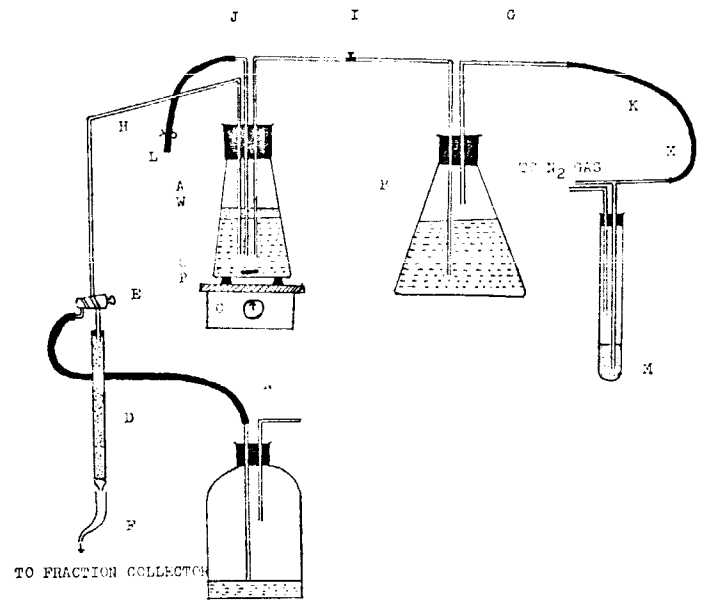


FIG. 1. Column chromatography apparatus: A. 500-ml wide-mouth Erlenmeyer flask; B. One-liter widemouthed Erlenmeyer flask; C. Magnetic stirrer; D. Silica-gel column; E. Two-way stopcock; F. Adaptor; G., H., I., J. Connection glass tubings; K., L. Rubber tubings; M. Mercury safety valve; N. Drainage tube; O. Corks; P. Asbestos plate; and W. Distilled water.

added immediately and thoroughly mixed with a glass rod to a homogenous, granular powder. To this hydrated silicic acid, a total of 60 ml of water-saturated chloroform are added slowly with continuous stirring until a uniform slurry is formed.

The slurry is poured down the column, filled up to the top each time and allowed to settle down with the aid of compressed air, until the chloroform layer almost coincides with the top of the silica gel. The previously extracted urine sample, containing the organic acids to be determined, is slowly poured down the top of the column. Again, the mixture is forced down, using gentle air pressure, until its meniscus coincides with the top of the silica gel. A filter paper disk is placed at the top of the column, which must be leveled. Next, 2 ml of water-saturated chloroform is placed on the column and forced

1731 A

down, using air current, until its level almost coincides with the top of the silica gel. The column is filled to the top with water-saturated chloroform and attached to the rest of the apparatus through tubing H.

A tank of nitrogen gas is used to maintain a constant pressure of approximately 80 mm Hg in the flasks and the column. Experience has shown that a micropump (Buchler) can also be used to maintain a constant flow of solvent through the column. The solvent system coming out of Flask A is fed into the micropump, which is adjusted to maintain a flow rate of 24 drops per minute. The time needed to collect the desired 180 drops of effluent can be accurately set with an automatic "timer" unit on the fraction collector.

*Titration of organic acids.* After all of the 180 test tubes have been filled with 180 drops in each, the collecting tubes are removed from the fraction collector and titrated against 0.0200 *N* sodium hydroxide by an automatic titrator (Aminco), using phenol red or any other suitable indicator. A true titration value for each fraction is obtained by subtracting a baseline value from a recorded titration value. This "baseline value" is obtained when the entire procedure is carried out without the adding of acids to the column, but substituting a "blank" sample.

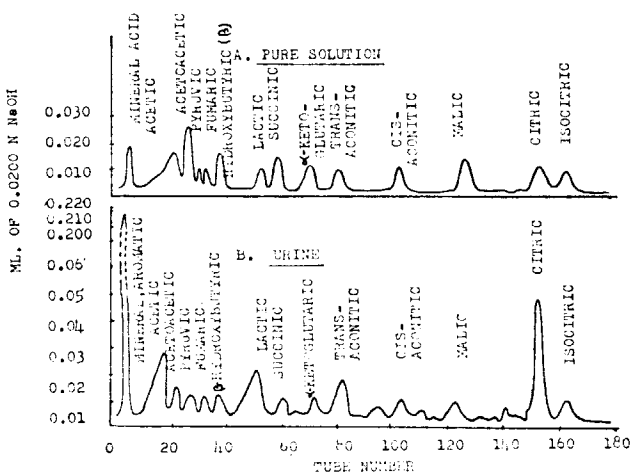


FIG. 2. Placement of acids; A, pure solution; B, urine.



*Chromatography of a mixture of organic acids.* Before using the above procedure for urine samples, in the authors' laboratory known mixtures of pure organic acids have also been successfully chromatographed by this method. For this purpose, a mixture of small amounts of known organic acids is prepared in *tert*-amyl alcohol. One ml of this mixture is taken and diluted with 1 ml of water-saturated chloroform, and it is placed in the column and chromatographed as described above. This procedure, of course, serves to determine the collecting tube placement of emerging peaks of each known organic acid, as well as to give the percentage "recovery" of each acid. The identity of each known organic acid isolated by the above procedure is confirmed by paper chromatography, using the method described by Ladd and Nossal (3).

## RESULTS AND DISCUSSION

Figure 2A is a typical graphic representation of a chromatogram showing positions of the various organic acids separated from pure solution. The graph shows that separation was good for most of the organic acids. It appears that the less polar organic acids have a tendency to be eluted earlier from the column compared to the more polar ones, which come out in later fractions. It should be noted that there was a clear separation, even of *cis*- and *trans*-aconitic, and citric and isocitric acids, which are rather difficult to separate by other current methods (*4, 6*). The percentage recoveries of the acids from pure solutions are given in Table 1. This table shows that there is an excellent recovery of all of the organic acids, with the exception of oxalosuccinic (data not reported) and oxaloacetic acids, which probably decompose quickly or are converted to some other intermediates.

To confirm the positions and homogeneity of the respective fractions of acids, an additional proof of their identity was needed. Although rechromatographing on the silica gel column could serve this purpose, paper chromatography as a supplementary tool for identification and also for separation was chosen, because of its simplicity and [illegible] sensitivity. By developing chromatograms of known mixtures of acids along with those of the extracts, and by reference to the $R_f$ values determined for single acids, the acids in the mixtures and extract were characterized. The $R_f$ values of the different organic acids, obtained by using Ladd and Nossal's method (3) are given in Table 2. Special

1731

TABLE 1

RECOVERIES OF ORGANIC ACIDS FROM MIXTURES OF 12 ACIDS ($\mu g$)

| Acid | Added | Recovered | Percentage recovered |
|---|---|---|---|
| | Mixture 1 | | |
| Fumaric | 75 | 74 | 98.5 |
| Succinic | 135 | 134 | 99.5 |
| Malic | 140 | 140 | 100.0 |
| Pyruvic | 190 | 185 | 97.5 |
| Lactic | 20 | 19 | 95.0 |
| *cis*-Aconitic | 118 | 115 | 94.5 |
| Citric | 47 | 46 | 98.5 |
| Oxaloacetic | 100 | 71 | 71.0 |
| α-Ketoglutaric | 32 | 30 | 93.5 |
| Isocitric | 46 | 44 | 96.0 |
| β-Hydroxybutyric | 42 | 40 | 95.5 |
| Acetic | 146 | 145 | 99.5 |
| | Mixture 2 | | |
| Fumaric | 150 | 147 | 98.0 |
| Succinic | 70 | 70 | 100.0 |
| Malic | 75 | 74 | 98.5 |
| Pyruvic | 29 | 27 | 93.0 |
| Lactic | 60 | 59 | 98.3 |
| *cis*-Aconitic | 40 | 38 | 95.0 |
| Citric | 120 | 122 | 103.0 |
| Oxaloacetic | 58 | 40 | 69.0 |
| α-Ketoglutaric | 108 | 104 | 96.5 |
| Isocitric | 23 | 22 | 95.6 |
| β-Hydroxybutyric | 25 | 23 | 92.0 |
| Acetic | 70 | 70 | 100.0 |

notice should be taken of the fact that the $R_f$ value of an individual organic acid was identical, whether pure acid or the mixture of pure acid, or organic acids separated from the urines were chromatographed. The data obtained by paper chromatography not only confirm the identity of the acids, but also show that separation by column chromatography, as outlined earlier, was complete. The appearance of only one spot, when a mixture of the acids and the corresponding pure acids were applied to paper, is further evidence of the identity of the acid. Likewise, it can be argued that the homogeneity



TABLE 2

$R_f$ VALUES OF THE ACIDS

| Acid | Pure acid | Separated by column chromatography from mixtures of pure acids | Separated from urine |
|---|---|---|---|
| A. Acidic solvent | | | |
| Acetoacetic | 0.83 | 0.83 | 0.83 |
| Fumaric | 0.81 | 0.81 | 0.81 |
| β-Hydroxybutyric | 0.73 | 0.73 | 0.73 |
| Lactic | 0.76 | 0.76 | 0.76 |
| Succinic | 0.74 | 0.74 | 0.74 |
| α-Ketoglutaric | 0.63 | 0.62 | 0.62 |
| trans-Aconitic | 0.67 | 0.68 | 0.67 |
| *cis*-Aconitic | 0.70 | 0.70 | 0.71 |
| Malic | 0.46 | 0.47 | 0.47 |
| Citric | 0.41 | 0.41 | 0.41 |
| Isocitric | 0.44 | 0.44 | 0.44 |
| B. Alkaline solvent | | | |
| Acetic | 0.06 | 0.06 | 0.06 |
| Pyruvic | 0.08 | 0.08 | 0.08 |
| Acetoacetic | 0.11 | 0.11 | 0.10 |

of the separated acids from columns is proved by the fact that not more than one spot on the chromatogram could be observed.

The method of silica-gel chromatography was next extended to the separation and quantitative estimation of the levels of different organic acids in normal human urine and normal rat urine. The presence in the urine of stable acids has been previously well established (*4, 6*). The data obtained on the human urine samples and the rat urine samples in the present study are presented in Tables 3 and 4, respectively. A graphic representation of the pattern of the urinary organic acids in human urine is also shown in Fig. 2B.

Having established the levels of the organic acids in the normal urine samples, recovery experiments were performed as before. In brief, known amounts of the acids, as their sodium salts, were added to exactly 100 ml of the same 24-hour human urine specimen. The volume was diluted to exactly 250 ml in a volumetric flask. A 25-ml aliquot of the resulting solution was used for analysis. The results of the recovery, presented in Table 5, clearly indicate that the method

1731

TABLE 3

ORGANIC ACIDS IN TYPICAL HUMAN URINE SAMPLES
(mg/24 hours)

| Acid | I | II |
|---|---|---|
| A. Non-Krebs cycle acids | | |
| Lactic | 198 | 136 |
| β-Hydroxybutyric | 34 | 101 |
| Pyruvic | 30 | 34 |
| Acetoacetic | 90 | 49 |
| Acetic | 37 | 49 |
| B. Krebs cycle acids | | |
| Citric | 514 | 550 |
| Aconitic | 36 | 79 |
| Isocitric | 75 | 66 |
| α-Ketoglutaric | 71 | 51 |
| Succinic | 13 | 35 |
| Fumaric | 32 | 40 |
| Malic | 21 | 44 |

TABLE 4

ORGANIC ACIDS IN TYPICAL RAT URINE SAMPLES
(mg/24 hours)

| Acid | I | II |
|---|---|---|
| A. Non-Krebs cycle acids | | |
| Lactic | 16 | 15 |
| β-Hydroxybutyric | 1 | 1 |
| Pyruvic | 2 | 2 |
| Acetoacetic | 4 | 5 |
| Acetic | 1 | 1 |
| B. Krebs cycle acids | | |
| Citric | 44 | 30 |
| Aconitic | 1 | 1 |
| Isocitric | 1 | 2 |
| α-Ketoglutaric | 1 | 1 |
| Succinic | 10 | 11 |
| Fumaric | 2 | 3 |
| Malic | 1 | 1 |



TABLE 5

RECOVERIES OF ACIDS FROM HUMAN URINE
(Av of 3 dets.)

| Acid | Acid added (meq/25 ml) | Acid recovered (meq/25 ml) | Recovery % |
|---|---|---|---|
| Acetic | 0.0136 | 0.0122 | 90.00 |
| Acetoacetic | 0.0057 | 0.0054 | 94.9 |
| Pyruvic | 0.0104 | 0.0105 | 100.0 |
| Fumaric | 0.0157 | 0.0140 | 89.0 |
| β-Hydroxybutyric | 0.0031 | 0.0028 | 91.0 |
| Lactic | 0.0098 | 0.0102 | 102.0 |
| Succinic | 0.0212 | 0.0194 | 91.5 |
| α-Ketoglutaric | 0.0073 | 0.0068 | 92.8 |
| Aconitic [a] | 0.0099 | 0.0092 | 92.7 |
| Malic | 0.0143 | 0.0141 | 98.5 |
| Critic | 0.0103 | 0.0095 | 92.2 |
| Isocitric | 0.0062 | 0.0062 | 96.8 |

[a] Includes both *cis*- and *trans*-aconitic acid.

was quite satisfactory for the extraction of the organic acids from urine.

It is evident from the data presented that silica-gel chromatographic method could be successfully used for the separation and quantitative determination of micro-quantities of the different organic acids present in the normal urine sample. This method has also been used successfully for determining the levels of the different urinary organic acids in certain physiological and pathological states (data being published elsewhere).

## SUMMARY

Silica-gel column chromatographic method for the quantitative determination of micro-quantities of 12 different, physiologically most important, organic acids in mixtures, as well as in human and rat urine samples, is described. This method is simple, accurate, rapid, and has a high degree of sensitivity. It yields a high percentage of recovery and the results are reproducible.

The identity of the acids separated by this technique has been repeatedly confirmed by paper chromatography; known pure acids were compared with those eluted from the silica-gel column used in this method.

1731

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PC364 *Citrate Inhibition of Ceruloplasmin* J. Biol. Chem. Vol. 239, No. 1

# Citric Acid as the Principal Serum Inhibitor of Ceruloplasmin*

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During an investigation of the effects of various compounds on the oxidase activity of ceruloplasmin, a strong inhibitory activity of undialyzed human serum was observed. Dialysis removed this inhibitor of ceruloplasmin from serum as shown in Figs. 1 and 4. However, dailysis did not diminish the strong inhibition by serum of the oxidation of ascorbate by $Cu^{++}$, possibly due to the presence of serum albumin. The dialysate had no effect on ascorbate oxidation catalyzed by $Cu^{++}$, indicating that: (*a*) there is a specific inhibitor(s) of ceruloplasmin in serum; and (*b*) the inhibitor(s) is dialyzable. When the dialysate was added back to the serum, the inhibition was restored as shown in Fig. 4. While this work was being completed, Walshe also reported the presence of an unidentified dialyzable inhibitor of ceruloplasmin in serum (1).

In order to identify the inhibitor in human serum, fresh samples of 80 to 100 ml of whole blood from five donors were collected and allowed to clot. The serum was separated by centrifugation and was treated according to the method of Huehns and Shooter (2) to obtain the ultrafiltrate. Each sample was shown to inhibit the ascorbate oxidase activity of purified ceruloplasmin (ultracentrifugally homogeneous and treated with Chelex 100). When a very small volume of serum ultrafiltrate (2.0 $\mu$l) was added to 3 ml of reaction mixture in the presence of $3.8 \times 10^{-5}$ M ascorbate and $2 \times 10^{-7}$ M ceruloplasmin, the rate was inhibited 25 to 50%. Preliminary experiments showed that the inhibitor(s) could pass through a column of CG-120, a strong cation exchange resin, but was retained on a column of CG-400, a strong anion resin, suggesting its acidic nature. Therefore, each ultrafiltrate was treated with a small column of CG-120. The strongly acidic solution obtained was then concentrated to a very small volume and subjected to examination by paper chromatography, with propionic acid-butanol-water (2:2:1) as the solvent. Two major spots and a minor spot were detected as acids on the paper strip. The mobility of the two major spots corresponds to that of authentic citric acid ($R_F$ 0.38) and lactic acid ($R_F$ 0.68) which were spotted on the same paper sheet. A major part of the inhibitory activity of the original dialyzed serum was associated with eluants from the two major spots. The ultrafiltrate of fresh human serum was examined by two other criteria to establish the presence of citric acid. Citrate was specifically detected by the formation of a pentabromoacetone-thiourea complex, described by Natelson *et al.* and modified by Elliot (3). Confirmatory data was also suggested by its conversion to ammonium citrazinate which gave strong fluorescence in the ultraviolet (4). We are also investigating the identity of any other inhibitors, although they are not in sufficient concentration to contribute significantly to ceruloplasmin inhibition in normal human serum.

The effect of citrate on the oxidation of ascorbate and other substrates by ceruloplasmin was tested, and the strong inhibitory activity of citrate, under appropriate conditions, was confirmed. Among the common organic acids tested as reported in Table I, citrate is the only acid which is sufficiently inhibitory and in sufficient concentration to account for the inhibition in serum. The amount of citrate in human serum is reported to be 1.1 to 3.2 mg/100 ml (5) or 0.7 to $1.7 \times 10^{-4}$ M. This has been con-

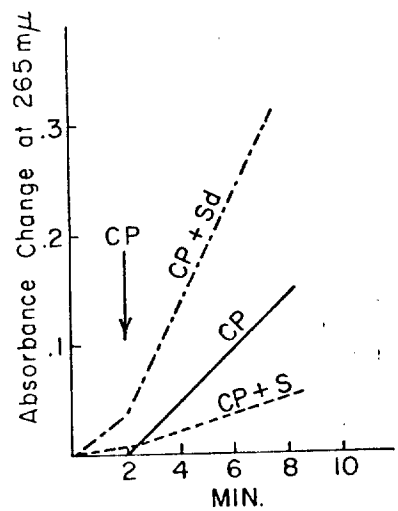


FIG. 1. Effect of dialysis on ceruloplasmin (*CP*) activity in serum. At zero time, fresh serum (*S*) and dialyzed serum (*Sd*) were added to $3.5 \times 10^{-5}$ M ascorbic acid in 0.2 M acetate buffer, pH 5.2. No addition was made to a third sample containing the same reaction mixture. At 2 minutes, $1.93 \times 10^{-7}$ M ceruloplasmin was added to all three reaction mixtures. As indicated ceruloplasmin can oxidize ascorbate only in serum which had been dialyzed.

TABLE I

*Inhibition of ceruloplasmin by organic acids*

No inhibition was observed by the following related acids at the indicated micromolarity: oxalosuccinic, 33; glyceric, 660; malonic, 660; pyruvic, 660; α-ketoglutaric, 660; dihydroxymaleic, 330; malic, 660; fumaric, 330; succinic, 330.

| Compound | Concentration | Inhibition* |
|---|---|---|
| | μM | % |
| Citric acid | 1 | 64 |
| | 100† | 95 |
| DL-Isocitric acid | 66 | 30 |
| | 330 | 80 |
| Oxalic acid | 660 | 25 |
| Oxaloacetic acid | 660 | 25 |
| *cis*-Aconitic acid | 660 | 0 |
| Lactic acid | >1000 | 0 |

* Ascorbate was $3.8 \times 10^{-5}$ M in a 3.0-ml volume containing 0.2 M acetate buffer at pH 5.2; temperature, 30°. Ceruloplasmin (homogeneous and treated with Chelex 100) concentration, $2.0 \times 10^{-7}$ M.

† This concentration corresponds to that of citrate in human blood.

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firmed in our laboratory; the estimated citrate concentration in 60 normal human serums was 0.4 to 2.6 × $10^{-4}$ M, as determined by the method of Elliot as described by Stern (3). Thus, the normal citrate concentration of serum is several hundred times greater than the $K_I$ value of 4 × $10^{-7}$ M reported below. Presumably, the oxidation of ascorbate and other ceruloplasmin substrates in the plasma will be strongly inhibited by citrate. The amount of serum isocitric acid, another powerful ceruloplasmin inhibitor (see Table I), was determined enzymically

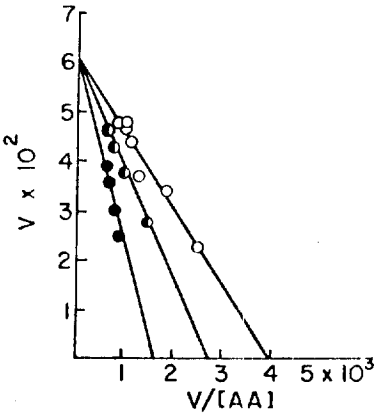


FIG. 2.[1] The competitive inhibition of ceruloplasmin by citrate as measured spectrophotometrically in a Beckman model DK-1 recording spectrophotometer at 30°. The velocity ($v$) of ascorbate oxidation by ceruloplasmin with or without presence of the inhibitor, citrate, at various concentrations of ascorbate ($AA$) were plotted against $v$/ascorbate concentration; $v$ in terms of absorbance change at 265 mμ per minute. ○——○, no citrate; ◐——◐, 2.0 × $10^{-7}$ M citrate; ●——●, 5.0 × $10^{-7}$ M citrate.

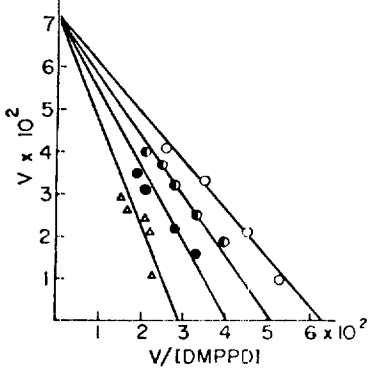


FIG. 3.[1] Competitive inhibition by citrate of the ceruloplasmin-catalyzed oxidation of $N,N$-dimethyl-$p$-phenylenediamine ($DMPPD$) as measured spectrophotometrically at 30°. The velocity ($v$) of $N,N$-dimethyl-$p$-phenylenediamine oxidation as measured in terms of the linear absorbance change at 550 mμ per minute by ceruloplasmin with and without citrate, at various concentrations of $N,N$-dimethyl-$p$-phenylenediamine was plotted against $v/(DMPPD)$. ○——○, no citrate; ◐——◐, 2.0 × $10^{-7}$ M citrate; ●——●, 5.0 × $10^{-7}$ M citrate; △——△, 10 × $10^{-7}$ M citrate.

[1] It is recognized that the treatment of the data usually implicit in Figs. 2 and 3 is obviously oversimplified because the concentrations of inhibitor and enzyme are approximately equal. The full appropriate kinetic treatment will be given in a subsequent paper.

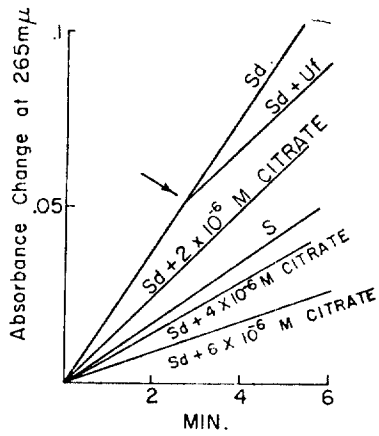


Fig. 4. Restoration of serum ceruloplasmin inhibition by the addition of both citrate and serum ultrafiltrate ($Uf$). Rates were determined with a Beckman model DK-1 recording spectrophotometer at a constant temperature of 30°. To a reaction mixture of 3.8 × $10^{-6}$ M ascorbate in 0.2 M acetate buffer, pH 5.2, 100 μl of serum ($S$) or dialyzed serum ($Sd$) were added to a total volume of 3.0 ml. The activity of undialyzed serum is increased by dialysis and is inhibited by both citrate and 100 μl of serum ultrafiltrate (addition indicated by the *arrow*) obtained from the same serum. In the assay, 1.2 × $10^{-4}$ M citrate, on dilution, gives a final concentration of 4 × $10^{-6}$ M in the reaction mixture.

on five different serums with the use of isocitric acid dehydrogenase (6) and was estimated to be less than $10^{-8}$ M.

Correlation of the total ceruloplasmin activity and the copper content of human serum precludes the possibility of a significant amount of a nondialyzable ceruloplasmin inhibitor in normal human serum. This is in agreement with Walshe (1), who reported a nondialyzable ceruloplasmin inhibitor only in serum from patients with Wilson's disease.

Further kinetic studies on the inhibition by citrate of the oxidase activity of ceruloplasmin are shown in Figs. 2 and 3. The inhibition is competitive for both ascorbate and $N,N$-dimethyl-$p$-phenylenediamine oxidation with $K_I$ values of 4 × $10^{-7}$ M and 7 × $10^{-7}$ M, respectively. Citrate also competitively inhibits $p$-phenylenediamine oxidation by ceruloplasmin with a $K_I$ value of 8 × $10^{-7}$ M. With the use of conventional Warburg techniques at 30° in a 3-ml reaction mixture containing 0.002 M ascorbate, 9.0 × $10^{-7}$ M ceruloplasmin, 1.7 × $10^{-3}$ M neocuproine, and 0.2 M acetate buffer (pH 5.2), 50% inhibition of the initial rate of oxygen uptake was produced by $10^{-4}$ M citrate. Greater concentrations of citrate are required to inhibit ceruloplasmin activity when determined by oxygen uptake, probably because the ascorbate concentration (0.002 M) is 100 times greater than in the spectrophotometric method. The partial inhibition of ceruloplasmin by high concentrations of citrate ($10^{-2}$ M) first reported by Holmberg and Laurell (7) was considered essentially an anionic effect and was not associated with the presence of citrate in serum heretofore. Broman (8) and Curzon (9) have reported earlier on the inhibition of ceruloplasmin by numerous dibasic acids, none of which inhibited as strongly as the tribasic acids reported here.

In Fig. 4, the amount of citric acid that will restore the inhibition to dialyzed serum to give the same amount of inhibition as undialyzed serum is estimated to be 1.2 × $10^{-4}$ M. The undialyzed serum used in this experiment contained 1.3 × $10^{-4}$ M citrate as measured by the formation of pentabromo-

acetone-thiourea complex (3); 1 ml of the same serum was dialyzed against 1 liter of 0.1 M acetate buffer, pH 5.5, and then checked for activity. The results of Fig. 4 show that the concentration of citric acid which gives the same amount of inhibition is very close to $1.3 \times 10^{-4}$ M. Both ceruloplasmin and the inhibitor of the serum were diluted 30-fold on addition to the final reaction mixture. Therefore, the original concentration of citrate in the serum anticipated from this kinetic study is $4 \times 10^{-6}$ M $\times$ 30 or $1.2 \times 10^{-4}$ M, which is in good agreement with the value, $1.3 \times 10^{-4}$ M, obtained by the pentabromoacetone-thiourea method. This result also strongly suggests that the major inhibitor in the blood is citric acid.

In a previous paper (10), we have discussed the ascorbate oxidase activity of ceruloplasmin and emphasized that ascorbate oxidation could not be due to $Cu^{++}$ contamination as suggested by Morrell, Aisen, and Scheinberg (11). For example, it was pointed out that several compounds which completely inhibited the $Cu^{++}$ catalysis of ascorbate, *e.g.* neocuproine and serum albumin, did not affect ceruloplasmin catalysis of ascorbate oxidation. Citrate inhibition of ceruloplasmin action provides a converse situation, since $10^{-5}$ M citrate does not affect ascorbate oxidation as catalyzed by $Cu^{++}$, but inhibits ceruloplasmin oxidation over 85%.

Finally, it should be emphasized that tests for ceruloplasmin activity in serum or plasma are valid only under conditions in which serum inhibitors have been removed. Obviously, anticoagulants such as citrate, oxalate, or EDTA, which are ceruloplasmin inhibitors, should be avoided or eliminated through exhaustive dialysis or other procedures. The implications of a common metabolite such as citrate in participating in the control of the activity of this serum enzyme are intriguing. The possible biological and clinical importance of the serum inhibitors of ceruloplasmin is now under further investigation.

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1755

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# Taste Interrelationships. V. Sucrose, Sodium Chloride, and Citric Acid in Lima Bean Purée[a]

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**SUMMARY**

**A highly trained panel of nine judges determined the taste interactions of suprathreshold concentrations of sucrose, sodium chloride, and citric acid in lima bean purée. The results were in good agreement with previous results with solutions of distilled water, in which: 1) sucrose and citric acid exhibited a mutual masking effect, and 2) sucrose and sodium chloride produced mutual masking except for low levels of sodium chloride (0.05 and 0.10%), which slightly enhanced the apparent sweetness of 0.4 and 0.8% sucrose. Sodium chloride generally depressed sourness, but citric acid significantly increased the saltiness of all levels of sodium chloride. The large amount of individual-judge variation, especially in the acid-salt comparisons, is discussed.**

## INTRODUCTION

The interaction of sweetness, saltiness, and sourness in solutions of distilled water has been reported by several investigators (Fabian and Blum, 1943; Kamenetzky and Pilgrim, 1958; Beebe-Center *et al.*, 1959; Kamen *et al.*, 1961; and Pangborn, 1960, 1961, 1962). There is no assurance, however, that combinations of pure compounds dispersed in aqueous mediums will elicit similar patterns of response in more complex food systems. For example, fructose is sweeter than sucrose on a weight basis. Although this relationship is valid for water solutions, it was not true when the medium was pear nectar (Pangborn, 1963b). It is much more difficult to ascertain the mechanisms by which primary tastes interact in natural and processed foods because olfactory, kinesthetic, and tactile attributes of the commodity complicate the taste stimuli.

The present investigation was made to establish the taste interrelationships of the following compounds in a natural food product, lima bean purée:

1) Sucrose-citric acid (Studies I & II).
2) Sucrose-sodium chloride (Studies III & IV).
3) Citric acid-sodium chloride (Studies V & VI).

**Preparation of samples.** Frozen lima beans (USDA Grade A all-green Fordhook variety; Oxnard Frozen Foods Coop., Oxnard, Calif.) were selected as the test material because of availability, uniformity, and freedom from salt or other additives. During the six months required to complete the study, the beans were stored at −15°F in 20-lb lots in polyethylene bags in sealed cardboard containers. Purées were prepared daily between 1 and 2 P.M. by adding 600 g of frozen beans to 500 ml of boiling distilled water and cooking for 20 min. The cooked beans were drained for 5 min, combined with 500 ml of distilled water, and puréed in an electric blender for 100 sec at 110 volts. Weighed quantities of sucrose, sodium chloride, or citric acid were added to weighed portions of the purée, which was placed in covered glass beakers in a water bath maintained at 174±2°F during the time of serving (2:30 to 3:00 P.M.). The concentrations of the additives, which had been pretested, ranged from threshold to very intense levels for lima beans. Samples were served in coded, black-lined, heavy porcelain cups that were placed in polystyrene blocks to maintain the temperature between 165 and 170°F during the tasting period.

**Taste panel.** Six men and seven women judges were used. All had extensive experience in tasting aqueous solutions and

[a] Presented in part at the 23rd IFT Annual Meeting, Detroit, Michigan, May 28, 1963.

one year of experience in tasting lima bean purée. The original selection was made on the basis of a judge's consistent ability to recognize small differences in sweetness, sourness, and saltiness in aqueous solutions (Pangborn, 1962). Judges were seated in individual, partitioned booths maintained at 70±2°F. Each judge received four pairs of purée in randomized order at each test session except in Study VI, where five pairs were presented. All testing was done under low, red illumination to eliminate visual clues, since the purées containing citric acid were a lighter green than those containing sucrose or salt. Distilled water was provided for oral rinsing, but judges were instructed not to swallow any water or purée. Testing sessions were held Monday through Friday, and panel members were informed of their progress after each session. Individuals who were unable to attend a testing period were required to have a "make-up" the following day so that, within a study, all samples were tested by all subjects an equal number of times. As indicated in Table 5, not all judges were available for all studies.

A method of paired-comparison constant-stimulus, described previously (Pangborn, 1961), was used. Within a pair, both samples contained the secondary taste compound whereas only one contained the primary compound. Judges were required to circle the number of the sample within each pair that contained the greater intensity of the secondary taste sensation. In addition, judges indicated the degree of difference in taste intensity within each pair on a four-point scale of "slight, moderate, large, or extreme." These descriptive terms were converted to integers of −7, −5, −3, −1, +1, +3, +5, and +7, and analysis of variance was applied to the data. Chi-square analysis was applied to determine significance of difference in the paired selections.

## RESULTS

**Study I. Effect of sucrose on apparent sourness of citric acid.** The frequency of selection of the sample with the greater sourness within each pair is given in Table 1. As the amount of sucrose was increased, a greater number of selections indicated that the sweetened sample was less sour. This reduction in sourness by sucrose occurred to the same degree in all four levels of citric acid. The average difference scores for this relationship (Fig. 1) were all negative, verifying that increasing amounts of sucrose decreased the apparent sourness of all levels of citric acid to a similar degree. Analysis of variance of the difference scores indicated that the judges contributed substantially to the amount of variation in the data and reacted differentially to both the citric acid and the sucrose (Table [illegible]). No variation was attributable to replications, indicative of the reproducibility of the responses. Table 5 shows that although all judges ascribed negative average total difference scores, the intensity of difference ranged from −0.31 to −2.[illegible].

**Study II. Effect of citric acid on apparent sweetness of sucrose.** Selections of samples with greater sweetness within pairs showed that levels of 0.1 and 0.2% citric acid significantly reduced apparent sweetness (Table 1). Higher levels of sucrose were reduced slightly more than were lower levels (Fig. 2). The analysis of variance

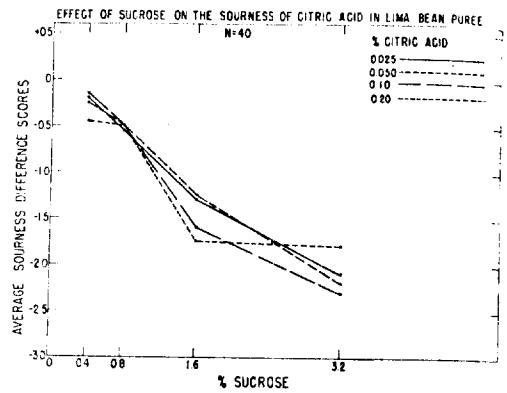


Fig. 1. Effect of sucrose on apparent sourness of citric acid in lime bean purée (n = 40).

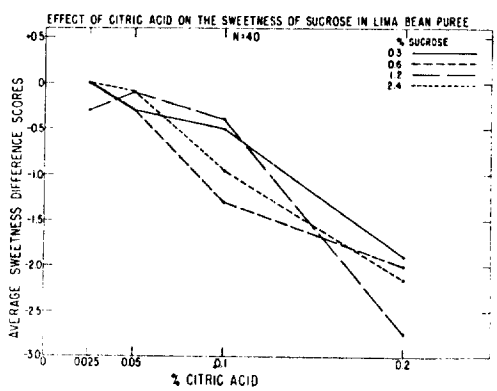


Fig. 2. Effect of citric acid on apparent sweetness of sucrose in lima bean purée (n = 40).

Table 1. Taste intensity of sucrose, sodium chloride, and citric acid in lima bean purée. Frequency of selection of sample without primary as most intense within each pair.

| Primary: Sucrose (%) | I. Sucrose—sourness (n=40) | | | | |
|---|---|---|---|---|---|
| | 0.4 | 0.8 | 1.6 | 3.2 | Total |
| % Citric acid | | | | | |
| .025 | 24 | 27[a] | 36[c] | 36[c] | 123[c] |
| .05 | 24 | 25 | 33[c] | 35[c] | 117[c] |
| .10 | 22 | 29[b] | 37[c] | 40[c] | 128[c] |
| .20 | 25 | 28[a] | 35[c] | 33[c] | 121[c] |
| Total | 95[a] | 109[c] | 141[c] | 144[c] | 489[c] |

| Primary: Citric acid (%) | II. Citric—sweetness (n=40) | | | | |
|---|---|---|---|---|---|
| | .025 | .05 | .10 | .20 | Total |
| % Sucrose | | | | | |
| 0.3 | 20 | 21 | 25 | 34[c] | 100[b] |
| 0.6 | 20 | 24 | 36[c] | 35[c] | 115[c] |
| 1.2 | 23 | 21 | 26 | 38[c] | 108[c] |
| 2.4 | 25 | 24 | 35[c] | 37[c] | 121[c] |
| Total | 88 | 90 | 122[c] | 144[c] | 444[c] |

| Primary: Sucrose (%) | III. Sucrose—saltiness (n=36) | | | | |
|---|---|---|---|---|---|
| | 0.2 | 0.4 | 0.8 | 1.6 | Total |
| % NaCl | | | | | |
| .05 | 18 | 17 | 27[b] | 31[c] | 93[c] |
| .10 | 22 | 23 | 25[a] | 32[c] | 102[c] |
| .20 | 20 | 18 | 32[c] | 32[c] | 102[c] |
| Total | 60 | 58 | 84[c] | 95[c] | 297[c] |

| Primary: NaCl (%) | IV. Sodium chloride—sweetness (n=36) | | | | |
|---|---|---|---|---|---|
| | .05 | .10 | .20 | .40 | |
| % Sucrose | | | | | |
| 0.4 | 13 | 10[a] | 16 | 16 | 55[b] |
| 0.8 | 17 | 15 | 18 | 20 | 70 |
| 1.6 | 14 | 14 | 20 | 24 | 72 |
| Total | 44[a] | 39[b] | 54 | 60 | 197 |

| Primary: Citric acid (%) | V. Citric—saltiness (n=45) | | | | | |
|---|---|---|---|---|---|---|
| | .025 | .05 | .10 | .20 | .40 | Total |
| % NaCl | | | | | | |
| .03 | 21 | 29 | 21 | 25 | 23 | 119 |
| .06 | 24 | 23 | 20 | 24 | 23 | 114 |
| .12 | 27 | 27 | 18 | 21 | 21 | 114 |
| .25 | 31[a] | 17 | 22 | 25 | 24 | 119 |
| Total | 103 | 96 | 81 | 95 | 91 | 466[a] |

| Primary: NaCl (%) | VI. Sodium chloride—sourness (n=36) | | | | |
|---|---|---|---|---|---|
| | .05 | .10 | .20 | .40 | Total |
| % Citric acid | | | | | |
| .035 | 29[c] | 26[a] | 22 | 24 | 101[c] |
| .070 | 28[b] | 26[a] | 24 | 23 | 101[c] |
| .140 | 26[a] | 24 | 27[b] | 21 | 98[c] |
| .280 | 25[a] | 22 | 29[c] | 21 | 97[c] |
| Total | 108[c] | 98[c] | 102[c] | 89[b] | 397[c] |

[a, b, c] Respectively significant differences within pairs at P = 0.05, 0.01, and 0.001.

1767

Table 1. Taste intensity of sucrose, sodium chloride, and citric acid in lima bean purée. Frequency of selection of sample without primary as most intense within each pair.

| Primary: Sucrose (%) | I. Sucrose—sourness (n=40) | | | | |
|---|---|---|---|---|---|
| | 0.4 | 0.8 | 1.6 | 3.2 | Total |
| % Citric acid | | | | | |
| .025 | 24 | 27[a] | 36[c] | 36[c] | 123[c] |
| .05 | 24 | 25 | 33[c] | 35[c] | 117[c] |
| .10 | 22 | 29[b] | 37[c] | 40[c] | 128[c] |
| .20 | 25 | 28[a] | 35[c] | 33[c] | 121[c] |
| Total | 95[a] | 109[c] | 141[c] | 144[c] | 489[c] |

| Primary: Citric acid (%) | II. Citric—sweetness (n=40) | | | | |
|---|---|---|---|---|---|
| | .025 | .05 | .10 | .20 | Total |
| % Sucrose | | | | | |
| 0.3 | 20 | 21 | 25 | 34[c] | 100[b] |
| 0.6 | 20 | 24 | 36[c] | 35[c] | 115[c] |
| 1.2 | 23 | 21 | 26 | 38[c] | 108[c] |
| 2.4 | 25 | 24 | 35[c] | 37[c] | 121[c] |
| Total | 88 | 90 | 122[c] | 144[c] | 444[c] |

| Primary: Sucrose (%) | III. Sucrose—saltiness (n=36) | | | | |
|---|---|---|---|---|---|
| | 0.2 | 0.4 | 0.8 | 1.6 | Total |
| % NaCl | | | | | |
| .05 | 18 | 17 | 27[b] | 31[c] | 93[c] |
| .10 | 22 | 23 | 25[a] | 32[c] | 102[c] |
| .20 | 20 | 18 | 32[c] | 32[c] | 102[c] |
| Total | 60 | 58 | 84[c] | 95[c] | 297[c] |

| Primary: NaCl (%) | IV. Sodium chloride—sweetness (n=36) | | | | |
|---|---|---|---|---|---|
| | .05 | .10 | .20 | .40 | Total |
| % Sucrose | | | | | |
| 0.4 | 13 | 10[a] | 16 | 16 | 55[b] |
| 0.8 | 17 | 15 | 18 | 20 | 70 |
| 1.6 | 14 | 14 | 20 | 24 | 72 |
| Total | 44[a] | 39[b] | 54 | 60 | 197 |

| Primary: Citric acid (%) | V. Citric—saltiness (n=45) | | | | | |
|---|---|---|---|---|---|---|
| | .025 | .05 | .10 | .20 | .40 | Total |
| % NaCl | | | | | | |
| .03 | 21 | 29 | 21 | 25 | 23 | 119 |
| .06 | 24 | 23 | 20 | 24 | 23 | 114 |
| .12 | 27 | 27 | 18 | 21 | 21 | 114 |
| .25 | 31[a] | 17 | 22 | 25 | 24 | 119 |
| Total | 103 | 96 | 81 | 95 | 91 | 466[a] |

| Primary: NaCl (%) | VI. Sodium chloride—sourness (n=36) | | | | |
|---|---|---|---|---|---|
| | .05 | .10 | .20 | .40 | Total |
| % Citric acid | | | | | |
| .035 | 29[c] | 26[a] | 22 | 24 | 101[c] |
| .070 | 28[b] | 26[a] | 24 | 23 | 101[c] |
| .140 | 26[a] | 24 | 27[b] | 21 | 98[c] |
| .280 | 25[a] | 22 | 29[c] | 21 | 97[c] |
| Total | 108[c] | 98[c] | 102[c] | 89[b] | 397[c] |

[a, b, c] Respectively significant differences within pairs at P = 0.05, 0.01, and 0.001.

17617

Table 2. Analysis of variance of difference scores. Interaction of sucrose and citric acid.

| Source of variation | Degrees of freedom | I. Sucrose—sourness[a] Mean square | II. Citric—sweetness[b] Mean square |
|---|---|---|---|
| (S) Sucrose | 3 | 116.62*** | 1.72 |
| (C) Citric | 3 | 0.42 | 151.72*** |
| (J) Judges | 9 | 30.59*** | 25.80*** |
| (R) Replications | 3 | 0.72 | 4.34* |
| S × C | 9 | 1.49 | 4.16** |
| S × J | 27 | 9.44*** | 1.78 |
| S × R | 9 | 1.01 | 1.02 |
| C × J | 27 | 5.30*** | 7.77*** |
| C × R | 9 | 2.17 | 1.31 |
| J × R | 27 | 2.39 | 1.70 |
| S × C × R | 27 | 1.27 | 1.18 |
| S × C × J | 81 | 1.70 | 3.23*** |
| S × R × J | 81 | 1.50 | 1.13 |
| C × R × J | 81 | 1.79 | 1.40 |
| Remainder | 243 | 1.58 | 1.45 |

[a] Effect of sucrose on sourness of citric acid.
[b] Effect of citric acid on sweetness of sucrose.
*, **, *** Respectively sig. at P = 0.05, 0.01, 0.001.

demonstrated a significant interaction between the two compounds (Table 2). Judges contributed significantly to the observed variation in the data and reacted differently to the acid but not to the sugar. The slight degree of significance due to replication suggests that some judges were inconsistent in their responses upon repeated tasting, or that some other factor influenced the scoring. Only one judge (Judge 9) indicated a general enhancement of sweetness by the acid (Table 5). Judges 1, 2, 3, 10, and 11 averaged high negative values, and greatly influenced the average panel response.

**Study III. Effect of sucrose on apparent saltiness of sodium chloride.** The higher levels of sucrose (0.8 and 1.6%) decreased the saltiness of 0.05, 0.10, and 0.20% salt (Table 1). As illustrated in Fig. 3, the lowest salt level was depressed the least. There was greater agreement in opinion between judges in this study than in any other, as evidenced by lower judge variation (Table 3) and a smaller range in the average total difference scores (Table 5).

**Study IV. Effect of sodium chloride on apparent sweetness of sucrose.** Lower levels of sodium chloride enhanced and higher levels depressed apparent sweetness (Table 1, Fig. 4). The apparent sweetness of the highest concentration of sucrose, 1.6%, was reduced substantially by additions of 0.2 and 0.4% sodium chloride. Although the same judges participated in both studies, the analysis of variance showed that variability attributable to judges was greater in Study IV than in Study III (Table 3). In addition, all interactions involving judges were significant. Table

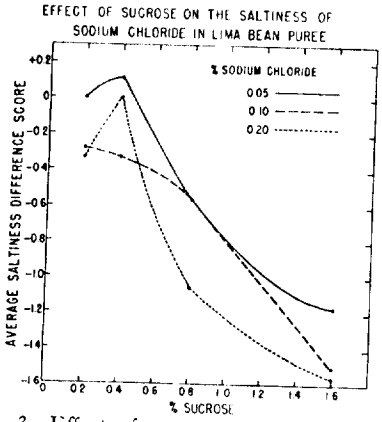


Fig. 3. Effect of sucrose on apparent saltiness of sodium chloride in lima bean purée (n = 36).

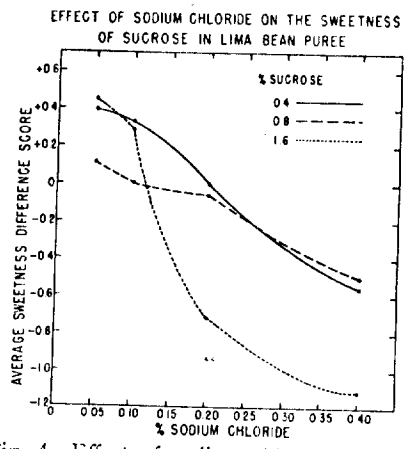


Fig. 4. Effect of sodium chloride on apparent sweetness of sucrose in lima bean purée (n = 36).

Table 3. Analysis of variance of difference scores. Interaction of sucrose and sodium chloride.

| Source of variation | III. Sucrose—saltiness[a] | | IV. Sodium chloride—sweetness[b] | |
|---|---|---|---|---|
| | Degrees of freedom | Mean square | Degrees of freedom | Mean square |
| (S) Sucrose | 3 | 39.62*** | 2 | 3.68 |
| (N) NaCl | 2 | 4.45* | 3 | 24.33*** |
| (J) Judges | 8 | 7.48*** | 8 | 78.23*** |
| (R) Replications | 3 | 0.21 | 3 | 0.63 |
| S × N | 6 | 1.07 | 6 | 2.85* |
| S × J | 24 | 4.48*** | 16 | 3.40** |
| S × R | 9 | 1.47 | 6 | 1.15 |
| N × J | 16 | 1.14 | 24 | 8.30*** |
| N × R | 6 | 0.55 | 9 | 1.01 |
| J × R | 24 | 1.49 | 24 | 2.20* |
| S × N × R | 18 | 0.97 | 18 | 0.98 |
| S × N × J | 48 | 1.50* | 48 | 2.72*** |
| S × R × J | 72 | 1.61** | 48 | 0.88 |
| N × R × J | 48 | 1.02 | 72 | 1.30 |
| Remainder | 144 | 1.01 | 144 | 1.27 |

[a] Effect of sucrose on saltiness of sodium chloride.
[b] Effect of sodium chloride on sweetness of sucrose.
*, **, *** Respectively sig. at P= 0.05, 0.01, and 0.001.

5 shows the range in average difference scores categorized by individual judges. Judges 2, 3, and 13 assigned high negative values, whereas the remaining six judges assigned positive difference scores.

**Study V. Effect of citric acid on apparent saltiness of sodium chloride.** In this study, judges were sharply divided in their reaction to apparent saltiness. As indicated in Table 5, five of the nine subjects found saltiness to be decreased by increasing amounts of citric acid, whereas four subjects reported marked enhancement. The magnitude of this subdivision can be seen in the following table:

| % citric acid | 0.025 | 0.05 | 0.1 | 0.2 | 0.4 |
|---|---|---|---|---|---|
| | | | average difference scores | | |
| Group A. n = 100 (5 subjects) | −0.16 | −0.18 | −0.52 | −1.60 | −1.62 |
| Group B. n = 80 (4 subjects) | −0.05 | 0 | +1.05 | +2.58 | +4.45 |

Although the majority of the panel reported depression of saltiness, the scores of the minority carried more weight, so that the average effect was enhancement (Fig. 5). Statistical analysis of these data showed the great extent to which judge variation influenced the results (Table 4). The insignificant variance for replications attests to the ability of the panel to replicate the responses; however, J × R was significant, as was the interaction of C × N × J. This study is currently being repeated with water solutions and green bean purée, using the same experimental subjects.

**Study VI. Effect of sodium chloride on apparent sourness of citric acid.** In general, sodium chloride decreased apparent sourness (Fig. 6). The lower levels of citric acid produced a pattern quite dissimilar to the patterns for higher levels. Analysis of variance suggested that the large judge-effects may have been responsible for the response pattern. Panel replications and the interaction of J × R were not significant, indicating a reproducibility of individual judgment, even though the interaction of judges × sodium chloride was significant.

## DISCUSSION

Responses to combinations of sucrose, sodium chloride, and citric acid in lima bean purée were in complete agreement with those obtained previously in solutions of distilled water (Pangborn, 1960, 1961, 1962). Although the range of concentrations tested was greater in water than in purée, the patterns of response were similar, if not identical.

The most clear-cut results were those involving interactions of sweetness and sourness, where mutual masking occurred in water (Pangborn, 1960, 1961, 1963a), in pear nectar (Pangborn, 1960), in canned tomato juice (Pangborn and Chrisp, 1964),

Table 4. Analysis of variance of difference scores. Interaction of citric acid and sodium chloride.

| Source of variation | V. Citric—saltiness[a] Degrees of freedom | Mean square | VI. Sodium chloride—sourness[b] Degrees of freedom | Mean square |
|---|---|---|---|---|
| (C) Citric | 4 | 42.43*** | 3 | 2.12 |
| (N) NaCl | 3 | 2.93 | 3 | 4.99 |
| (J) Judges | 8 | 192.21*** | 8 | 82.42*** |
| (R) Replications | 4 | 0.96 | 3 | 1.64 |
| C × N | 12 | 2.58* | 9 | 1.83 |
| C × J | 32 | 50.20*** | 24 | 3.18 |
| C × R | 16 | 0.80 | 9 | 3.04 |
| N × J | 24 | 2.93** | 24 | 10.72*** |
| N × R | 12 | 2.12 | 9 | 2.36 |
| J × R | 32 | 3.21*** | 24 | 1.32 |
| C × N × R | 48 | 0.89 | 27 | 1.80 |
| C × N × J | 96 | 2.25** | 72 | 3.79** |
| C × R × J | 128 | 1.61 | 72 | 1.27 |
| N × R × J | 96 | 1.33 | 72 | 1.58 |
| Remainder | 384 | 1.34 | 216 | 2.25 |

[a] Effect of citric acid on saltiness of sodium chloride.
[b] Effect of sodium chloride on sourness of citric acid.
*, **, *** Respectively sig. at P = 0.05, 0.01, and 0.001.

as well as in lima bean purée. In variance with the results obtained in our laboratories are those of Fabian and Blum (1943), who used a matching technique, and Kamen *et al.* (1961), who presented samples singly. Although those investigators agreed that sucrose reduced the intensity of sourness in water solutions, they observed that citric acid generally increased the sweetness of sucrose. In Fabian and Blum's study, various acids affected the sweetness of glucose, sucrose, and fructose differentially, but all sugars decreased the sourness of all acids. Pangborn (1963a) retested much of those data, using a paired-comparison constant-stimulus procedure, and observed that citric, tartaric, lactic, and acetic acids significantly reduced the sweetness of glucose, sucrose, lactose, and fructose to approximately the same extent.

Agreement on the general interaction-effects of sweetness and saltiness was excellent among various carriers, but not between investigators. In our laboratory, in

Table 5. Average difference scores assigned by individual judges.[a]

| Judge | Sex | I Sucrose—sourness | II Citric—sweetness | III Sucrose—saltiness | IV NaCl—sweetness | V Citric—saltiness | VI NaCl—sourness |
|---|---|---|---|---|---|---|---|
| | | n = 64 | n = 64 | n = 48 | n = 48 | n = 100 | n = 64 |
| 1 | F | −1.56 | −1.44 | −0.79 | 1.25 | −1.06 | −2.75 |
| 2 | F | −1.25 | −1.13 | −0.58 | −1.38 | 0.88 | 0.34 |
| 3 | F | −1.63 | −1.75 | −1.29 | −1.71 | −1.42 | −1.50 |
| 4 | F | −0.56 | −0.75 | ... | ... | −0.48 | −0.31 |
| 5 | F | −0.78 | −0.34 | ... | ... | 2.08 | 0.03 |
| 6 | F | −0.09 | −0.22 | −0.71 | 0.46 | ... | ... |
| 7 | F | ... | ... | −0.13 | 0.46 | ... | ... |
| 8 | M | −0.72 | −0.19 | −0.04 | 0.83 | −0.88 | −1.66 |
| 9 | M | −0.31 | 0.13 | −0.54 | 0.71 | −0.24 | 0.63 |
| 10 | M | −2.16 | −1.41 | ... | ... | 2.32 | −1.72 |
| 11 | M | −1.81 | −1.06 | ... | ... | 1.14 | −1.34 |
| 12 | M | ... | ... | −0.96 | 0.54 | ... | ... |
| 13 | M | ... | ... | −0.38 | −2.21 | ... | ... |
| Δ | | 2.07 | 1.88 | 1.25 | 3.46 | 3.74 | 3.38 |

[a] Negative scores indicate the compound depressed apparent taste intensity. Positive scores indicate the compound enhanced apparent taste intensity.

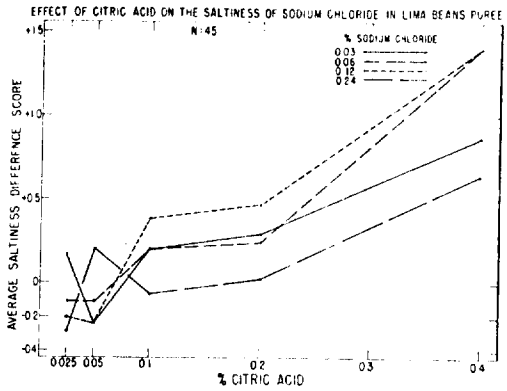


Fig. 5. Effect of citric acid on apparent saltiness of sodium chloride in lime bean purée (n = 45).

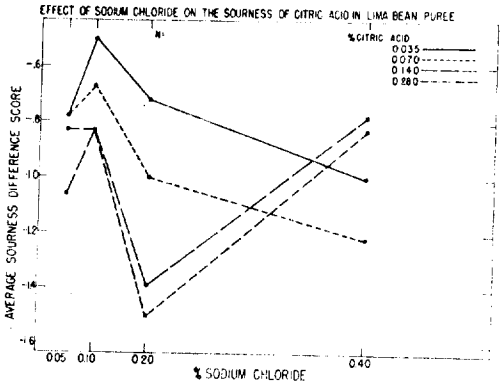


Fig. 6. Effect of sodium chloride on apparent sourness of citric acid in lima bean purée (n = 36).

water solutions, canned tomato juice, and lima bean purée, sucrose and sodium chloride exhibited mutual masking effects, except for a slight enhancement of low levels of sucrose by low levels of sodium chloride. For solutions of distilled water, Fabian and Blum (1943) reported that sodium chloride increased the sweetness of all sugars but all sugars decreased saltiness. Beebe-Center *et al.* (1959) observed mutual masking except for slight enhancement of solutions of 1% sucrose by up to 3% sodium chloride. Kamen *et al.* (1961) concluded that the reduction of the sweetness of sucrose by sodium chloride occurred primarily in solutions that were high in both salt and sugar. For the lower sugar concentrations, the various levels of salt had relatively little effect. Those investigators further reported an absence of an overall enhancing or masking effect in their study of sucrose on saltiness.

Citric acid increased the saltiness of sodium chloride in water solutions (Fabian and Blum, 1943; Kamen *et al.*, 1961), in canned tomato juice (Pangborn and Chrisp, 1964), and in lima bean purée. Sodium chloride, on the other hand, generally decreased sourness in all mediums. Kamen *et al.* (1961) noted that the highest levels of salt tended to enhance the sourness of the lower concentrations of citric acid but reduced the sourness of the higher acid concentrations. They were unable to give an explanation for this non-monotonic function but observed that the results of the effect of salt on sourness were more complex than those from other interactions.

As early as 1899 Heymans reported that sodium chloride raised the threshold for hydrochloric acid, and vice versa. Only one subject was employed in his investigation. Subsequently, Cragg (1937), using an undisclosed number of subjects, reported that the addition of 0.2*M* sodium chloride had no effect on the sourness of hydrochloric acid. According to Hahn and Ulbrich (1948), thresholds for hydrochloric or acetic acids were unaffected by the addition of threshold levels of sodium chloride. Thresholds for sodium chloride, however, were decreased in one out of three subjects for hydrochloric acid and in two out of four subjects for acetic acid. Anderson (1950) observed that tartaric acid raised the threshold for sodium chloride in both experimental subjects, but the acid threshold increased in the presence of salt for only one of the subjects.

It is apparent that a lack of agreement between subjects could be the major contributing factor in producing divergent results between laboratories. To our knowledge, none of the investigators cited herein has separated their data for analysis of individual judge effects. In some studies, only one or two judges were used. Fabian and Blum (1943) used results from ten subjects but did not analyze for judge or replication effects. Kamen *et al.* (1961) drew his 960 judgments from 700 participants, so that few people tasted more than

once. Nevertheless they stated there was a significant between-judge variation in their sodium chloride-sourness study. Using the same single-stimulus method as did Kamen *et al.*, Pangborn (1962) observed highly significant variation attributable to treatments, judges, replications, and all interactions. However, when the same judges used the paired-stimulus technique, significant F ratios were obtained for treatments and judges but not for replications (d.f. = 9) or for any interactions involving replications.

Thus it can be concluded that the factors accounting for contradictory responses to taste mixtures between laboratories include: 1) individual variability in response to taste mixtures; 2) lack of reproducibility of judgment upon repeated testing; 3) range of concentrations tested; and 4) the psychophysical tests used.


## ACKNOWLEDGMENTS

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# CITRIC ACID CONTENT OF MILK IN MANITOBA*

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(With 1 Figure)

Though important, citric acid seems to be one of the least investigated of the minor constituents of milk. Henkel(1) isolated and identified citric acid as a normal constituent of cow's milk as early as 1888. A search of the literature reveals, however, that at the present time, 70 years later, there are still too few published results of investigations to get a true picture of the citric acid content of milk. The available data are summarized in Table 1.

Table 1. *Published values for citric acid content of milk*

| Authority | No. of samples | and description | Average citric acid content (%) | Highest value (%) | Lowest value (%) |
|---|---|---|---|---|---|
| Scheibe(2) | 32 | — | 0·132 | 0·298 | 0·082 |
| Sommer & Hart(3) | 16 | — | 0·231 | 0·248 | 0·191 |
| Hess, Unger & Supplee(4) | 2 | Cows stall fed | 0·08 | — | — |
| | | Cows pasture fed | 0·130 | — | — |
| Supplee & Bellis(5) | 17 | Winter and summer | 0·145 | 0·182 | 0·121 |
| | | Winter | 0·142 | — | — |
| | | Summer | 0·148 | — | — |
| | 8 | Evaporated and condensed milks, reconstituted | 0·112 | 0·151 | 0·078 |
| | 15 | Milk powder, reconstituted | 0·137 | 0·177 | 0·102 |
| Kieferle, Schwaibold & Hackmann(6) | 104 | Individual quarter samples from 26 cows | — | 0·4 | 0·2 |
| Sherwood & Hammer(7) | 335 | From individual cows in various stages of lactation, of various breeds and on various feeds | 0·18 | 0·33 | 0·07 |
| Hartman & Hilling(8) | 58 | Bulk supplies of 14 cities | 0·160 | 0·190 | 0·140 |
| Rumments(9) | | Cows, mid-lactation | 0·213 | — | — |
| | | Same cows towards end of lactation | 0·134 | 0·180 | 0·038 |
| Arup(10) | 25 | — | — | 0·206 | 0·150 |
| | 11 | Defatted milk solids, reconstituted | — | 0·184 | 0·151 |
| Heinemann(11) | 27 | Defatted milk solids, dry wt. basis | 1·995 | 2·09 | 1·88 |
| Babad & Shtrikman(12) | 8 | — | 0·193 | 0·245 | 0·180 |
| | 2 | Milk powder, dry wt. basis | 2·52 | — | — |
| | | | 1·91 | — | — |

The present investigation was a part of a study of the composition of milk in Manitoba. The purpose was to obtain information on the level of citric acid in milk in Manitoba and its variability. The study was carried out on bulk milk over the period January to December 1955, and on the mixed milk of the University Holstein herd and on the milk of individual cows from this herd over the period April 1956 to March 1957.

* A study supported by the Canada Department of Agriculture.

## EXPERIMENTAL

The samples of bulk milk were obtained from the three largest dairies in the City of Winnipeg. The samples of mixed milk of the University herd were taken at fortnightly intervals. In addition, samples were taken on 5 days each week during 8 weeks in May and June and during 10 weeks in September to November at the time the feeding of the cows in the herd was changed from stall feeding to pasture and vice versa. Samples were also taken at weekly intervals throughout the lactations of three individual cows in the University herd. In a further experiment samples were taken once a week from the milk of each of five cows, for 12 weeks at the beginning and 12 weeks at the end of lactation.

For the determination of the citric acid content of milk a modification of the colorimetric method of Saffran & Denstedt was used. A description and discussion of the method is reported elsewhere (13).

Table 2. *Citric acid content of bulk milk and of milk of the University Holstein herd, %*

| | Dairies 1955 | | | Univ. of Man. herd milk |
|---|---|---|---|---|
| | 1 | 2 | 3 | 1956–57 |
| Jan. I | 0·185 | 0·181 | 0·176 | 0·182 |
| II | 0·174 | 0·174 | 0·175 | 0·176 |
| Feb. I | 0·175 | 0·178 | 0·172 | 0·185 |
| II | 0·169 | 0·172 | 0·174 | 0·186 |
| Mar. I | 0·173 | 0·176 | 0·173 | 0·175 |
| II | 0·171 | 0·162 | 0·162 | 0·175 |
| Apr. I | 0·169 | 0·176 | 0·174 | 0·165 |
| II | 0·177 | 0·180 | 0·178 | 0·153 |
| May I | 0·175 | 0·182 | 0·186 | 0·164 |
| II | 0·182 | 0·181 | 0·174 | 0·157 |
| June I | 0·171 | 0·164 | 0·159 | 0·151 |
| II | 0·179 | 0·176 | 0·172 | 0·158 |
| July I | 0·165 | 0·166 | 0·161 | 0·159 |
| II | 0·164 | 0·166 | 0·164 | 0·184 |
| Aug. I | 0·162 | 0·165 | 0·160 | 0·142 |
| II | 0·165 | 0·164 | 0·162 | 0·155 |
| Sept. I | 0·167 | 0·171 | 0·175 | 0·160 |
| II | 0·175 | 0·176 | 0·178 | 0·184 |
| Oct. I | 0·175 | 0·176 | 0·175 | 0·159 |
| II | 0·168 | 0·173 | 0·173 | 0·169 |
| Nov. I | 0·180 | 0·174 | 0·173 | 0·153 |
| II | 0·170 | 0·168 | 0·171 | 0·181 |
| Dec. I | 0·162 | 0·176 | 0·177 | 0·173 |
| II | 0·162 | 0·169 | 0·172 | 0·176 |
| Average | 0·171 | 0·173 | 0·172 | 0·168 |
| Range | 0·159–0·186 | | | 0·142–0·186 |
| S.D. | | 0·006 | | 0·013 |
| C.V. | | 3·49 | | 7·74 |

## RESULTS AND DISCUSSION

The numbers of samples analysed were, bulk milk, 72; mixed herd milk, 114; individual cow's milk, 254.

The average citric acid content of the bulk milk (Table 2) was 0·172 % with a minimum of 0·159 % and a maximum of 0·186 %. The average citric acid content of the herd milk was 0·168 % with a minimum of 0·142 % and a maximum of 0·186 %. The analysis

Table 3. *Analysis of variance*

| Source | D.F. | M.S. |
|---|---|---|
| Dairies and Univ. herd | 3 | 0·000121 |
| Housing and grazing seasons | 1 | 0·001023* |
| Error | 91 | 0·000062 |
| Total | 95 | — |

* $P < 0·01$.

Table 4. *Citric acid percentage in the milk of individual cows during a lactation period*

| Cow ... | Cl | W | T |
|---|---|---|---|
| No. of samples ... | 53 | 53 | 50 |
| Colostrum* | 0·093 | 0·110 | 0·118 |
| Average (colostrum incl.) | 0·171 | 0·179 | 0·189 |
| Range in normal milk | 0·136–0·219 | 0·135–0·233 | 0·159–0·231 |
| Standard deviation | 0·023 | 0·024 | 0·019 |
| Coefficient of variation | 13·5 | 13·4 | 10·1 |

Differences between average values

| | | | |
|---|---|---|---|
| Cl–W | 0·008 ± 0·005 | (104 D.F.) | $P > 0·05$ |
| Cl–T | 0·018 ± 0·004 | (101 D.F.) | $P < 0·01$ |
| W–T | 0·010 ± 0·004 | (101 D.F.) | $0·05 > P > 0·01$ |

* Sample taken from second and third milking after calving.

Table 5. *Coefficients of variation of various constituents of milk in Manitoba*

| | Bulk milk | Herd milk | Milk of indiv. cows |
|---|---|---|---|
| Fat | 3·97 | 5·33 | 16·02* |
| Protein | 4·05 | 3·71 | 14·95* |
| Lactose | 1·57 | 3·63 | 5·61* |
| Ash | 1·51 | 2·60 | 6·65* |
| Calcium (CaO) | 2·00 | 5·75 | — |
| Total phosphorus ($P_2O_5$) | 3·40 | 4·55 | — |
| Magnesium (MgO) | 8·33 | 10·00 | — |
| Chlorine (Cl) | 1·85 | 9·65 | — |
| Citric acid | 3·49 | 7·74 | 10·6–13·5 |

* Reported by Overman *et al.* (14).

of variance (Table 3) indicates that the differences between the citric acid contents of the bulk milk from the different dairies and that of the milk of the University herd are not significant ($P > 0·05$). The average citric acid content of the milk of 3 individual cows (Table 4) for whole lactations were: 0·171, 0·179 and 0·189%. The difference between the citric acid contents of the milks of cows Cl and W is not significant ($P > 0·05$), but the difference for the milks of cows W and T is significant ($P < 0·05$), and the difference for the milks of cows Cl and T is highly significant ($P < 0·01$).

The average citric acid content of all these milks was quite uniform. However, the variations in their citric acid contents were different. The coefficient of variation of herd milk (7·74) was over twice the coefficient of variation in bulk milk (3·49). The coefficients of variation of the milk of individual cows (13·5, 13·4 and 10·6) were approximately three times the coefficient of variation of the bulk milk.

Table 5 gives the coefficients of variation of various constituents of milk as found in this laboratory. It may be seen that in bulk milk the variations in citric acid content are similar to the variations in phosphorus content but smaller than the variations in fat, protein and magnesium contents and larger than the variations in the contents of the other constituents. In mixed herd milk the variations in the citric acid content were

smaller than the variations in magnesium and chlorine contents, but larger than those in the contents of other constituents. For the milk of individual cows, results on the citric acid content are lacking. However, compared with the results reported by Overman, Sanman & Wright(14) for the major constituents of the Holstein milk, the variations in the citric acid content were smaller than the variations in fat and protein contents but larger than those in lactose and ash contents.

The citric acid content of milk varies from month to month (Table 2). In the present study the variations also showed a seasonal character. The citric acid content of milk in the grazing season was slightly lower and somewhat more variable than in the housing season. The differences were small, averaging 0·005% in bulk and 0·012% in herd milk,

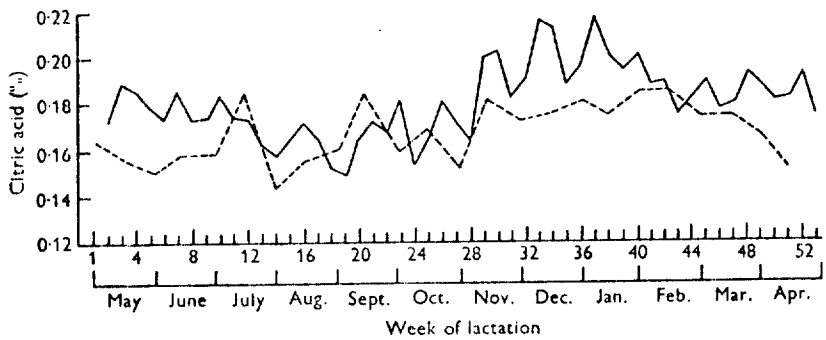


**Fig. 1. Average citric acid contents of mixed herd milk (----) and mixed milk from 3 cows (———) during 1 year.**

but significant ($P < 0·01$) (Table 3). This is in disagreement with some earlier reports(4) which stated that summer feeding increases the citric acid content of milk. To study more closely the effect of change of feeding of cows from stall feeding to pasture and reverse, samples of herd milk were examined five times weekly in spring and autumn before and after changes in feeding of the cows. Altogether 40 samples were examined in the spring and 50 in the autumn. In all these 90 spring and autumn milk samples the citric acid content ranged from 0·142 to 0·193% and averaged 0·167%. The standard deviation was 0·012 and the coefficient of variation 7·38. These values are almost identical to those given for the herd milk in Table 2. Table 6 gives the citric acid content of the milk of 14 samples before and 14 samples after the change of feeding of the cows in spring and autumn. It can be seen that in spring, after the cows went to pasture, the citric acid content of milk decreased and in autumn, after they returned to stall feeding, it increased. A '$t$' test reveals that the decrease in spring was significant ($0·05 > P > 0·01$) and the increase in autumn was not ($P > 0·05$).

Fig. 1 shows the variations in the average citric acid content of weekly samples of milk from 3 individual cows throughout the lactation period together with the citric acid content of the mixed herd milk in the same period. It may be seen that the citric acid content of milk was similar at the beginning and at the end of lactation. It was lower in the summer and early autumn months and higher in winter. Table 7 shows the citric acid content of the milk of 5 individual cows during 12 weeks at the beginning and 12 weeks at the end of lactation period. It can be seen that there are some fluctuations in the citric acid content of milk in the different periods; however, these fluctuations do not show any

regularity or relationship with the stage of lactation. Further, it can be seen that the variations in the citric acid content of milk during the lactation are different in the milk of different cows. The citric acid content in the milk of the cows Ap and Sh was remarkably lower at the end of lactation than at the beginning. The reason may be that the lactation periods of these cows ended in summer when the citric acid content of the milk was

Table 6. *Citric acid content of milk of the University Holstein herd, stall and pasture feeding*

| Stall feeding | | Pasture | | Pasture | | Stall feeding | |
|---|---|---|---|---|---|---|---|
| Date | % | Date | % | Date | % | Date | % |
| 10 May | 0·167 | 5 June | 0·170 | 23 Oct. | 0·175 | 10 Nov. | 0·171 |
| 11 | 0·158 | 6 | 0·155 | 24 | 0·187 | 12 | 0·192 |
| 12 | 0·169 | 7 | 0·151 | 25 | 0·189 | 13 | 0·186 |
| 15 | 0·157 | 8 | 0·155 | 26 | 0·187 | 14 | 0·181 |
| 16 | 0·164 | 12 | 0·166 | 27 | 0·173 | 15 | 0·175 |
| 17 | 0·170 | 13 | 0·164 | 30 | 0·187 | 16 | 0·176 |
| 18 | 0·168 | 14 | 0·174 | 31 | 0·171 | 19 | 0·175 |
| 19 | 0·175 | 15 | 0·165 | 1 Nov. | 0·153 | 20 | 0·166 |
| 22 | 0·174 | 19 | 0·158 | 2 | 0·153 | 21 | 0·178 |
| 23 | 0·159 | 20 | 0·158 | 3 | 0·156 | 22 | 0·172 |
| 24 | 0·154 | 21 | 0·155 | 5 | 0·149 | 23 | 0·172 |
| 25 | 0·166 | 22 | 0·147 | 6 | 0·163 | 24 | 0·193 |
| 26 | 0·163 | 26 | 0·152 | 7 | 0·157 | 26 | 0·173 |
| 29 | 0·177 | 27 | 0·147 | 8 | 0·152 | 27 | 0·173 |
| Average | 0·166 | | 0·158 | | 0·168 | | 0·177 |
| S.D. | 0·007 | | 0·008 | | 0·015 | | 0·008 |

Difference between average values

Stall feeding – pasture
$0{\cdot}008 \pm 0{\cdot}003$ (26 D.F.) $0{\cdot}05 > P > 0{\cdot}01$

Pasture – stall feeding
$-0{\cdot}009 \pm 0{\cdot}005$ (26 D.F.) $P > 0{\cdot}05$

Table 7. *Citric acid content of milk of individual cows at the beginning and end of lactation*

| Cow ... | Cl | | W | | T | | Ap | | Sh | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Month | % | Month | % | Month | % | Month | % | Month | % |
| Beginning of lactation | May | 0·177 | June | 0·176 | June | 0·192 | Jan. | 0·195 | Jan. | 0·203 |
| | June | 0·169 | July | 0·170 | July | 0·192 | Feb. | 0·197 | Feb. | 0·186 |
| | July | 0·160 | Aug. | 0·174 | Aug. | 0·188 | Mar. | 0·201 | Mar. | 0·168 |
| End of lactation | Feb. | 0·164 | Mar. | 0·183 | Feb. | 0·204 | July | 0·168 | July | 0·157 |
| | Mar. | 0·167 | Apr. | 0·200 | Mar. | 0·200 | Aug. | 0·147 | Aug. | 0·159 |
| | Apr. | 0·195 | May | 0·169 | Apr. | 0·192 | Sept. | 0·158 | Sept. | 0·160 |

Each figure is an average of 4 samples in a 4-week period.

lowest. The variations in the citric acid content of the milk of individual cows in general are similar to the variations in the citric acid content of the mixed herd milk. The citric acid content of the colostrum was remarkably lower than in normal milk. In the colostrum of these 5 individual cows it varied from 0·093 to 0·118%.

The citric acid content of the evening and morning milk of 5 individual cows was examined in five 2-day periods (Table 8). The average content was, in 50 samples of evening milk, 0·166% and in 50 samples of morning milk 0·167%. The standard deviations were 0·021 and 0·020 respectively. Thus the citric acid content was similar in both milks. This indicates that the time of the milking had no influence on the citric acid content of milk. In the citric acid content of evening and morning milk from two successive milkings of one individual cow, however, there were remarkable differences. The highest difference in this experiment was 0·031% or 23·6% of the lowest value.

Table 8. *Citric acid content of evening and morning milk of individual cows*

| Cow ... | Cl | | T | | W | | Ap | | Sh | |
|---|---|---|---|---|---|---|---|---|---|---|
| Date of sampling | E. | M. | E. | M. | E. | M. | E. | M. | E. | M. |
| 26–27 June | 0·141 | 0·151 | 0·184 | 0·187 | 0·156 | 0·160 | 0·132 | 0·136 | 0·144 | 0·145 |
| 27–28 | 0·145 | 0·142 | 0·193 | 0·181 | 0·157 | 0·152 | 0·136 | 0·142 | 0·145 | 0·147 |
| 3–4 July | 0·158 | 0·168 | 0·205 | 0·195 | 0·176 | 0·174 | 0·172 | 0·162 | 0·164 | 0·183 |
| 4–5 | 0·157 | 0·158 | 0·178 | 0·181 | 0·178 | 0·177 | 0·144 | 0·157 | 0·169 | 0·169 |
| 18–19 July | 0·149 | 0·149 | 0·210 | 0·200 | 0·188 | 0·173 | 0·160 | 0·169 | 0·155 | 0·182 |
| 19–20 | 0·151 | 0·150 | 0·199 | 0·170 | 0·188 | 0·156 | 0·143 | 0·152 | 0·195 | 0·189 |
| 20–21 Aug. | 0·171 | 0·167 | 0·199 | 0·189 | 0·189 | 0·160 | 0·182 | 0·198 | 0·186 | 0·164 |
| 21–22 | 0·145 | 0·153 | 0·168 | 0·190 | 0·145 | 0·138 | 0·156 | 0·158 | 0·148 | 0·139 |
| 4–5 Sept. | 0·155 | 0·153 | 0·194 | 0·217 | 0·143 | 0·172 | 0·144 | 0·178 | 0·144 | 0·160 |
| 5–6 | 0·153 | 0·170 | 0·212 | 0·218 | 0·157 | 0·159 | 0·180 | 0·175 | 0·154 | 0·139 |

Average: Morning milk = 0·167%
Evening milk = 0·166%

S.D.: Morning milk = 0·020
Evening milk = 0·021

C.V.: Morning milk = 12·00
Evening milk = 12·65

Difference between average values
Morning - evening milk 0·001 ± 0·004 (98 D.F.) $P > 0·05$


## SUMMARY

The citric acid content of 440 samples of milk has been determined. The average citric acid content of bulk milk of an estimated 10,000 cows was 0·172%. The average citric acid content of the mixed milk of a Holstein herd of 35 cows was 0·168%, and the average citric acid content of the milk of individual cows was 0·171–0·189%. The coefficients of variation of the citric acid content were: in bulk milk 3·49, in mixed herd milk 7·74 and in the milk of individual cows 10·6–13·5. The citric acid content of the milk of individual cows may differ significantly. The citric acid content of milk in the grazing season was slightly lower and more variable than in the housing season. The stage of lactation had no significant effect on the citric acid content of milk. The time of day of the milking had no influence on the citric acid content of milk. Marked differences may occur in the citric acid content of milk from two successive milkings of an individual cow, and in this study differences up to 0·034% or 23·6% of the lowest value have been found.


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2099

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# CHEMICAL COMPOSITION OF POTATOES. VI. EFFECT OF VARIETY AND LOCATION ON ACID CONCENTRATIONS[1]

J. H. Schwartz, M. I. Fow, R. L. Raskin, and W. L. Porter[2]

## Introduction

In recent years, with the increasing use of chromatographic techniques, knowledge of potato composition has been greatly extended. Of special interest are constituents that play a role in problems of the potato processing industry. The involvement of amino acids and reducing sugars in non-enzymatic browning (Maillard reaction) has been known for some time. Andreotti (1) showed that, among the amino acids, serine and glutamic and aspartic acids are most active. Some non-nitrogenous acids also take part in this type of browning. Haas and Stadtman (4), Anet and Reynolds (2), and Andreotti (1) showed this for malic and citric acids, Lewis et al. (9) and Kapur et al. (8) for oxalic and other acids. Chlorogenic acid and citric acid are involved in the problem of after-cooking blackening (Hughes and Swain (6), Heisler et al. (5)).

One approach to ameliorating these problems could lie in either growing or selecting potatoes high or low in particular constituents, for example high in citric acid and low in chlorogenic acid for the purpose of controlling after-cooking blackening. To be able to do this it is important to know how constituent concentrations vary among different varieties and growing areas and to know in general the relative influence of varietal and environmental factors. This matter has been little studied with potato constituents. Stevenson et al (12) found that there are varietal differences in total solids content, but that environmental influences are often more important than varietal. Hyde (7) studied variety and location effects on ascorbic acid concentrations and lists earlier work on the subject. He found that varietal differences in concentration are relatively unaffected by location. As part of a larger study being carried out at this Laboratory on the effects of variety and place of growth on the concentrations of potato constituents, the concentration of glutamic, aspartic, pyroglutamic, malic, citric, oxalic, and phosphoric acids in potatoes from several important varieties and growing areas were investigated.

## Materials and Methods

Five commercially important varieties of fall-crop potatoes (Cobbler, Katahdin, Kennebec, Red Pontiac, and Russet Burbank) were chosen for investigation. These varieties were grown at each of six State Experiment Stations in 1961 through 1963 (see Table 2, footnote 3). Cultural conditions were similar to those typically employed in the various locations. Upon harvest in late September or early October a 100-pound sample of each variety was randomly selected. All varieties at each location

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were stored the same length of time at 40 F (one week to one month) and then shipped to this Laboratory by Railway Express. Upon arrival they were sampled, peeled, and made into a slurry with ethanol. Portions of the slurry were taken for determining solids by oven-drying and for making extracts of the potato acids. The methods of sampling and extraction were those described by Talley et al. (13) except that the potatoes were not classified according to specific gravity. The extracts from three representative samples of each batch of potatoes were stored in a cold room at 34-38 F until ready for analysis. Each contained 20% ethanol by weight to prevent alcoholic fermentation.

In order to obviate the effects of any changes in acid content during storage of the extracts, the order of analysis of the different extracts was randomized with the help of a table of random numbers. For the same reason the three extracts from each batch of potatoes were classified into an A, B, and C series, and after analysis of the A series was complete the B series was analyzed in the same order. The C samples were kept as a reserve. For the 1963 crop, only the A series was run. Because 2 days were required for the analysis of each extract, approximately 2 to 3 months elapsed between analyses of an extract in the A series and the replicate extract in the B series. Little or no change in acid concentrations occurred during this interval, as indicated by the replicate values. The acids were determined by titration after gradient elution from an ion-exchange column (Schwartz 10, Schwartz et al. 11). The same batch of ion-exchange resin was used for all 3 years. Of the acids determined, pyroglutamic is probably, at least in part, an artifact derived from glutamine. The term "total acids," as used below, refers to the sum of the concentrations of the known acids; an unidentified acidic material which was eluted last from the ion-exchange column is not included.

## Results and Discussion

The data were subjected to a statistical analysis of variance by two methods. For both, missing plot values (Idaho Katahdins and Pontiacs in 1961, Red River Valley Katahdins in 1963) were estimated in order to make possible a complete factorial treatment of the data, and the degrees of freedom in the error terms were adjusted for the missing data. In the first method, upon which the results shown in Tables 1 and 2 are based, a separate analysis was made for each year. Since in this case the purpose was to compare important varieties and locations rather than to regard the varieties and locations selected as a random sample, residual mean squares were used in the F-ratio denominators when determining significance. The results of this analysis of variance are given in Table 1. In all but a few cases variety and location mean squares were found to be significant at the 5% level for the years 1961 and 1962. In 1963, significance was found in only half the cases possibly because replicates were not run that year.

The means computed for each variety by averaging the acid concentrations of potatoes grown in all six locations, and for each location by averaging the results for all five varieties grown therein, are subsequently referred to simply as "averages" or "average values." Duncan's Multiple Range Test (Duncan (3) was applied to these values in order to determine whether the differences between the individual averages were

TABLE 1.—*Analysis of variance (mean squares x $10^3$).*[1]

| Source | df | Glutamic acid | Aspartic acid | Pyro-glutamic acid | Malic acid | Citric acid | Phos-phoric acid | Oxalic acid |
|---|---|---|---|---|---|---|---|---|
| 1961 | | | | | | | | |
| Varieties | 4 | 0.49* | 0.13* | 0.86[2] | 1.02* | 2.86* | 0.76* | 0.014 |
| Locations | 5 | 1.94* | 0.22* | 1.71* | 5.30* | 38.68* | 0.16* | 0.047* |
| Var. x Loc. | 18[3] | 0.35 | 0.26 | 0.37 | 0.60 | 3.01 | 0.13 | 0.031 |
| Replicates | 1 | 0.69 | 1.25 | 3.44 | 0.01 | 5.08 | 0.00 | 0.061 |
| Residual | 25[3] | 0.13 | 0.04 | 0.33 | 0.06 | 0.28 | 0.01 | 0.015 |
| 1962 | | | | | | | | |
| Varieties | 4 | 0.85* | 0.19* | 0.50[2] | 2.19* | 9.80* | 0.12* | 0.020*[4] |
| Locations | 5 | 1.03* | 1.03* | 1.25* | 6.22* | 43.29* | 0.58* | 0.080* |
| Var. x Loc. | 20 | 0.11 | 0.08 | 0.40 | 0.35 | 5.09 | 0.07 | 0.028 |
| Replicates | 1 | 0.03 | 0.08 | 2.55 | 0.31 | 0.20 | 0.34 | 0.001 |
| Residual | 29 | 0.01 | 0.01 | 0.23 | 0.10 | 0.57 | 0.02 | 0.007 |
| 1963 | | | | | | | | |
| Varieties | 4 | 0.44* | 0.16 | 1.63 | 0.42 | 1.15 | 0.17* | 0.051* |
| Locations | 5 | 0.26* | 0.09 | 1.28 | 2.57* | 11.59*[4] | 0.12* | 0.025 |
| Residual | 19[3] | 0.04 | 0.21 | 0.98 | 0.33 | 3.96 | 0.04 | 0.013 |

*Denotes varieties and locations significant at the 5% level (F-test); results of F-tests based on dry-weight data were similar except where noted.
[1]Based on fresh-weight data.
[2]Significant at the 5% level when based on dry-weight data.
[3]Degrees of freedom corrected for missing data.
[4]Not significant at the 5% level when based on dry-weight data.

significant. The averages and the results of the Multiple Range Test are given in Table 2. Because of the large volume of data, Tables 1 and 2 list only the results of calculations made on a fresh-weight basis; results on a dry basis were similar. Both fresh- and dry-basis tables of individual data, and the results of dry-basis Multiple Range Tests, will be furnished upon request.

Table 2 shows that in any given year varieties can be found which, when averaged over the six locations, are significantly higher or lower in particular acids than all or most of the other varieties. A similar statement can be made about locations. In addition, several varieties and locations are high or low in particular acids for 2 or 3 years, although the differences may not be statistically significant in all 3 years. The more pronounced examples of this among the varieties are Cobbler, high in glutamic and low in phosphoric and total acids (for total acids see Figs. 1 and 2); Russet Burbank, high in malic; Kennebec, high in citric; Katahdin, low in aspartic acid. Examples of this among the locations are Maine, high in malic and low in glutamic and phosphoric acids; Idaho, high in oxalic; Long Island, low in malic, citric, and total acids.

In order to facilitate comparison of the three crop-years, average data calculated on a fresh-weight basis are presented as yearly profiles (Figs. 1 and 2). In these figures the "curves" do not represent functions of a variable, but connect variety or location means of the same crop in order that the three crops may be compared as to the sequence

TABLE 2.—*Mean acid concentrations in varieties and locations; per cent on fresh weight.*[1]

| Acid | Varieties[2] 1961 | Varieties[2] 1962 | Varieties[2] 1963 | Locations[3] 1961 | Locations[3] 1962 | Locations[3] 1963 |
|---|---|---|---|---|---|---|
| Glutamic | COB .055 a | COB .077 a | COB .077 a | RRV .073 a | PEN .079 a | PEN .073 a |
| | RUS .052 ab | PON .060 b | RUS .064 b | IDA .054 b | RRV .065 b | LI .071 a |
| | KEN .051 ab | KEN .059 b | KAT .062 bc | PEN .045 bc | LI .065 b | RRV .062 b |
| | PON .043 bc | RUS .058 b | PON .060 bc | WIS .042 c | IDA .059 c | WIS .062 b |
| | KAT .040 c | KAT .057 b | KEN .054 c | MAI .039 c | WIS .058 c | IDA .059 b |
| | | | | LI .034 c | MAI .048 d | MAI .055 b |
| Aspartic | PON .068 a | RUS .047 a | RUS .048 a | PEN .070 a | PEN .060 a | MAI .047 a |
| | KEN .064 ab | COB .047 a | COB .043 a | LI .069 a | RRV .045 b | PEN .043 a |
| | COB .064 ab | KEN .043 b | KEN .042 a | IDA .063 b | WIS .043 b | RRV .042 a |
| | RUS .062 b | KAT .039 c | PON .036 a | MAI .061 b | IDA .043 b | IDA .037 a |
| | KAT .059 b | PON .038 c | KAT .036 a | RRV .060 b | LI .036 c | LI .037 a |
| | | | | WIS .059 b | MAI .030 d | WIS .036 a |
| Pyroglutamic | KEN .088 a | RUS .069 a | KEN .124 a | LI .101 a | IDA .073 a | WIS .127 a |
| | KAT .081 ab | KAT .066 ab | KAT .122 a | PEN .087 ab | RRV .068 ab | LI .121 a |
| | PON .080 ab | KEN .060 ab | RUS .111 a | RRV .075 bc | PEN .064 ab | IDA .112 a |
| | RUS .077 ab | PON .057 ab | PON .102 a | IDA .070 bc | LI .064 ab | RRV .095 a |
| | COB .065 b | COB .053 b | COB .082 a | MAI .070 bc | MAI .056 b | MAI .093 a |
| | | | | WIS .068 c | WIS .041 c | PEN .093 a |
| Malic | PON .115 a | RUS .099 a | KAT .084 a | RRV .139 a | MAI .113 a | MAI .114 a |
| | RUS .112 a | KAT .087 b | RUS .081 a | MAI .122 b | IDA .097 b | IDA .079 b |
| | COB .103 b | PON .079 c | KEN .073 a | WIS .107 c | RRV .095 b | RRV .071 bc |
| | KAT .096 c | KEN .076 c | PON .067 a | IDA .094 d | WIS .071 c | WIS .065 bc |
| | KEN .094 c | COB .062 d | COB .063 a | LI .084 e | PEN .063 c | PEN .054 bc |
| | | | | PEN .080 e | LI .046 d | LI .052 c |

2099

| | | | | | | |
|---|---|---|---|---|---|---|
| Citric | KEN .427 a | KEN .426 a | RUS .391 a | WIS .514 a | IDA .471 a | RRV .416 a |
| | RUS .414 ab | PON .413 a | KEN .377 a | IDA .430 b | PEN .467 a | IDA .398 a |
| | KAT .410 b | COB .391 b | KAT .371 a | MAI .404 c | RRV .384 b | WIS .376 a |
| | COB .401 b | RUS .371 c | COB .363 a | RRV .395 c | WIS .382 b | PEN .374 a |
| | PON .386 c | KAT .357 c | PON .350 a | PEN .375 d | MAI .339 c | MAI .368 a |
| | | | | LI .328 e | LI .308 d | LI .277 b |
| Phosphoric | KAT .061 a | KEN .050 a | KAT .057 a | IDA .059 a | IDA .056 a | LI .058 a |
| | KEN .059 ab | KAT .048 a | KEN .049 b | PEN .057 ab | RRV .053 a | IDA .048 b |
| | PON .058 b | PON .048 a | PON .048 b | WIS .055 bc | LI .048 b | WIS .047 b |
| | COB .046 c | RUS .044 b | RUS .045 b | LI .052 cd | PEN .043 c | RRV .046 b |
| | RUS .044 c | COB .042 b | COB .043 b | MAI .050 de | WIS .039 cd | MAI .045 b |
| | | | | RRV .048 e | MAI .037 d | PEN .045 b |
| Oxalic | KEN .02[illegible] | COB .023 a | KEN .020 a | IDA .024 a | PEN .026 a | IDA .019 a |
| | RUS .02[illegible] | RUS .023 a | RUS .017 ab | WIS .022 ab | IDA .026 a | PEN .017 ab |
| | COB .020 a | KEN .023 a | KAT .016 ab | MAI .021 ab | LI .022 b | WIS .017 ab |
| | PON .020 a | KAT .023 a | PON .014 b | LI .019 b | RRV .021 bc | RRV .015 ab |
| | KAT .020 a | PON .020 b | COB .013 b | PEN .019 b | WIS .020 bc | MAI .014 ab |
| | | | | RRV .018 b | MAI .019 c | LI .013 b |

[1]Means followed by same letter not significantly different from each other, 5% level.
[2]COB - Cobbler, KAT - Katahdin, KEN - Kennebec, PON - Red Pontiac, RUS - Russet Burbank.
[3]IDA - Idaho (Aberdeen), LI - Long Island (Riverhead, New York), MAI - Maine (Presque Isle), PEN - Pennsylvania (State College), RRV - Red River Valley (East Grand Forks, Minnesota), WIS - Wisconsin (Sturgeon Bay).

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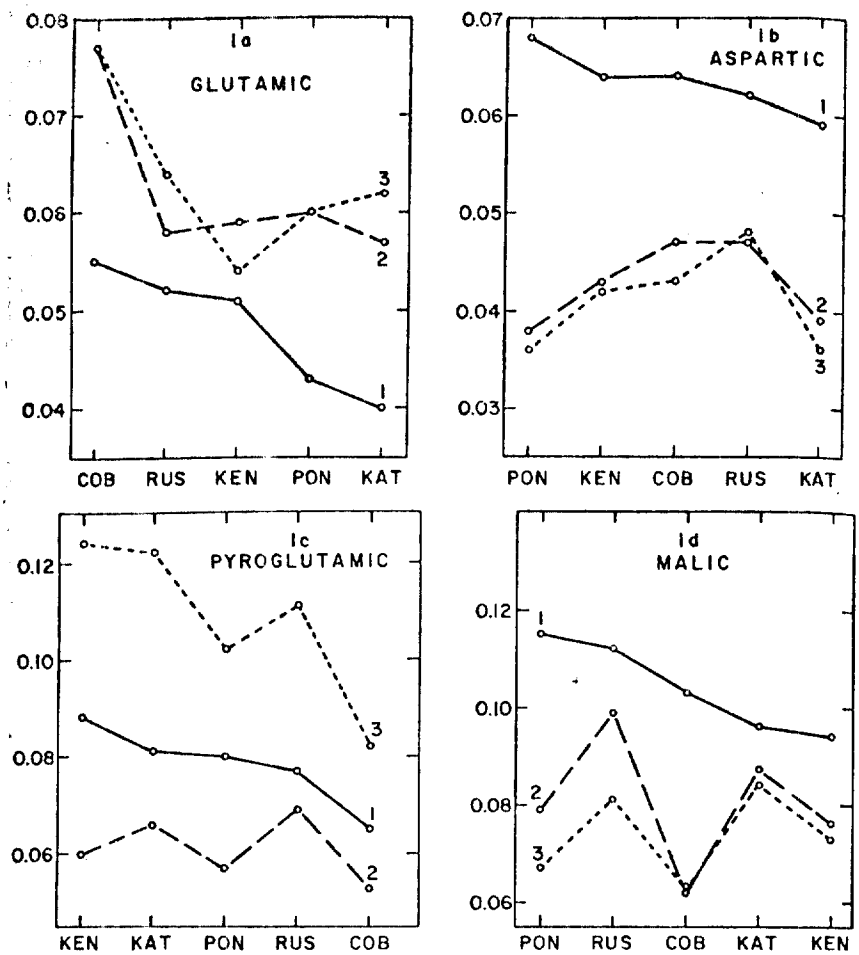


FIG. 1.—Profiles of acid concentrations (%, fresh basis) in three crops of five varieties. 1 = 1961, 2 = 1962, 3 = 1963. For abbreviations see Table 2, footnote 2.

of varieties or locations when arranged in order of acid content. With some acids it was necessary to choose different scales for the variety and location profiles because of differences in range and the exigencies of illustration. With this is mind, no difficulties should arise in making the above-mentioned comparison. The profiles for "total acids" included in the figures were obtained by adding the concentrations of the known acids. Values for 1961 are shown in order of decreasing magnitude; for 1962 and 1963, varieties and locations are in the same order as for 1961 rather than in order of magnitude. This results in rather jagged profiles for most of the acids, but notable for 1962 and 1963 is the remarkable

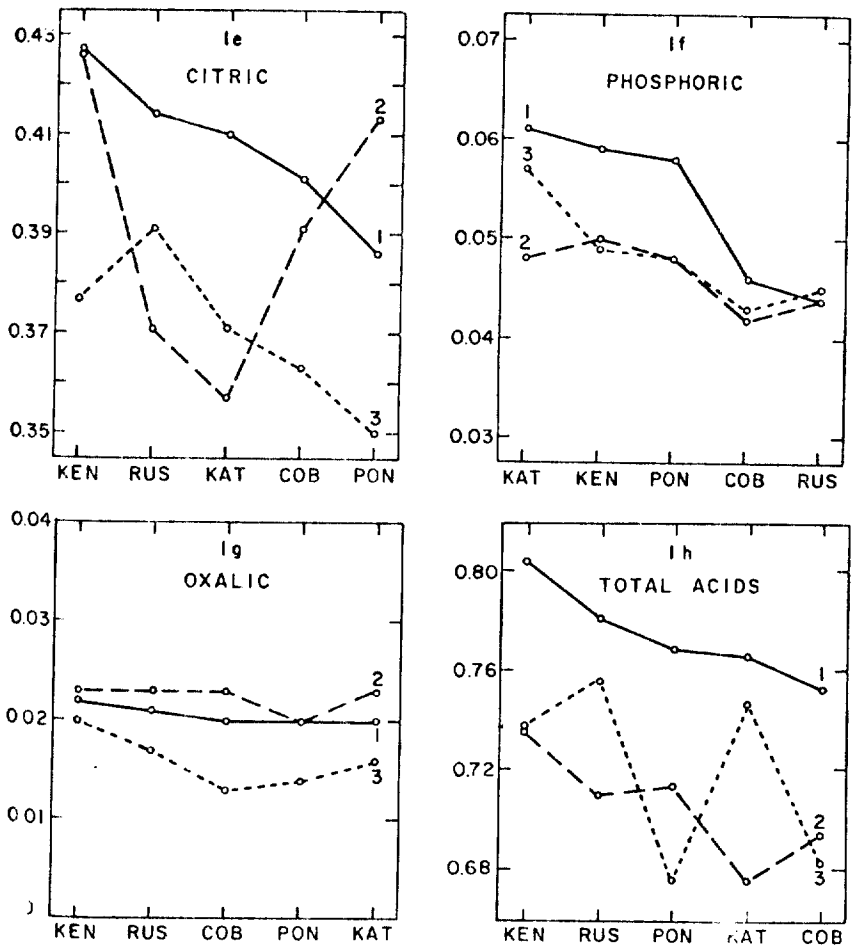


FIG. 1.—*Continued.*

similarity in profile for almost all acids, indicating a high degree of consistency in relative acid concentrations. This also indicates greater reliability for results for 1963, which were not as often significant by the Multiple Range Test (Table 2) as results for other years, because samples for 1963 were not run in replicate.

Not only does the 1961 crop differ in profile shape, but it is also higher in total acids and in most of the individual acids than the other crops. It is higher in aspartic, malic, phosphoric, and the unidentified acid. It is also high in citric, with some exceptions among the locations. The 1961 crop lies between the other two in pyroglutamic and oxalic acid and is low only in glutamic acid. Acid concentrations in 1962 and 1963 are about equal in most cases.

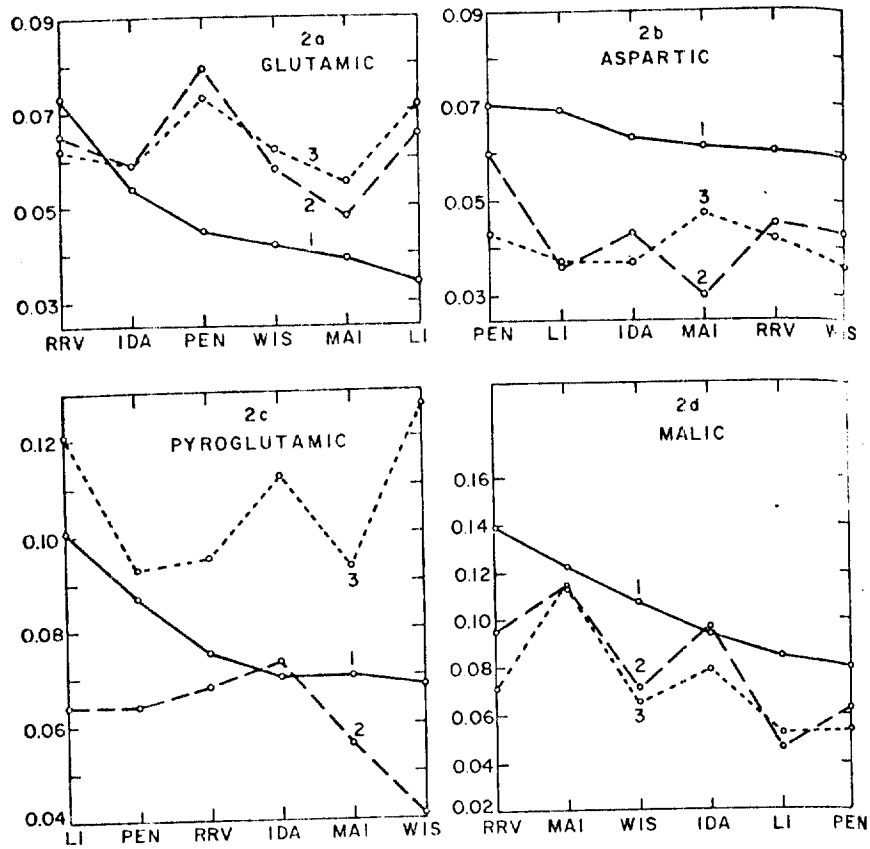


FIG. 2.—Profiles of acid concentrations (%, fresh basis) in three crops of potatoes from six locations. 1 = 1961, 2 = 1962, 3 = 1963. For abbreviations see Table 2, footnote 3.

The above-mentioned unidentified acid ranged from approximately 1.2 milliequivalents per 100 g in Russet Burbanks to approximately 1.6 milliequivalents per 100 g (fresh basis) in Katahdins. Its profiles for varieties resembled each other for all 3 years, but in the case of the location averages only the profiles for 1962 and 1963 were similar. As with most of the other acids, there was more of the unidentified acid present in 1961 than in the other 2 years.

An indication of the reason for the differences between the 1961 crop and the other two crops is shown in Table 3, which lists the deviations from the normal total rainfall and normal average temperature, for the months May through August, in the localities where the potatoes were grown. In all areas, except Sturgeon Bay, Wisconsin, rainfall in 1961 was either the highest or the lowest of the three years. Deviations from normal temperature were more pronounced in the western localities and in 1961 were in a direction tending to aggravate the effects of the drought

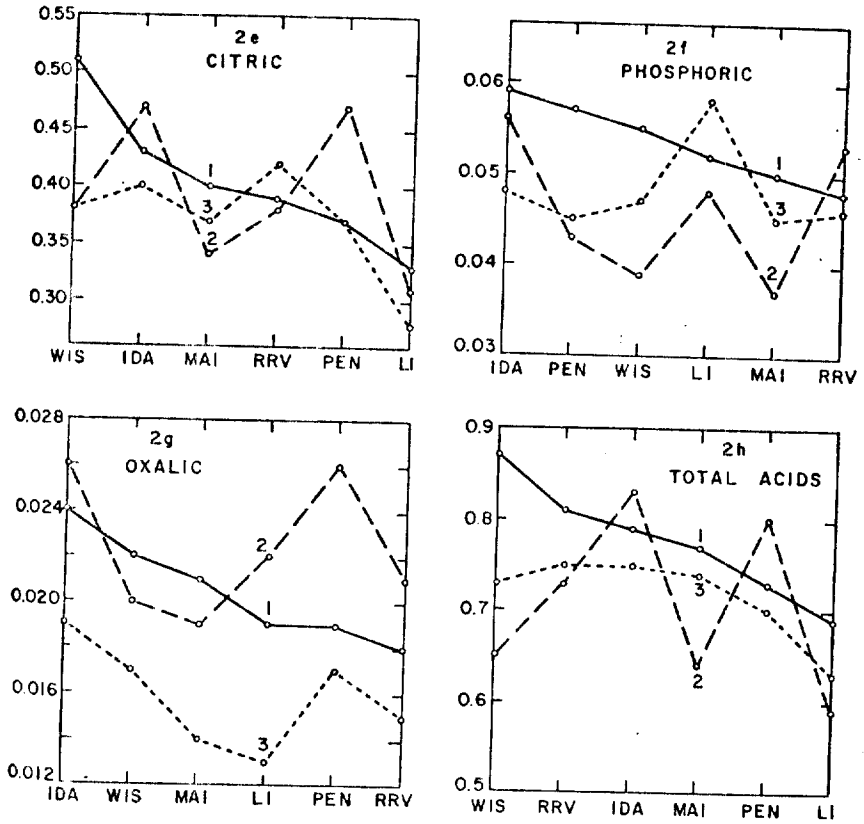


FIG. 2.—*Continued.*

then occurring. These facts help explain the differences in profiles but not necessarily the tendency of 1961 potatoes to be higher in acid concentrations, since the deviations from normal were in two directions, eastern localities having more rain than normal and western localities less. Weather data are presented in this paper for possible future reference and are not intended to indicate that any numerical relation has been presently found between the data and acid concentrations.

It appears so far that variety, location, and differences in weather conditions in different years all have an influence on the concentration of acids in potato tubers. With the object of gaining some idea about the relative importance of these factors, the data were given a furher statistical treatment which assumed that varieties, locations, and years were variables randomly selected from a larger population. Conclusions derived from this treatment, however, would be expected to be most applicable to commercial fall-crop varieties and growing areas. Since the 1963 samples were not run in replicate, the averages of each set of replicates in 1961 and 1962 were used as raw data for these years. Per cent of the total variance contributed by each of the variables was estimated.

TABLE 3.—*Deviations from normal rainfall and temperature.*[1]

| Location | Rain Normal[2] | Rain 1961 | Rain 1962 | Rain 1963 | Temperature Normal[2] | Temperature 1961 | Temperature 1962 | Temperature 1963 |
|---|---|---|---|---|---|---|---|---|
| Maine | 13.8 | +4.9 | +2.3 | +1.6 | 60.5 | —0.7 | —1.5 | +0.2 |
| Long Island | 14.2 | —0.1 | —1.1 | —1.5 | 68.6 | —1.2 | —2.4 | —1.1 |
| Pennsylvania | 15.3 | +0.7 | —9.2 | —2.3 | 67.6 | —1.6 | +0.2 | —1.5 |
| Wisconsin | 11.9 | +1.3 | +6.6 | —3.5 | 62.7 | —0.3 | —0.3 | —0.1 |
| Red River Valley | 11.4 | —6.1 | +1.7 | +1.0 | 62.3 | +2.8 | +0.9 | +2.5 |
| Idaho | 2.4 | +0.6[4] | +2.2 | +2.5 | 63.5 | +2.0 | —3.2 | —2.4 |

[1]Calculated from data obtained from Weather Bureau publications.
[2]Normal total rainfall (inches) for May through August.
[3]Normal average temperature (°F) for May through August.
[4]Over 70% of this fell in May. Rain scanty during the summer.

TABLE 4.—*Per cent of total variance contributed by variables and interactions.*[1]

| Source | df | Glutamic acid | Aspartic acid | Pyroglutamic acid | Malic acid | Citric acid | Phosphoric acid | Oxalic acid |
|---|---|---|---|---|---|---|---|---|
| Varieties | 4 | 13.7 | 0.2 | 5.8 | 3.2 | 0.0 | 21.6 | 3.9 |
| Locations | 5 | 7.8 | 1.0 | 0.1 | 35.9 | 25.9 | 2.5 | 7.7 |
| Years | 2 | 19.9 | 51.3 | 42.9 | 21.9 | 1.2 | 8.3 | 34.9 |
| Var. x Loc. | 20 | 0.0 | 4.9 | 0.0 | 0.0 | 4.1 | 8.6 | 0.0 |
| Var. x Years | 8 | 2.0 | 0.0 | 0.0 | 2.0 | 0.0 | 4.1 | 0.0 |
| Loc. x Years | 10 | 25.2 | 6.7 | 7.6 | 8.1 | 26.1 | 20.6 | 1.7 |
| Residual | 37[2] | 31.4 | 35.9 | 43.6 | 28.8 | 42.8 | 34.4 | 51.8 |

[1]Based on fresh-weight data.
[2]Degrees of freedom corrected for missing data.

Results calculated on a fresh-weight basis are shown in Table 4; dry-basis results were similar. The table indicates that for some of the acids (glutamic, pyroglumatic, and phosphoric) variety was the more important factor; for other acids (malic, citric, and oxalic) location was more important. Also, in accordance with what one might expect from the yearly profiles, years were a relatively important factor with all acids except citric and phosphoric. These conclusions on the relative importance of different factors in determining acid concentration must be considered tentative because of the limited nature of the data. However, they should be of interest for comparison with other work which may be done in the field.

## SUMMARY

A study was made of the variation of glutamic, aspartic, pyroglutamic, malic, citric, phosphoric, and oxalic acid concentrations in Cobbler, Katahdin, Kennebec, Red Pontiac, and Russet Burbank potatoes. Each variety was grown in six different locations (Idaho, Maine, Pennsylvania, Wisconsin, Long Island, and the Red River Valley) during the years 1961 through 1963.

The varieties and locations studied were found to have a statistically significant effect in determining concentrations of the various acids. The few exceptions to this were almost entirely in 1963, probably because replicates were not run that year. The more pronounced trends in acid concentration noted for all 3 years included, among the varieties, Cobbler, high in glutamic, low in phosphoric; Russet Burbank, high in malic; Kennebec, high in citric; Katahdin, low in aspartic. Among the locations were Maine, high in malic, low in glutamic and phosphoric; Idaho, high in oxalic; Long Island, low in malic and citric.

The years 1962 and 1963 strongly resembled each other in relative concentration of the various acids but differed from 1961, a year in which abnormal weather conditions prevailed.

In a separate statistical treatment in which the importance of variety, location, and year in determining acid concentrations was estimated, indications were obtained that for some acids (glutamic, pyroglutamic, and phosphoric) variety was the most important factor; for other acids (malic, citric, and oxalic) location was more important. Also, years were a relatively important factor with all acids except citric and phosphoric.

## Acknowledgments

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They also wish to thank Mr. J. W. Garvin, Mr. G. H. Eppley, and Mr. R. O. Pierce for carrying out the many tedious extractions, Dr. T. J. Fitzpatrick for determining solids contents, and the National Weather Records Center of the U. S. Department of Commerce for supplying appropriate publications of weather data.

## Resumen

Se hizo un estudio de la variación en concentración de los ácidos glutámico, piroglutámico, malico, cítrico, fosfórico, y oxálico en las variedades de papa Cobbler, Katahdin, Kennebec, Red Pontiac, y Russet Burbank. Cada variedad se cultivó en seis localidades diferentes (Idaho, Maine, Pennsylvania, Wisconsin, Long Island, y la Red River Valley) durante los ános 1961 - 1963.

Las variedades y localidades tuvieron un efecto estatisticamente significativo en la determinacion de la concentración de los ácidos. Las pocas excepciones a esto fueron casi todos durante el año 1963, posiblemente porque no hubo replicaciones en ese año. Los más pronuciados tendencias en concentraciones de ácido anotado para los tres años incluidos, entre los variedades, Cobbler, alto en ácido glutamico, bajo en fosfórico; Russet Burbank, alto en málico; Kennebec, alto en cítricó, Katahdin, bajo en aspártico. Entre los localidades, Maine estuvo alto en málico, bajo en glutámico y fosfórico; Idaho alto en oxálico; Long Island bajo en málico y en cítrico.

Los años 1962 y 1963 fueron muy similares en concentración relativa

de los ácidos, pero diferente de 1961, un año en lo cual prevalecieron ambientales anormales.

En un tratamiento estadistico en lo cual fue estimado la importancia de variedad, localidad, y año en determinación de concentración de ácido, se encontró indicaciones que para alqunos ácidos (glutámico, piroglutámico, y fosfórico) variedad era lo más importante: para otros ácidos (málico, cítricó, y oxálico) localidad era mas importante. Además, año fue un factor relativamente importante con todo los ácidos con excepción del cítrico y fosfórico.

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# THE METABOLISM OF ORALLY ADMINISTERED CITRIC ACID*

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The increase in total organic acids of the urine following the ingestion of large amounts of citrus fruit juices has been generally interpreted as a measure of the amount of citric acid escaping oxidation in the body (Blatherwick and Long, 1922; Chaney and Blunt, 1925; Saywell and Lane, 1933). This is an unsatisfactory criterion for several reasons. By the Van Slyke and Palmer (1920) titration method for organic acids only a part of the citric acid present may be determined.[1] Furthermore, it has been demonstrated that the production of a more alkaline urine by the ingestion of fruit juices or of alkalies may, in itself, induce an augmented excretion of citric acid (Fasold, 1930; Östberg, 1931; Schuck, 1934) and of other organic acids (Booher and Killian, 1924; Haldane, 1924; Fasold, 1930). Finally, even when the addition of citric acid is the only dietary change, the "total organic acids" is not an accurate measure of the citric acid content of the urine.[2] Thus a

* The data in this paper were taken from a dissertation presented by Caroline C. Sherman in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Yale University, 1935. A part of the expense of this investigation was borne by the William Gilman Thompson Fund and the Russell H. Chittenden Fund for Research in Physiological Chemistry.

† Louise Hart Van Loon Fellow of Vassar College, 1934–35.

[1] The calcium hydroxide added as a preliminary step in the Van Slyke and Palmer procedure may cause considerable precipitation of citrate. The "total organic acids" titration value of a 0.1 per cent solution of citric acid was 40 per cent less after the calcium treatment than before, and direct determination of citric acid in the filtrate indicated a comparable loss.

[2] The following data for the pH, total organic acids, and citric acid content of the 24 hour urine collection of Dog 2 on control days and following





satisfactory estimate of the ability of the organism to destroy ingested citric acid requires not only a direct determination of the citrate reappearing unchanged, but also the exclusion of factors disturbing the acid-base equilibrium.

Gonce and Templeton (1930) observed no increase in the citric acid output of four normal children when 0.6 gm. of citric acid per kilo of body weight was given daily for 3 days. Of five normal individuals ingesting numerous daily doses of 10 to 40 gm. of citric acid, Östberg (1931) found that only one exhibited any considerable increase in urinary citric acid. The administration of the material to the other subjects caused slight variations in the citric acid excretion, in general paralleling changes in the urinary pH. Kuyper and Mattill (1933) reported that when 2 to 20 gm. of citric acid were administered either to fasting individuals or in addition to a constant diet, 1.5 to 2.5 per cent escaped oxidation. Three young women who ingested 12 gm. of citric acid daily for 8 days excreted no more citric acid than in a control period; whereas three others excreted "extra" citric acid corresponding to 1.6, 3.2, and 3.6 per cent of the acid ingested (Schuck, 1934). The hog has been reported to utilize completely more than 2.0 gm. of citric acid per kilo administered orally (Fürth *et al.*, 1934; Woods, 1927).

An increase in the serum concentration of citric acid of 0.7 to 1.5 mg. per cent was noted following the administration of 5 to 15 gm. of citric acid to normal human subjects, and to individuals with disturbed citrate metabolism (Thunberg, 1933; Lennér,

the administration of large doses of citric acid illustrate the lack of parallelism between the Van Slyke and Palmer titration figure and the citric acid content of the sample. The increase in the total organic acids following the ingestion of large amounts of citric acid is also to be noted.

| Date | Citric acid ingested | pH | Total organic acids | Citric acid | |
|---|---|---|---|---|---|
| | *gm. per day* | | *cc. 0.1 N acid* | *cc. 0.1 N acid* | *mg.* |
| Oct. 14 | 35.2 | 5.7 | 536 | 31 | 198 |
| " 16 | 0 | 5.7 | 232 | 1.0 | 6 |
| " 18 | 17.6 | 5.7 | 420 | 1.1 | 7 |
| " 20 | 13.2 | 5.6 | 320 | 6.2 | 40 |
| " 21 | 0 | 5.7 | 256 | 0.6 | 4 |
| " 22 | 26.4 | 5.9 | 414 | 41 | 262 |
| " 24 | 0 | 5.8 | 272 | 1.3 | 8 |

2136

1934); similar blood studies on rabbits have been complicated by anesthesia, but have shown large rises in the blood level (to as high as 20 to 40 mg. per cent) (Salant and Wise, 1916–17; Kuyper and Mattill, 1933). There have been no reports of tolerance studies in which simultaneous determinations of the citric acid concentration in blood and urine of the same individual were made, which might give some insight into the question of the renal threshold for citrate.

The present study concerns the effect of the ingestion of large doses of citric acid by dogs on the amounts of the acid present in the urine, blood, and feces, and on the urinary pH, organic acid, and nitrogen excretion. In some instances, blood and urine determinations were made at frequent intervals in an attempt to establish the relationship between the concentration of citrate in the blood and its rate of excretion by the kidney.

## EXPERIMENTAL

The animals used in this study were adult dogs of both sexes, weighing 8 to 22 kilos, which received a constant daily allowance of one of the basal diets described in the preceding paper (Sherman *et al.*, 1936). Solutions of citric acid were administered by stomach tube. With the exception of Dog 4, urine collections were made by catheterization. Blood was drawn from the jugular vein, oxalated, and the blood filtrates immediately prepared for analysis. Carmine suspended in the citric acid solution was used to mark the feces corresponding to the experimental period. Citric acid was determined by the method previously described (Pucher *et al.*, 1936), and the pH by an electrometric measurement with the antimony-antimony trioxide electrode (Roberts and Fenwick, 1928).

Following the ingestion of 8 to 35 gm. of citric acid (0.5 to 2.0 gm. per kilo of body weight), there occurred in some cases no increased excretion of citrate by the kidney, and in other cases elimination of as much as 450 mg. of citric acid in excess of the basal value (Table I). In twenty experiments of this sort, the excess citric acid in the urine corresponded, at most, to 5 per cent, and, on the average, to 0.7 per cent of the ingested material. The variations in the citric acid tolerance of the different animals were quite marked: thus, of four animals ingesting 0.5 gm. of citric



acid per kilo of body weight, one excreted none, a second 0.5 per cent, a third 1.7 per cent, and the fourth, 5.0 per cent of the material as "extra" citric acid in the urine. At least 95 per cent of this citrate which escaped oxidation was excreted within about 7 hours, and the remainder usually within less than 24 hours.

The administration of citric acid to fasting dogs was followed promptly (within less than half an hour) by a decided increase in

TABLE I

*Effect of Ingested Citric Acid on Urinary Citric Acid*

| Dog No. | Citric acid ingested | | Citric acid in urine, excess over basal value | |
|---|---|---|---|---|
| | *gm. per kg. body weight* | *gm.* | *mg.* | *per cent of acid administered* |
| 1 | 0.5 | 9.0 | 450 | 5.0 |
| 2 | 0.5 | 8.8 | 6.0 | 0.07 |
| | 1.0 | 17.6 | 5.5 | 0.03 |
| | 2.0 | 35.2 | 192 | 0.5 |
| | 1.0 | 17.6 | 0 | 0 |
| | 1.0 | 17.6 | 0 | 0 |
| | 1.5 | 26.4 | 0 | 0 |
| | 0.75 | 13.2 | 40 | 0.3 |
| | 1.5 | 26.4 | 256 | 1.0 |
| | 0.5 | 8.8 | 0 | 0 |
| | 1.0 | 17.6 | 170 | 1.0 |
| 3 | 1.0 | 8.0 | 0 | 0 |
| 4 | 1.0 | 16.0 | 40 | 0.3 |
| | 1.0 | 16.0 | 110 | 0.7 |
| | 1.0 | 16.0 | 30 | 0.2 |
| | 1.0 | 16.0 | 135 | 0.8 |
| | 1.0 | 16.0 | 90 | 0.6 |
| | 0.5 | 8.0 | 40 | 0.5 |
| | 1.0 | 16.0 | 80 | 0.5 |
| 5 | 0.5 | 11.0 | 190 | 1.7 |

the citrate concentration of the blood, which reached a maximum of 2 to 4 times the basal level within $\frac{1}{2}$ to $3\frac{1}{2}$ hours and returned practically to normal within $3\frac{1}{2}$ to $7\frac{1}{2}$ hours (Fig. 1). The failure of the citrate concentration of the general circulation to reach more than 9 mg. per cent when as much as 17.6 gm. was ingested and the comparatively short time required for the blood level and urinary excretion to return to normal suggest a highly efficient mechanism for the disposal of orally administered citric acid in the dog.

2136

Simultaneous determinations of citrate in blood and urine in hourly periods following oral administration of citric acid indicated the existence of a renal threshold for citrate in the dog. The

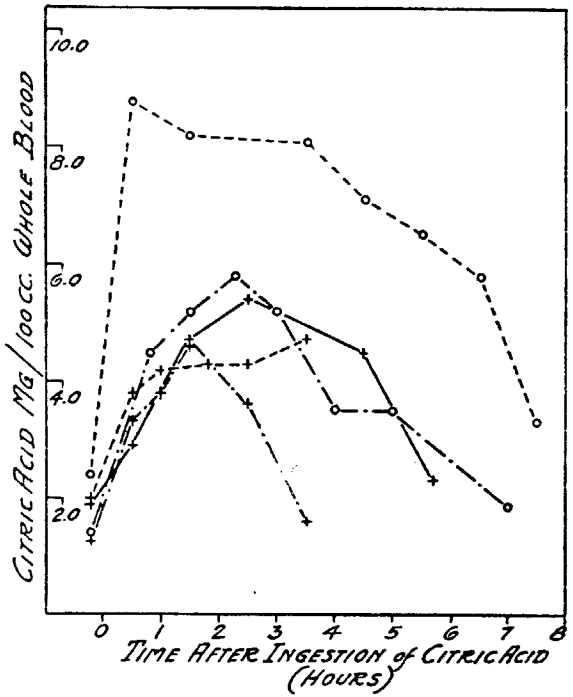


FIG. 1. The effect of ingestion of citric acid upon the concentration of citric acid in the blood. The data for Dog 1 are shown by the solid line; for Dog 2 by the dash line; for Dog 4 by the dash and dot line. The cross denotes 0.5 gm. of citric acid; the circle, 1.0 gm. of citric acid per kilo of body weight.

differences between two dogs in this respect are illustrated by Fig. 2. In the case of Dog 1, the urinary excretion of citrate was greatly increased when the concentration of citrate in the blood reached about 2.3 mg. per cent; with Dog 2, on the other hand, no increased excretion occurred when the blood level rose as high as 4.7 mg. per cent, and the kidney threshold was only exceeded when, with larger doses of the acid, blood citrate concentrations as great as 5.8 to 6.5 mg. per cent were attained. Such differences in the



renal threshold for citrate offer a probable explanation for the considerable individual variations in citric acid tolerance shown in Table I.

No increased amounts of citric acid were found in the feces when large doses of the substance had been administered. It is, of course, possible that a part of the ingested citric acid failed to be

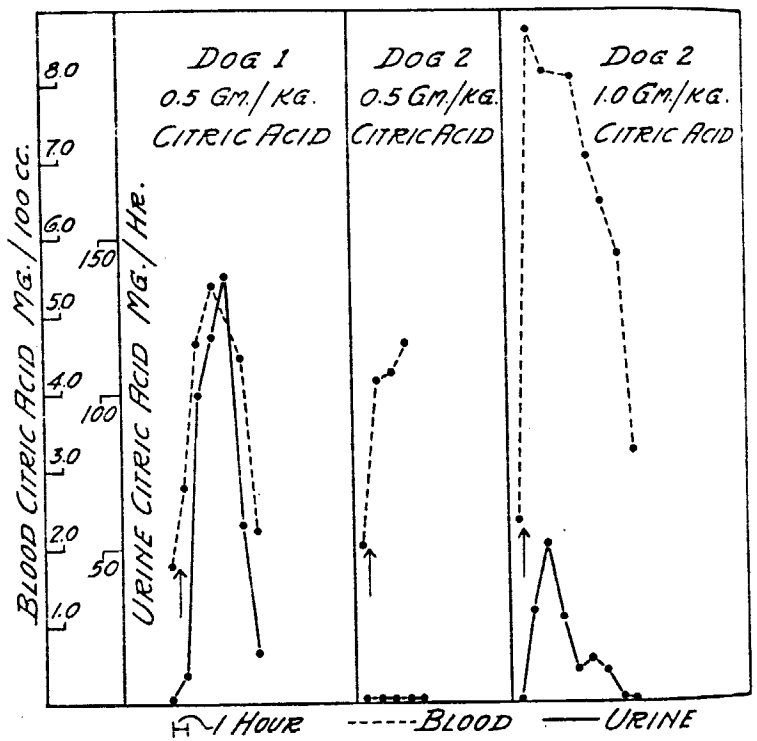


FIG. 2. The relation of the blood level to the urinary excretion of citric acid following the ingestion of citric acid.

absorbed and was destroyed by microorganisms in the gastro-intestinal tract.

The ingestion of large quantities of citric acid was without effect on the pH of the 24 hour collection of urine, although a temporary drop was occasionally noted in the pH of short time collections containing much "extra" citric acid.

2136

No decrease in the urinary nitrogen, such as that reported by Fürth *et al.* (1934) for the hog, was observed when 0.5 or 1.0 gm. of citric acid per kilo of body weight was administered to dogs in nitrogen equilibrium or in negative nitrogen balance.

## SUMMARY

The dog has the ability to destroy nearly completely large amounts of ingested citric acid. Following the oral administration of 0.5 to 2.0 gm. of citric acid per kilo of body weight, an average of 0.7 per cent of the acid given escaped oxidation and appeared in the urine; a rise in the blood citrate level was maintained for $3\frac{1}{2}$ to $7\frac{1}{2}$ hours; and no extra citric acid appeared in the feces.

Apparent renal threshold values varying from about 2.2 to about 6.0 mg. of citric acid per 100 cc. of whole blood have been observed.

Ingestion of citric acid in addition to a constant diet did not affect the pH or the total nitrogen of the 24 hour urine collection.

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2247

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# ORGANIC ACID, AMINO ACID, AND ASCORBIC ACID CONTENT OF POTATOES AS AFFECTED BY STORAGE CONDITIONS

J. P. SWEENEY, P. A. HEPNER, AND S. Y. LIBECK[1]


## ABSTRACT

A study of the changes in organic acids and amino acids occurring during storage of tubers at 70 and 55 F was made on several varieties grown in 1965 and 1966. Citric acid tended to increase and malic acid usually decreased. Pungo potatoes showed decreases in aspartic and glutamic acids and increases in several other amino acids. Increases or decreases in both organic and amino acids were usually greater in potatoes stored at 70 F than those stored at 55 F. Temperature of storage appeared to have a greater effect on organic and amino acids than would be indicated by results of previous workers. Considerable amounts of pyroglutamic acid were found in some potato varieties. There were significant decreases in ascorbic acid in potatoes stored at both 70 F and 55 F.

## RESUMEN

Se hizo un estudio de los cambios en ácidos orgánicos y amino ácidos que tienen lugar en tubérculos almacenados a 70 y 55 F de algunas variedades cultivadas en 1965 y 1966. Acido cítrico tendía a aumentar y el ácido málico generalmente disminuía. Papas Pungo dieron indicaciones de disminución en ácidos aspártico y glutámico y de incrementos en varios otros amino ácidos. Tanto los incrementos como las disminuciones en ácidos orgánicos y amino ácidos fueron generalmente mayores en papas almacenadas a 70 F que en las almacenadas a 55 F. La temperatura de almacenamiento pareció tener un efecto myor sobre los ácidos orgánicos y amino ácidos del indicado por los resultados obtenidos por los investigadores hasta la fécha. Cantidades considerables de ácido piroglutánico fueron encontradas en algunas variedades de papa. Hubo una disminución significante en ácido ascórbico en papas almacenadas tanto a 70 F como a 55 F.


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The preservation of the palatability and nutritive value of potatoes during storage depends to a considerable extent on storage conditions. Studies of changes in composition of potatoes may provide an insight into storage problems. Previous investigations of compositional changes during storage have dealt chiefly with carbohydrates and total solids (Schwimmer et al 15), or with nitrogenous matter (Cotrufo and Levitt 5).

An indication of metabolic changes occurring in potatoes is provided by a study of organic acid and amino acid content. Organic acids are known to play a central role in metabolism (Burris 4) and may also be involved in nonenzymic browning reactions and after-cooking discoloration (Heisler et al 8). Amino acids are important because of their nutritive value and because they may be involved in nonenzymic browning (Talley et al 16).

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Except for ascorbic acid, the organic acids in potatoes have not been extensively studied. Schwartz et al (14), working with Maine Katahdin potatoes stored eight months at 38 F, followed by one month at 45 F and an additonal month at 50 F, have related several organic acids to specific gravity and storage time and presented evidence for the conversion of malic acid to citric acid during storage.

Heisler (8) found the stem end of several potato varieties to be lower in organic acids than the bud end and reported an association of organic acid content to after-cooking discoloration. Minina (10) investigated the concentrations of both citric and malic acids during 7 months storage. He concluded that there was an initial decrease in citric acid content followed by an increase, and a decrease in malic acid toward the end of the storage period.

Very little information is available on changes in free amino acids during storage. Talley et al (16) working with the same potato extract used by Schwartz et al. (14) have reported an increase in proline to be the only significant change in potatoes stored at 38 F and classified according to specific gravity.

Potatoes are generally stored in the home at approximately 70 F. Investigations have revealed that potatoes stored at 50 to 60 F have better color, texture and flavor when cooked or made into chips than do those stored at lower temperatures (Heinze et al. 7). But commercially potatoes are usually stored at temperatures near 40 F in order to prevent sprouting over a period of several months. Much of the previous research on organic and amino acid content has been carried out on potatoes stored at 38 F. Storage changes at higher temperatures might be expected to differ from those obtained at 38 F.

The work described here was carried out to obtain more information regarding changes in organic acids and amino acids during storage at 70 and 55 F. Possible relationships between increases in organic acid content and increases or decreases in amino acid content were also considered.

## Materials and Methods

Potato varieties studied in 1965 were Pungo from Virginia, Rosemont Cobbler from Minnesota and Russet Burbank from Washington.

In 1966 Pungos were obtained from Virginia, and Irish Cobblers from Minnesota. The Pungo potatoes were transported to the laboratory on the day of harvest. Other varieties investigated were received in the laboratory from 7 to 10 days after harvest.

Determinations were made on the potatoes when received, after 1 month at 70 F, and after periods of 2 and 5 months at 55 F. Changes during storage were investigated by determination of titratable acids, citric, malic and pyroglutamic acids and ascorbic acid. Amino acids were determined only on the Pungos grown in 1966.

*Preparation of sample extracts:* Sample extracts were prepared by blending 50 g samples, taken from 15 to 20 pared potatoes, with 200 ml cold 80% ethyl alcohol. An electric blender was used. These extracts were filtered and stored at 0 F until used for determination of organic and titratable acids.



*Non volatile organic acids*: A 50 ml aliquot for analysis was evaporated under reduced pressure to remove alcohol. The sample was passed through Dowex 50 W-X8 ($H^+$ form, 20 to 50 mesh) and diluted to 200 ml. A 50 ml aliquot was chromatographed on Dowex 1-X8 (formate form, 200-400 mesh), and eluted by gradient elution with formic acid. Acids determined were citric, malic and pyroglutamic. The procedure used was a modification of that of Palmer (11) and Hulme and Woolterton (9).

*Titratable acids:* The alcohol was removed from a 50 ml aliquot of the sample extract, the sample rediluted with water and titrated to pH 8, as determined with a pH meter.

*Ascorbic acid:* The indophenol titration procedure of Bessey and King (3) was used for ascorbic acid determination.

*Amino acids and nitrogen:* Amino acids and nitrogen were determined by a commercial laboratory. Extracts for amino acids were prepared by blending 100 g potatoes with 190 ml 100% ethyl alcohol. The mixture was filtered and the pulp reextracted four times with 70% alcohol. The extract and washings were combined and diluted to 1000 ml with 70% alcohol. The chromatographic procedure of Moore et al (12) was used for amino acids. Nitrogen was determined as described in Association Official Agricultural Chemists (1).

*Total solids:* A procedure similar to that described in Association Official Agricultural Chemists (2) was used for total solids determination. Samples were heated to constant weight in a vacuum oven at 70 F.

*Specific gravity:* Specific gravity was determined by loss of weight in water.

## Results and Discussion

### *Non volatile organic acids*

The potatoes stored 1 month at 70 F tended to show an increase in citric acid. Malic acid generally decreased (Table 1). In potatoes stored at 55 F citric acid content also tended to increase while that of malic acid usually decreased. However, changes in acid content at 55 F were not as large as at 70 F. Citric was the predominant acid at all times.

These results differ somewhat from those obtained by Schwartz et al. (14), who worked with potatoes stored at lower temperatures than those described above. They reported that potatoes stored at 38 F, followed by storage at 45 and 55 F had an initial decrease in citric acid, followed by an increase. Malic acid followed a reverse pattern, first increasing and then decreasing. Raising the storage temperature, they reported, made no significant difference in the pattern of organic acid change.

Schwartz et al. (14) have speculated on a possible interconversion of malic and citric acids. Our results on potatoes stored at 70 F show generally decreases in malic acid and increases in citric acid. However, the citric acid changes at both 55 and 70 F were too large to be accounted for entirely by malic acid changes. Also for the 1966 Pungos there were significant increases in both malic and citric acids during the same storage period. This would seem to indicate that factors other than citric-malic interconversion were also involved.

2302

TABLE 1.—*Acid content of potatoes as affected by storage time and temperature.*[1]

| Year and section | Storage time, months | Storage Temp. °F | Titratable acids % | | | | Malic acid mg/100 g | | | | Citric acid mg/100 g | | | | Ascorbic acid mg/100 g | | | | Pyroglutamic acid mg/100 g | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Pungo | Rosemont Cobbler | Russet Burbank | Pungo | Rosemont Cobbler | Russet Burbank | Pungo | Rosemont Cobbler | Russet Burbank | Pungo | Rosemont Cobbler | Russet Burbank | Pungo | Rosemont Cobbler | Russet Burbank | Pungo[2] | Rosemont Cobbler | Russet Burbank |
| 1965 | | | | | | | | | | | | | | | | | | | | | | |
| Stem | 0 | ... | 0.104b | 0.066ab | 0.081a | 91b | 40a | 75a | 174b | 116a | 224a | 22.2a | 21.2a | 18.3a | ... | 4 | 4 | ... | 4 | 4 |
| | 1 | 70 | 0.122a | 0.078a | 0.071a | 110ab | 34a | 57ab | 258a | 150a | 224a | 16.5b | 15.5b | 14.8b | ... | 6 | 8 | ... | ... | ... |
| | 2 | 55 | 0.120a | 0.069ab | 0.080a | 120a | 31ab | 62a | 204b | 96a | 193a | 13.0b | 13.4b | 13.7b | 12 | ... | 4 | ... | 4 | 4 |
| | 5 | 55 | 0.085c | 0.072ab | 0.075a | 75b | 31ab | 54b | 247a | 104a | 197a | 8.5c | 9.2c | 10.8c | ... | 3 | 10 | ... | 3 | 10 |
| Bud | 0 | ... | 0.128b | 0.085ab | 0.103a | 114ab | 60a | 74a | 237ab | 169a | 324a | 25.6a | 25.5a | 22.9a | ... | 4 | 5 | ... | 4 | 5 |
| | 1 | 70 | 0.154ab | 0.099a | 0.103a | 117ab | 42ab | 67a | 341a | 220a | 370a | 17.9b | 17.6b | 18.0b | ... | 4 | 12 | ... | 4 | 12 |
| | 2 | 55 | 0.159a | 0.101a | 0.111a | 139a | 57a | 75a | 253ab | 183a | 340a | 14.2b | 15.4b | 16.2b | 18 | 2 | 6 | 18 | 2 | 6 |
| | 5 | 55 | 0.114b | 0.094ab | 0.100a | 112b | 51a | 59ab | 329a | 190a | 329a | 10.0c | 12.0c | 11.5c | ... | 9 | 16 | ... | 9 | 16 |
| 1966 | | | Pungo | Irish Cobbler | | Pungo | Irish Cobbler | | Pungo | Irish Cobbler | | Pungo | Irish Cobbler | | Pungo | Irish Cobbler | | Pungo | Irish Cobbler | | | |
| Stem | 0 | ... | 0.120a | 0.088a | | 50ab | 82a | | 274c | 340ab | | 34.1a | 24.8a | | ... | 34 | | | | | | |
| | 1 | 70 | 0.112ab | 00.89a | | 34b | 68a | | 355b | 375ab | | 24.8b | 18.4b | | ... | 53 | | | | | | |
| | 2 | 55 | 0.092ab | 0.092a | | 52ab | 68a | | 313c | 380a | | 21.0b | 13.1c | | 55 | 53 | | | | | | |
| | 5 | 55 | 0.122a | 0.084a | | 97a | 71a | | 487a | 381a | | 12.6c | 10.8d | | 5 | 54 | | | | | | |
| Bud | 0 | ... | 0.150a | 0.098a | | 69bc | 100a | | 407b | 412ab | | 47.6a | 29.4a | | 6 | 38 | | | | | | |
| | 1 | 70 | 0.139ab | 0.122a | | 50c | 106a | | 438b | 460a | | 31.2b | 22.6b | | 9 | 61 | | | | | | |
| | 2 | 55 | 0.126ab | 0.103a | | 91b | 100a | | 433b | 454a | | 24.3b | 16.4c | | 59 | 60 | | | | | | |
| | 5 | 55 | 0.152a | 0.100a | | 138a | 98a | | 639a | 483a | | 14.1c | 13.2d | | 6 | 64 | | | | | | |

[1]a differs from b at 1% level, a differs from ab at 5% level.
[2]... indicates no detectable amount.



Pyroglutamic acid content did not appear to follow any regular pattern. In the Pungo potatoes it was present in considerable amounts after 2 months storage and declined thereafter. In the other varieties the amount present generally increased with storage time. Schwartz et al. (14) have reported consistent decreases in this acid with storage time of potatoes at 38, 45 and 50 F. Several workers have postulated that this acid may be formed from glutamine.

*Titratable Acids*

Titratable acids tended to increase in potatoes stored 1 month at 70 or 2 months at 55 F. During the storage interval between 2 and 5 months at 55 F there was usually a decrease. Since titratable acids were determined by direct titration, only free acids would be measured. In contrast, extracts for non volatile acids were passed through Dowex 50, and titration values would include salts of the acids as well as the free acids.

*Free amino acids:*

Free amino acids content, specific gravity and total solids of Pungo potatoes harvested in 1966 are shown in Table 2. Because of the interval between sampling times there is a possibility that fluctuations in amino acids occurred that were not detected by determinations carried out after 0, 1, 2 and 5 months storage.

During the first 2 months storage at 55 F there were increases in all free amino acids investigated except glutamic. During 1 month's storage at 70 F all except aspartic and glutamic acids showed increases. During the interval between 2 and 5 months storage at 55 F all amino acids except leucine decreased in amount. In general the pattern of change during 1 month at 70 F or 2 months at 55 is similar to that reported by Gee et al. (6) for foam-mat dried tomato powders during storage.

The considerable changes in amino acid content reported here are in contrast to those reported by Talley et al. (16). Investigating potatoes stored at 38, 45 and 50 F they found that proline content appeared to increase during storage, but changes in other amino acids were quite indefinite.

Increases or decreases in amino acids were of interest because of their possible relationship to increases or decreases in organic acid content of the potatoes.

Results on the 1966 Pungo potatoes show that both organic and amino acid increases or decreases were generally greater at 70 than at 55 F. The results obtained would seem to show that temperature of storage has a greater effect on both organic acid and amino acid content than would appear from the work of Schwartz et al. (14) on organic acids, and that of Talley et al. (16) on amino acids.

*Ascorbic acid:*

There were significant decreases in ascorbic acid with each storage period (Table 1). Decreases in ascorbic acid content of potatoes with storage have been reported by several investigators.

23 0 2

TABLE 2.—*Free amino acids, nitrogen, specific gravity and total solids in Pungo potatoes as affected by storage time and temperatures (1966).*[1]

| | As Received | Two Months 55 F | Five Months 55 F | One month 70 F |
|---|---|---|---|---|
| Alanine | 1.01 | 6.71 | 3.94 | 5.53 |
| Arginine | 5.00 | 8.29 | 5.88 | 7.99 |
| Aspartic acid | 11.38 | 31.97 | 8.96 | 5.59 |
| Cystine[2] | ..... | ..... | ..... | ..... |
| Glutamic acid | 14.71 | 9.80 | 4.33 | 6.29 |
| Glycine | 1.36 | 2.62 | 1.62 | 3.35 |
| Histidine | 2.96 | 4.80 | 3.08 | 4.37 |
| Isoleucine | 2.90 | 8.01 | 4.00 | 6.13 |
| Leucine | 0.87 | 2.57 | 2.68 | 2.22 |
| Lysine | 3.92 | 4.01 | 3.50 | 4.92 |
| Methionine | 2.10 | 3.54 | 2.39 | 2.43 |
| Phenylalanine | 2.11 | 7.40 | 3.69 | 8.12 |
| Proline | 5.38 | 13.82 | 12.68 | 8.95 |
| Serine[3] / Threonine | 14.54 | 25.13 | 20.29 | 14.73 |
| Tryptophan[4] | ..... | | | |
| Tyrosine | 2.22 | 5.27 | 4.55 | 5.38 |
| Valine | 5.96 | 15.27 | 13.04 | 13.99 |
| Nitrogen, % | 0.15 | 0.24 | 0.19 | 0.20 |
| Specific gravity | 1.088 | 1.089 | 1.095 | 1.089 |
| Total solids, % | 24.0 | 23.6 | 25.4 | 24.9 |

[1]Amino acids expressed as micromoles per gram potato (dry weight).
[2]None found.
[3]Serine and Threonine not separated. Calculated as serine.
[4]Not determined.

*Specific gravity and total solids:*

Specific gravity and total solids content of the potatoes did not appear to vary greatly over the storage intervals investigated. Results shown for the 1966 Pungo potatoes (Table 2) were typical.

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2302

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# Some Observations on the Tolerance of Large Amounts of Citric Acid

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In regard to the consumption of lemons and citric acid, opinions are popularly expressed, such as "citric acid keeps one trim" or "citric acid dilutes the blood". Such and other similar concepts are devoid of any scientific basis. In view of its composition (Table 1), lemon juice is used both in normal and dietetic nutrition due to its refreshing properties and pleasant taste, and also in view of its content of vitamins and minerals. However, it never occupies a dominant position in the diet, so that "a course of treatment with lemon juice" is no longer encountered today. In the fruit juice cures recommended as a means of keeping trim, which often contain lemon juice as a component, it is not the fruit juice which causes a weight loss, but rather the absence of high-calorie nutrient products.

In regard to pure lemon juice cures, C.v. Noorden and H. Salomon (i) have reported at one time that such cures were used at the end of the past century with patients suffering from gout and uric acid diathesis, or in chronic rheumatic diseases, whereby the juice of up to 20 lemons was prescribed; however, the negative result of such a treatment was soon established. Except for an initial laxative effect, which stopped already after a few days, no other shortcomings of such a cure were noted, according to the information supplied by these authors; it is mentioned further that a daily consumption of the juice from 5-7 lemons was quite common in lemon-growing countries, although not in an undiluted state.

According to tests performed by C. C. Sherman et al. (3) on the feeding of relatively large amounts of citric acid to dogs, in a dosage of 0.5-2.0 g per kg body weight, citric acid undergoes an almost complete metabolic breakdown, and only 0.7% of the acid resists oxidation and appears in an unmodified form in the urine; additional citric acid was not found in the feces. The expected increase of the blood citrate level lasted for about 3.5 to 7.5 hours. S. L. Bonting (4)

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Table 1. Composition of Lemon Juice (according to J. F. Kefford (2))

| | | |
|---|---|---|
| pH Value | 2,1-2,3 | |
| Acids | | |
| Total Acid Content (as citric acid) | 4-9 | g/100 ml |
| including Citric Acid | 91,8% | |
| Malic Acid | 4,9% | |
| Quinic Acid + Phosphoric Acid | 0,5% | |
| Unidentified Acids | 2,8% | |
| Vitamins | | |
| m-Inositol | 85 | mg/100 g |
| Ascorbic Acid | 37 | mg/100 g |
| Nicotinamide | 0,029-0,196 | mg/100 g |
| Thiamine | 0,004-0,125 | mg/100 g |
| Riboflavine | 0,005-0,073 | mg/100 g |
| Folic Acid | 0,001 | mg/100 g |
| Minerals | | |
| Potassium | 99 -128 | mg/100 g |
| Sodium | 1 - 5 | mg/100 g |
| Calcium | 6 - 28 | mg/100 g |
| Magnesium | 9 - 11 | mg/100 g |
| Phosphorus | 5 - 17 | mg/100 g |
| Sulfur | 2 - 8 | mg/100 g |
| Chlorine | 2 - 4 | mg/100 g |
| Iron | 0,14-0,69 | mg/100 g |

fed rats, over 2-3 generations, amounts of phosphoric and citric acid corresponding to a continuous excessive consumption of refreshing beverages acidified with such acids; the animals were checked in regard to growth, reproduction, nature (condition) of the blood picture, tissues, bones and teeth, and also in regard to the composition of blood, urine and feces; no injurious effects of these acids could be detected.

During the wartime and post-war years, citric acid assumed therapeutic interest in combating rickets. Thus, E. Rominger (5) showed that severe rickets in nursing infants, which did not respond to treatment with vitamin D, could be cured in about 3 weeks by daily oral administration of 10-20 g sodium citrate; according to observations made by F. Glanzmann et al. (6), daily amounts of 1-1.25 g free citric acid are already sufficient for this purpose. K. Taufel and F. Krusen (7) confirmed these results in model tests on rats, and were able to show, in addition, that although a subcutaneous application of sodium citrate again causes a substantial increase of reduced bone citrate values, rickets cannot be affected in this manner. The rachitogenic effect (activity) of citric acid, which has not yet been conclusively explained from a causal standpoint and which has been accepted in the literature not without contradiction, is attributed, among other things, to the ability of citric acid to form easily soluble and well absorbable calcium complexes (8-10). According to measurements performed by W. Hennig et al. (11), the monovalent negative calcium citrate ion is preferentially formed, and, surprisingly, was found to be more stable in acid solution than in a neutral reaction.

Formulation of the Problem

The studies described here were prompted by the opinion, occasionally subject to discussion, that an abundant supply of citric acid could remove calcium from the body and thereby could cause a further deterioration of the negative calcium balance frequently observed in our nutrition. It is known that the amounts of citric acid normally taken up with the food are subject to great variations depending upon the type and composition of the meals; according to studies performed by R. Pohloudek-Fabini (12), daily amounts of 2-10 g citric acid must be considered. in order to further clarify these relationships, our goal was to determine experimentally whether an increased calcium excretion in the urine takes place in the healthy human organism as a result of an extremely high supply of citric acid, regardless of other deviations from the norm.

1. Materials and Methods

Experimental Procedure. Four healthy male subjects, ranging in age from 22 to 32 years, were available for our tests; female test subjects were not selected since, according to E. Shorr et al. (13), the citric acid excretion in the urine

undergoes changes during the course of the menstruation cycle.

In order to determine normal values (tests without citric acid loading), the 24-hour urine is collected on the 1st, 3rd and 5th day of the test period and analyzed for titration acidity, citric acid and calcium content. From the 7th to the 12th day, 50 g of citric acid monohydrate (corresponding to 45.7 g water-free acid) is administered daily. For this purpose, citric acid is dissolved in water, diluted with a bottle of fruit syrup (mostly strawberry syrup - 450 g) to 1-1.5 liter, and drunk in portions during the course of the day. In this form, the drinking of the strongly acid preparation is to a certain extent tolerable. On the 8th, 10th and 12th day the titration acidity, citric acid and calcium content are again determined in 24-hour urine (tests with citric acid loading). Nutrition during the test period is normal.

Urine processing. The urine is collected in a refrigerator in a bottle containing a few drops of toluene; on the following day, its temperature is brought to 20°C and the volume is determined.

Determination of the degree of acidity. 10 ml urine are diluted to about 100 ml and titrated in the usual manner with 0.1N caustic soda and phenolphthalein as indicator.

Determination of citric acid. 40 ml urine are mixed with 10 ml of 20% trichloracetic acid and 0.8 g active carbon, which has been previously washed with acid and dried. The mixture is allowed to stand, with occasional shaking, and is filtered after 20 min. From the filtrate (according to the citric acid content), 10 ml are diluted to 100-1000 ml, and 5 ml of this diluted filtrate are used for the photometric determination by the pentabromoacetone method (14).

Calcium determination. In 5 ml of the active carbon filtrate calcium is precipitated as oxalate by the method of D. Van Slyke and I. Sendroy Jr., and its amount is determined by titration with 0.01N potassium permanganate (15).

## 2. Results

The quite large amount of citric acid consumed was well tolerated by all 4 test subjects, without manifestation of any adverse effects on the subjects' good health; in particular, no irritation of the gastrointestinal tract or diarrhea was noted in any of the subjects.

Table 2. Urine Analyses Without and With Citric Acid Loading

1. Titration Acidity

ml 0.1N Caustic Soda per 24 Hours

| Person | Without Loading | | | With Loading | | |
|---|---|---|---|---|---|---|
| | 1 day | 3 days | 5 days | 8 days | 10 days | 12 days |
| A | 174 | 191 | 282 | 237 | 178 | 246 |
| B | 401 | 342 | 344 | 371 | 222 | 292 |
| C | 320 | 357 | 396 | 405 | 388 | 526 |
| D | 355 | - | 435 | 296 | 280 | 326 |
| Average values | | 327 | | | 314 | |

2. Citric Acid

mg/24 Hours

| Person | Without Loading | | | With Loading | | |
|---|---|---|---|---|---|---|
| | 1 day | 3 days | 5 days | 8 days | 10 days | 12 days |
| A | 373 | 310 | 232 | 435 | 419 | 372 |
| B | 945 | 1190 | 1074 | 1305 | 1400 | 1495 |
| C | 325 | 639 | 566 | 666 | 641 | 633 |
| D | 280 | - | 392 | 384 | 345 | 480 |
| Average values | | 575 | | | 720 | |

3. Calcium

mg/24 Hours

| Person | Without Loading | | | With Loading | | |
|---|---|---|---|---|---|---|
| | 1 day | 3 days | 5 days | 8 days | 10 days | 12 days |
| A | 122 | 163 | 248 | 179 | 298 | 251 |
| B | 171 | 190 | 172 | 158 | 277 | 173 |
| C | 173 | 315 | 274 | 210 | 371 | 188 |
| D | 104 | - | 153 | 130 | 140 | 149 |
| Average values | | 190 | | | 210 | |

The results obtained in parallel tests are summarized in Table 2. The time course to be expected in tests with citric acid loading could not be detected, so that the analytical data can be considered as parallel values. In order to judge the results on hand of the three characteristics investigated (titration acidity, citric acid and calcium content of urine) in tests with and without citric acid loading, variance analysis was used as a statistical evaluation method (16).

At first, the material to be investigated, in this case the urine samples from the 4 subjects without citric acid loading, was tested for homogeneity (only occasional deviations were found). The application of the F-test (Table 3) shows that the urine samples of the different subjects can be considered homogeneous only in regard to the calcium content. In the case of the titration acidity and the citric acid content, the calculated F-values are greater than the test value established in tables (16) for a 99% statistical certainty, i.e. significant differences are present between the test participants (Note: $F_{calc} > F_{1\%}$ : variance (discrepancy) is significant; statistical certainty > 99%; $F_{calc} < F_{5\%}$ : variance is accidental; statistical certainty < 95%; $F_{1\%} > F_{calc} > F_{5\%}$ : variance can be significant and should be checked again. The % data in the index stand for "error probability" (% statistical certainty + % error probability = 100).).

Table 3. Homogeneity Testing of Urine Samples (Without Citric Acid Loading)

| | Calculated F-values | Tabulated F-values | Result |
|---|---|---|---|
| Titration acidity | F = 13,75 | $F_{1\%}$ = 7,59 | Samples are nonhomogeneous |
| Citric acid | F = 30,10 | | |
| Calcium | F = 3,34 | $F_{5\%}$ = 4,07 | Samples are homogeneous |

Table 4. Testing for Significant Differences of Urine Samples due to Citric Acid Loading

| | Calculated F-values | Tabulated F-values | Result |
|---|---|---|---|
| Titration acidity | F = 2,06 | $F_{5\%}$ = 4,30 | Differences are accidental |
| Citric acid | F = 1,07 | | |
| Calcium | F = 1,19 | | |

Further, it was also checked whether the differences shown in Table 2 (with and without citric acid loading) are statistically secure. The results of calculations are listed in Table 4, which shows that in each case the differences observed are accidental and that neither the tritration acidity nor the citric acid and calcium content are significantly affected by the citric acid loading.

3. Discussion

In a restrictive sense it should be pointed out that the results obtained here, in spite of their critical statistical evaluation, should not be given a general interpretation, since for this purpose the number of test subjects and findings obtained (duration of test) is still much too small. Further, we must account for the fact that, in the determination of the titration acidity and citric acid content, a homogeneous test material is not present; however, this fact does not exert a significant influence on the differences between the values of the tests with and without citric acid loading (Table 2). The considerable differences and variations of the citric acid values of the urine tests in the test subjects are quite striking. Apparently, the values of the citric acid level lie within a higher concentration range than the one occasionally reported in the literature. The amounts of citric acid excreted in 24 hours by a healthy person are equal, according to H. D. Cramer (17), to 0.15-0.3 g, according to A. Komarkova et al. (18), to 0.3-1.0 g, according to H. Otto (19), to 0.6-1.0 g, and according to K. Hinsberg (20), to 1.0-1.5 g; our own findings, obtained only with 4 persons, show variations ranging from 0.23 to 1.2 g (Table 2).

Therefore, recourse to the level of citrate excretion in urine at a diagnostic means, for example in case of renal insufficiency (19), tetany (18), etc., must be done with caution.

As mentioned above, the literature contains many references (5-11, 18) to the connections present between citric acid and calcium transport and absorption processes. Our own studies, which should be carefully evaluated, show that calcium is not excreted in larger amounts in the urine during citric acid loading. The already discussed stability of the calcium citrate complex exhibits a maximum (peak) in the stronger acid range, where more favorable conditions are also found for calcium absorption. Clinical findings have shown, in addition, that complex bound calcium is pharmacologically most effective in the presence of excess citric acid.

In agreement with the literature, we can conclude, with appropriate reservations, that a large supply (intake) of citric acid does not exert a damaging effect on the healthy organism. Therefore, there should be no objection to the consumption of fresh lemon juice (without exceeding extremely large amounts) with its components valuable from a nutritional-physiological standpoint, such as vitamins and minerals. Whether and in what manner citric acid supplied from the outside takes part in "the citric acid cycle" and thereby in the oxidative final breakdown of nutrient products is a problem which can only be pointed out here.

Summary

1. On hand of literature data, the importance of lemon juice and citric acid in nutrition, dietetics and therapy is discussed.

2. In a loading test, 4 healthy male subjects were given a daily oral dose of 50 g citric acid. The titration acidity, citric acid and calcium content were determined in 24-hour urine, and the results are compared with those obtained in tests without citric acid loading.

3. According to a statistical evaluation of the results, none of the 3 determined components of urine are significantly affected by citric acid loading.

4. The tests show that, in agreement with published articles, an abundant supply of lemon juice or citric acid, which does not exceed extreme limits, has no detrimental effect on the healthy organism, according to our present state of knowledge.

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# Einige Bemerkungen zur Verträglichkeit größerer Mengen an Citronensäure

K. Täufel und U. Behnke*

Was den Verzehr von Citronen bzw. Citronensäure betrifft, so gehen im Volksmund Meinungen um, wie „Citronensäure macht schlank" oder „Citronensäure verdünnt das Blut". Solche und ähnliche Auffassungen entbehren jeder wissenschaftlichen Grundlage. Citronensaft wird auf Grund seiner Zusammensetzung (Tab. 1)

Tabelle 1
Zusammensetzung von Citronensaft
(nach J. F. Kefford [2])

| | | | |
|---|---|---|---|
| pH-Wert | | 2,1—2,3 | |
| Säuren | | | |
| Gesamtsäure (als Citronensäure) | | 4—9 | g/100 ml |
| davon | Citronensäure | 91,8% | |
| | Äpfelsäure | 4,9% | |
| | Chinasäure + Phosphorsäure | 0,5% | |
| | nicht identifizierte Säuren | 2,8% | |
| Vitamine | | | |
| m-Inosit | | 85 | mg/100 g |
| Ascorbinsäure | | 37 | mg/100 g |
| Nicotinsäureamid | | 0,029—0,196 | mg/100 g |
| Thiamin | | 0,004—0,125 | mg/100 g |
| Riboflavin | | 0,005—0,073 | mg/100 g |
| Folsäure | | 0,001 | mg/100 g |
| Mineralstoffe | | | |
| Kalium | | 9 — 128 | mg/100 g |
| Natrium | | 1 — 5 | mg/100 g |
| Calcium | | 6 — 28 | mg/100 g |
| Magnesium | | 9 — 11 | mg/100 g |
| Phosphor | | 5 — 17 | mg/100 g |
| Schwefel | | 2 — 8 | mg/100 g |
| Chlor | | 2 — 4 | mg/100 g |
| Eisen | | 0,14 — 0,69 | mg/100 g |

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bei der normalen wie bei der diätetischen Ernährung wegen der erfrischenden Eigenschaften, des Wohlgeschmackes sowie des Gehaltes an Vitaminen und an Mineralstoffen verwendet. Er nimmt jedoch bei Diäten niemals eine dominierende Stellung ein, so daß man heute „Citronensaftkuren" nicht mehr begegnet. Bei den als Schlankheitsmittel empfohlenen Obstsaftkuren, die häufig Citronensaft als Bestandteil enthalten, ist es nicht der Fruchtsaft, der eine Abmagerung herbeiführt, sondern das Fehlen von kalorienreichen Nährstoffen.

Über reine Citronenkuren haben C. v. NOORDEN und H. SALOMON [1] dahingehend berichtet, daß sie gegen Ende des vorigen Jahrhunderts bei Kranken mit Gicht, harnsaurer Diathese oder bei chronisch-rheumatischen Leiden angewendet worden sind; dabei ist täglich der Saft von bis zu 20 Citronen verordnet worden; bald aber stellte sich das negative Ergebnis dieser Therapie heraus. Außer einer zu Anfang meist abführenden Wirkung, die schon nach wenigen Tagen aufhörte, machten sich nach Angaben der Autoren sonstige Nachteile nicht bemerkbar; erwähnt wird ferner, daß in den Ländern des Citronenanbaues ein täglicher Genuß des Saftes von 5—7 Citronen durchaus üblich ist, allerdings nicht im unverdünnten Zustand.

Nach Versuchen von C. C. SHERMAN und Mitarb. [3] über die Verfütterung relativ großer Mengen von Citronensäure an Hunde — Dosen von 0,5—2,0 g je kg Körpergewicht — erfolgt deren fast vollständiger metabolischer Abbau; nur 0,7% widerstanden der Oxydation und erschienen unverändert im Harn; in den Faeces wurde zusätzliche Citronensäure nicht gefunden. Der zu erwartende Anstieg des Blut-Citratspiegels hielt etwa 3,5 bis 7,5 Std. an. S. L. BONTING [4] verfütterte an Ratten über 2 bis 3 Generationen derartige Mengen an Phosphor- und Citronensäure, wie sie einem dauernd übermäßigen Verzehr an damit angesäuerten Erfrischungsgetränken entsprechen; die Tiere wurden auf Wachstum, Fortpflanzung, Beschaffenheit des Blutbildes, der Gewebe, der Knochen und Zähne sowie auf die Zusammensetzung von Blut, Harn und Faeces geprüft; schädigende Einflüsse ließen sich nicht feststellen.

Während der Kriegs- und Nachkriegsjahre erlangte die Citronensäure zur Bekämpfung der Rachitis therapeutisches Interesse. So zeigte E. ROMINGER [5], daß schwere, auf Vitamin D nicht ansprechende Rachitis beim Säugling durch Verabreichung von 10—20 g Natriumcitrat per os je Tag in etwa 3 Wochen zur Heilung gebracht werden kann; nach Beobachtungen von E. GLANZMANN und Mitarb. [6] sollen bereits Mengen von täglich 1—1,25 g freie Citronensäure dazu ausreichen. K. TÄUFEL und F. KRUSEN [7] bestätigten diese Ergebnisse in Modellversuchen an Ratten; sie zeigten darüber hinaus, daß eine subcutane Applikation von Natriumcitrat zwar die abgesunkenen Knochencitratwerte wieder beträchtlich erhöht, daß die Rachitis aber auf diese Weise nicht beeinflußbar ist. Die kausal noch nicht schlüssig deutbare und in der Literatur nicht widerspruchslos hingenommene rachitogene Wirksamkeit der Citronensäure wird u. a. auf ihre Fähigkeit zur Bildung leicht löslicher und gut resorbierbarer Calciumkomplexe zurückgeführt [8—10]. Nach Messungen von

2336



W. HENNIG und Mitarb. [11] wird das einwertig negative Calciumcitrat-Ion bevorzugt gebildet; es ist auffallenderweise in saurer Lösung beständiger als bei neutraler Reaktion.

### *Fragestellung*

Anlaß für die nachstehend beschriebenen Untersuchungen war die gelegentlich einer Diskussion vertretene Meinung, daß eine reichliche Zufuhr von Citronensäure aus dem Körper Calcium ausschwemmen und dadurch die in unserer Ernährung nicht selten bestehende negative Calciumbilanz zusätzlich verschlechtern könne. Es ist bekannt, daß die normalerweise mit der Nahrung aufgenommene Citronensäure in Abhängigkeit von Art und Zusammenstellung der Mahlzeiten mengenmäßig großen Schwankungen unterliegen kann; nach Untersuchungen von R. POHLOUDEK-FABINI [12] kommen Mengen von 2—10 g je Tag in Betracht. Zur weiteren Klärung der Verhältnisse sollte experimentell festgestellt werden, ob durch eine extrem erhöhte Zufuhr von Citronensäure — abgesehen von sonstigen Abweichungen von der Norm — beim gesunden menschlichen Organismus eine vermehrte Calciumausscheidung im Harn erfolgt.

### *1. Materialien und Methoden*

*Versuchsanordnung.* Für die Versuche stellten sich 4 gesunde männliche Personen im Alter von 22 bis 32 Jahren zur Verfügung; auf weibliche Versuchsteilnehmer wurde verzichtet, da nach E. STORR und Mitarb. [13] die Citronensäureausscheidung im Harn während des Menstruationscyclus Änderungen unterworfen ist.

Zur Ermittlung der Normalwerte (Versuche ohne Belastung) wird am 1., 3. und 5. Tag der Versuchsperiode der 24 Std.-Harn gesammelt und auf Titrationsaciditat, Citronensäure- sowie Calciumgehalt analysiert. Vom 7. bis 12. Tag werden täglich 50 g Citronenensäure-Monohydrat — entsprechend 45,7 g wasserfreie Säure — verabfolgt. Hierzu wird die Citronensäure im Wasser gelöst, mit einer Flasche Fruchtsirup — meist Erdbeersirup (450 g) — auf 1 bis 1½ l verdünnt und im Verlauf eines Tages portionsweise getrunken; in dieser Form ist die Aufnahme der stark sauren Zubereitung einigermaßen erträglich. Am 8., 10. und 12. Tag werden im 24 Std.-Harn wieder Titrationsacidität, Citronensäure- und Calciumgehalt bestimmt (Versuche mit Belastung). Die Ernährung ist während der Untersuchungsperiode normal.

*Aufbereitung des Harns.* Der Harn wird im Kühlschrank in einer Flasche gesammelt, die mit einigen Tropfen Toluol versetzt ist; am nächsten Tag temperiert man auf 20°C und bestimmt das Volumen.

*Bestimmung des Säuregrads.* 10 ml Harn werden auf etwa 100 ml verdünnt und in üblicher Weise mit 0,1 n-Natronlauge gegen Phenolphthalein als Indicator titriert.

*Bestimmung der Citronensäure.* 40 ml Harn werden mit 10 ml 20%iger Trichloressigsäure und 0,8 g Aktivkohle versetzt; letztere wird vorher mit Säure gewaschen und getrocknet. Man läßt unter gelegentlichem Umschütteln stehen und filtriert

nach 20 Min. Vom Filtrat werden — je nach Gehalt an Citronensäure — 10 ml auf 100 bis 1000 ml verdünnt und davon 5 ml zur Bestimmung auf photometrischem Wege nach der Pentabromacetonmethode [14] herangezogen.

*Bestimmung des Calciums.* In 5 ml des Aktivkohle-Filtrats wird das Calcium nach D. VAN SLYKE und I. SENDROY jr. als Oxalat gefällt und seine Menge durch Titration mit 0,01 n-Kaliumpermanganat ermittelt [15].

**Tabelle 2**

**Harnanalysen ohne und mit Citronensäurebelastungen**

1. Titrationsacidität

| Person | ml 0,1 n-Natronlauge je 24 Std. | | | | | |
|---|---|---|---|---|---|---|
| | ohne Belastung | | | mit Belastung | | |
| | 1. Tag | 3. Tag | 5. Tag | 8. Tag | 10. Tag | 12. Tag |
| A | 174 | 191 | 282 | 237 | 178 | 246 |
| B | 401 | 312 | 314 | 371 | 222 | 292 |
| C | 320 | 357 | 306 | 405 | 388 | 526 |
| D | 355 | — | 435 | 296 | 280 | 326 |
| Mittelwerte | | 327 | | | 314 | |

2. Citronensäure

| Person | mg je 24 Std. | | | | | |
|---|---|---|---|---|---|---|
| | ohne Belastung | | | mit Belastung | | |
| | 1. Tag | 3. Tag | 5. Tag | 8. Tag | 10. Tag | 12. Tag |
| A | 373 | 310 | 232 | 435 | 419 | 372 |
| B | 945 | 1190 | 1074 | 1365 | 1400 | 1495 |
| C | 325 | 639 | 566 | 666 | 641 | 633 |
| D | 280 | — | 392 | 384 | 315 | 480 |
| Mittelwerte | | 575 | | | 720 | |

3. Calcium

| Person | mg je 24 Std. | | | | | |
|---|---|---|---|---|---|---|
| | ohne Belastung | | | mit Belastung | | |
| | 1. Tag | 3. Tag | 5. Tag | 8. Tag | 10. Tag | 12. Tag |
| A | 122 | 163 | 248 | 179 | 298 | 251 |
| B | 171 | 190 | 172 | 158 | 277 | 173 |
| C | 173 | 315 | 274 | 210 | 371 | 188 |
| D | 104 | — | 153 | 130 | 140 | 149 |
| Mittelwerte | | 190 | | | 210 | |

## 2. *Ergebnisse*

Die verzehrte, recht große Menge an Citronensäure wird von allen 4 Versuchspersonen gut vertragen, ohne daß sich eine Beeinflussung des Wohlbefindens manifestiert; insbesondere sind in keinem Fall Reizung des Magen-Darm-Kanals oder Durchfall aufgetreten.

Die aus Parallelversuchen ermittelten Befunde sind in Tab. 2 zusammengestellt. Der bei den Versuchen mit Belastung zu erwartende zeitliche Gang ist nicht erkennbar, so daß die Analysendaten als Parallelwerte angesehen werden können. Zur Beurteilung der Ergebnisse anhand der 3 untersuchten Merkmale (Titrationsacidität, Citronensäure und Calcium im Harn) — Versuche ohne Belastung, Versuche mit Belastung — wird als statistisches Auswertungsverfahren die Varianzanalyse herangezogen [16].

Zunächst wird das Untersuchungsmaterial — hier die Harnproben der 4 Personen ohne Belastung — auf Homogenität (Vorliegen von nur zufälligen Abweichungen) geprüft. Die Anwendung des F-Testes (Tab. 3) zeigt, daß die Harnprobe der verschiedenen Personen nur in Bezug auf den Calciumgehalt als homogen angesehen werden können. Bei der Titrationsacidität und dem Gehalt an Citronensäure sind die berechneten F-Werte größer als die für eine 99%ige statistische Sicherheit in Tabellen [16] festgelegte Prüfgröße, d. h. es liegen signifikante Unterschiede zwischen den Versuchsteilnehmern vor[1].

Tabelle 3

Homogenitätsprüfung der Harnproben (ohne Belastung)

| | berechnete F-Werte | tabellierte F-Werte | Ergebnis |
|---|---|---|---|
| Titrationsacidität | $F = 13{,}75$ | $F_{1\%} = 7{,}59$ | Proben sind inhomogen |
| Citronensäure | $F = 30{,}10$ | | |
| Calcium | $F = 3{,}34$ | $F_{5\%} = 4{,}07$ | Proben sind homogen |

Tabelle 4

Prüfung auf signifikante Unterschiede der Harnproben durch Citronensäurebelastungen

| | berechnete F-Werte | tabellierte F-Werte | Ergebnis |
|---|---|---|---|
| Titrationsacidität | $F = 2{,}06$ | $F_{5\%} = 4{,}30$ | Unterschiede sind zufällig |
| Citronensäure | $F = 1{,}07$ | | |
| Calcium | $F = 1{,}19$ | | |

[1] $F_{ber.} > F_{1\%}$: Abweichung ist signifikant; statistische Sicherheit $>99\%$
$F_{ber} < F_{5\%}$: Abweichung ist zufällig; statistische Sicherheit $<95\%$
$F_{1\%} > F_{ber.} > F_{5\%}$: Abweichung kann signifikant sein und sollte nochmals geprüft werden
Die %-Angaben im Index bedeuten „Irrtumswahrscheinlichkeit" (% statistische Sicherheit + % Irrtumswahrscheinlichkeit = 100).

Weiterhin wird geprüft, ob die in Tab. 2 ersichtlichen Unterschiede (ohne und mit Belastung) statistisch gesichert sind. Die Berechnungsergebnisse sind in Tab. 4 zusammengestellt. Es zeigt sich, daß in jedem Fall die auftretenden Unterschiede zufällig sind, weder die Titrationsacidität noch der Citronensäure- und Calciumgehalt werden durch die Citronensäurebelastung signifikant beeinflußt.

### 3. *Diskussion*

Einschränkend ist zu betonen, daß die erhaltenen Resultate trotz der kritisch statistischen Auswertung nicht verallgemeinert werden dürfen; dafür ist die Anzahl der Versuchspersonen und der ermittelten Befunde (Versuchsdauer) noch viel zu gering. Zu berücksichtigen ist ferner, daß bei der Bestimmung von Titrationsacidität und Citronensäuregehalt ein homogenes Versuchsmaterial nicht vorliegt; dies hat jedoch auf die Unterschiede zwischen den Werten der Proben ohne und mit Belastung keinen wesentlichen Einfluß (Tab. 2). Sehr auffällig sind die erheblichen Differenzen und Schwankungen der Citronensäurewerte der Harnproben bei den Versuchsteilnehmern. Die Höhe des Citronensäurespiegels bewegt sich offenbar in größeren Konzentrationsbereichen, als sie in der Literatur z. T. angegeben sind. Die vom gesunden Menschen je 24 Std. ausgeschiedenen Mengen betragen nach H. D. CREMER [17] 0,15—0,3 g, nach A. KOMÁRKOVÁ und Mitarb. [18] 0,3—1,0 g, nach H. OTTO [19] 0,6—1,0 g und nach K. HINSBERG [20] 1,0—1,5 g; unsere eigenen Befunde an nur 4 Personen weisen Schwankungen zwischen etwa 0,23 und 1,2 g auf (Tab. 2). Einer Heranziehung der Höhe der Citratausscheidung im Harn als eines diagnostischen Mittels, z. B. bei Niereninsuffizienz [19], bei Tetanie [18] usw., ist daher mit Vorsicht zu begegnen.

Auf die Zusammenhänge zwischen Citronensäure und Calciumtransport- und -resorptions-Vorgänge wird, wie erwähnt, in der Literatur mehrfach hingewiesen [5—11, 18]. Die vorsichtig auszuwertenden eigenen Untersuchungen zeigen, daß Calcium bei Citronensäure-Belastungen nicht vermehrt im Harn ausgeschieden wird. Die schon diskutierte Stabilität des Calcium-Citrat-Komplexes weist im stärker sauren Gebiet ein Maximum auf, in dem auch die Calcium-Resorption begünstigt wird. Klinische Befunde haben zusätzlich ergeben, daß sich komplex gebundenes Calcium bei einem Überschuß an Citronensäure pharmakologisch am wirksamsten erweist [11].

In Übereinstimmung mit der Literatur darf — mit der gebotenen Zurückhaltung — gefolgert werden, daß eine reichliche Citronensäurezufuhr ohne beeinträchtigenden Einfluß auf den gesunden Organismus ist. Dem Genuß von frischem Citronensaft — ohne Überschreitung extremer Mengen — mit seinen ernährungsphysiologisch wertvollen Inhaltsstoffen (Vitamine, Mineralstoffe) stehen damit Bedenken nicht im Wege. Ob und in welcher Weise die exogen verabfolgte Citronensäure in den „Citronensäure-Cyclus" und damit in den oxydativen Endabbau der Nährstoffe einbezogen wird, ist eine Problematik, auf die hier nur hingewiesen sei.

## Zusammenfassung

1. Anhand der Literatur werden Citronensaft bzw. Citronensäure in ihrer Bedeutung für Ernährung, Diätetik und Therapie erörtert.

2. An 4 gesunde männliche Personen werden im Belastungsversuch peroral täglich 50 g Citronensäure verabfolgt. Im 24 Std.-Harn erfolgt Bestimmung von Titrationsacidität, von Citronensäure- und Calciumgehalt; die Ergebnisse werden denjenigen aus den Versuchen ohne Belastung gegenübergestellt.

3. Nach der statistischen Auswertung der Befunde wird durch die Belastung keines der 3 ermittelten Bestandteile des Harns in signifikanter Weise beeinflußt.

4. Die Versuche lehren, daß im Einklang mit dem Schrifttum eine reichliche, extreme Grenzen nicht übersteigende Zufuhr von Citronensaft bzw. Citronensäure nach dem heutigen Stand der Kenntnisse ohne nachteiligen Einfluß auf den gesunden Organismus ist.



## Summary

1. Referring to literature lemon juice respectively citric acid are discussed with regard to the importance of which as to nutrition, dietetics, and therapy.

2. To 4 healthy male persons forming part of a charge test 50 g. citric acid are delivered daily per os. In the 24 hour's urine titration acidity, content of citric acid, and calcium content are determined. The results are compared with the tests without charge.

3. According to the evaluation of the findings no one of the urine's 3 tested constituents has been significantly influenced.

4. The investigations show that corresponding to literature and according to the present state of knowledge a healthy organism is not enduringly influenced by a copious supply of lemon juice respectively citric acid not exceeding extreme limits.



## Резюме

1. На основании материалов из литературы обсуждаются лимонный сок и лимонная кислота в отношении их значения для питания, диэтетики и терапии.

2. Четырем здоровым лицам мужского пола в порядке опытной нагрузки давали вовнутрь принимать ежедневно по 50 г лимонной кислоты. В выделенной за 24 часа моче производилось определение титрационной кислотности и содержания лимонной кислоты и кальция; результаты противопоставлялись данным по опытам без предшествующей нагрузки.

3. Из статистической оценки полученных результатов явствует, что предпринятая нагрузка не влияет заслуживающим внимания образом ни на одну из установленных трех составных частей мочи.

4. Опыты учат, что соответственно опубликованиям в литературе обильное, крайних границ не превышающее потребление лимонного сока или лимонной кислоты, согласно настоящему уровню знаний, остается без влияния на здоровый организм, не вызывая в нем радикальных последствий. —


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# Occurrence and Significance of Citric Acid in the Animal Organism

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IN 1784 THE SWEDISH APOTHECARY Scheele (52) reported to the Swedish Academy of Sciences that he had obtained an organic acid in crystalline form from lemon juice, which had not previously been described. It was natural to name the substance citric acid. The lemon juice previously available at the pharmacist had been of an unsatisfactory nature, sometimes fermented, or even moldy. Scheele's crystalline acid, on the other hand, was dry and could be kept for an unlimited time. It was obvious that Scheele had obtained a very valuable preparation. Quite a long time afterwards it became clear that when an aqueous solution of citric acid was substituted for the pharmacist's lemon juice, from the therapeutic point of view the juice simultaneously lost its most valuable ingredient, vitamin C (ascorbic acid).

Citric acid was found particularly in the form of its salt, the citrate, as a constituent of a large number of plants. Franzen and Helwert (14) found reports in the literature of the occurrence of citrate in not less than 137 different plants. One must, however, consider the possibility that the analytical procedures in many cases were unsatisfactory. When greater claims were made on the reliability of the methods, the accepted number of 'citric acid plants' fell to about 15.

The properties of citric acid had long been considered to be those of a typical plant acid, but in 1888, Henkel (24, 24a) showed that milk contained citric acid. Widening the investigation to extend to other animal products was not successful for a long time. At the beginning of the present century, opinion among physiologists was fixed that the occurrence of citric acid in the animal kingdom was limited to milk, and that its physiological importance had something to do with this product.

In 1893 Wehmer (69) noted that citric acid could be obtained in considerable quantities from sugar, through the fermentative processes of certain microorganisms. It was by the use of molds, especially *Penicillium*, that Wehmer prepared citric acid. Wehmer's observation became the basis for the industrial preparation of citric acid.

Since citric acid has been found to be present in considerable quantities in potatoes, the use of this material for the technical production of the acid has been suggested (57).

An important step forward in our knowledge of citric acid was taken in 1881, when the synthesis was carried out for the first time by Grimaux and Adam (17). The citric acid prepared by these workers was identical in every respect with that

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obtained by Scheele from lemon juice. Both the naturally occurring and the synthetic acids crystallized out with one molecule of water of crystallization which was given off at 130°C.

The negative result of the search for citric acid in the fluids and tissues of the animal kingdom seems to have been due most of all to the lack of a reliable and sensitive method for its detection. Such a method was made available at the turn of the century by the Swedish pharmacist, Stahre (56). 'Stahre's reaction' depends upon the production of a characteristic precipitate when a solution containing citric acid is added to potassium permanganate and bromine water. The chemical nature of this precipitate was soon made clear by the Danish chemist, Wöhlk (71), who proved that it was due to the formation of a pentabromacetone. The extensive critical examination to which the Stahre reaction has been subjected bears out its surprisingly high specificity. There is scarcely a substance which, occurring with citric acid under natural conditions, interferes with its determination. The definitive demonstration of pentabromacetone in the Stahre reaction can be made in different ways by gravimetric, titrimetric and colorimetric methods. Up to now the different modifications put forward, however valuable they may be, are in general only simplifications. Nowadays excellent micromethods, based on the Stahre reaction, are available.

The enzymatic method devised by Thunberg (62) occupies a special place. It is based on the occurrence in certain types of seeds, e.g. *Cucumis sativus* (cucumber), of an enzyme specific for citric acid. It was above all with this method that the general occurrence of citric acid in animal fluids and tissues was demonstrated. The method is particularly useful in laboratories that are especially equipped for enzyme research, and in these laboratories is still put to good use. To a large extent though it has been replaced by the ordinary chemical methods.

From 1910 onwards Thunberg (60) carried out a systematic investigation of the behavior of different organic acids when added to a homogenate of various body tissues, especially muscle. He found that some acids, among them citric acid, which up till then had been regarded as typical plant acids, were able to bring about a considerably increased respiration in the muscle homogenate. According to Thunberg these acids represented normal intermediary substances in animal metabolism; in other words, to use a later expression, they were metabolites.

Citric acid was first shown to be a normal constituent of human urine by Amberg and McClure (2). Boothby and Adams (6) found that the excretion of citric acid in the urine of dogs persisted even in fasting. The amount of citric acid excreted certainly fell after some time of fasting, but then rose again. A higher rate of excretion can be reached under fasting than under normal conditions, and this in spite of the fact that the organism is receiving no citric acid. After hepatectomy, the amount of excreted citric acid at first falls, but rises again a short time before the animal's death.

The study of the behavior of citric acid added to the organism now made progress, and intense investigations were published by such authors as Batelli and Stern (3) and Meyerhof (41). That citric acid was synthesized from certain organic substances added to homogenates was shown by Gemill (15), Krebs and Johnson (28) and Hallman and Simola (21). Citric acid had now fought its way to an acknowledged position in the world of metabolites.

## General Survey of Citric Acid Content of Human Tissues and Fluids

The following table gives the amounts of citric acid in mammalian organisms, with special reference to human fluids and tissues. The values are given as micro-

grams per milliliter or as parts per million (p.p.m.). The concentration figures are given to the nearest whole number to avoid the use of decimals.

| ORGAN OR BODY FLUID | μG/ML. |
|---|---|
| Whole blood | 15 |
| Blood plasma | 25 |
| Red blood corpuscles | 10 |
| Milk | 500–1250 |
| Urine | 100–750 |
| Semen | 2000–4000 |
| Cerebrospinal fluid (lumbar puncture) | 50 |
| Cerebrospinal fluid (cisternal puncture) | 25 |
| Mammary gland | 3000 |
| Thyroid gland | 750 |
| Kidney and some other organs | 20 |
| Liver, muscle | 5 |
| Bone substance | 7500 |

All values are approximate. The following comments may be added to the above table.

**Serum, Blood.** The occurrence of citric acid in human blood was first shown by Benni, Scherstén and Östberg (5) and Nordbö and Scherstén (47).

The greater part of citric acid determinations in blood, in this and other series, refers to serum from venous blood, taken from the cubital vein. The samples were taken before rising and on an empty stomach. Because of the high rate of blood flow through the skin of the forearm and the low arteriovenous difference the citric acid value which is so obtained can be considered to hold for arterial blood also. The citric acid value of venous blood is different in different organs, and differs to a greater or or lesser extent from the mean arterial value. The arteriovenous difference is particularly marked in the kidneys. The citric acid value in the renal veins is, according to Mårtensson (44), 20 to 30 per cent lower than the corresponding arterial value.

The values given in the above table were generally obtained with Thunberg's enzymatic method. These determinations were intended primarily to obtain normal values for clinical use. The mean value was found to be 20.5 ± 1.8 μg. ml. (55). For practical clinical use we now take 30 μg. as the upper limit for normal cases and 17 μg. as the lower.

The concentration of citric acid is greater in serum (plasma) and lower in the corpuscles. Sufficient data for the estimation of the citrate content of the white blood corpuscles have not been obtained.

If a soluble citrate, for example sodium citrate, is added to whole blood, it will remain for the most part, judging from the hematocrit determination, in the serum. On the other hand, only a small amount of citrate seems to enter into the red blood corpuscles (59). An investigation directed particularly to the relationship between the citrate content of corpuscles and serum gave the result that the concentration in serum is five to six times greater than in the corpuscles (44). The small amount of citric acid found in centrifuged corpuscles could perhaps be looked upon as originating from the serum and only adhering to the corpuscles. As a result of this, the citric acid content of whole blood changes according to the percentage of red blood corpuscles even though the level in the serum is constant (63).

Among the factors which affect the level of serum citrate is the parathyroid hormone. An increased parathyroid function produces a corresponding increase in the citric acid content of the blood (55).

Owing to its deionizing action on calcium, citrate is poisonous if its concentra-

tion in serum rises over a certain value. In a case studied by Martius an attack of tetany was produced when the serum citrate value rose from its normal level of 22 to 80 p.p.m. (40).

Children generally show the same citrate values as adults (35, 38).

During fasting the citric acid content of the organism is only slightly reduced, the reduction not being of the order which might be expected from a substance of this character (see 6). However Lindholm has found a distinct decrease in citrate values for the first days of fasting in rabbits (34). Even the starved animal can maintain a high level of citric acid excretion, especially under the stimulating influence of alkalosis (72).

Physical work generally raises the serum citrate value (32, 1).

The serum citrate figures in pregnant women show no distinct difference from the normal values (33). Immediately before the beginning of labor the citrate content will increase. This increase can reach 9 p.p.m. and the value will remain at this high level until after the delivery. A rapid fall then takes place which is most pronounced on the 4th day. At this time the citrate value is below normal. The low level is maintained until about the 11th day after parturition, when it begins to increase slowly and after about a further 10 days reaches the normal value for nonpregnant women.

The serum citrate value is influenced by different pathological conditions (54). It is low in pneumonia and thrombosis and high in hepatitis. On the basis of the last mentioned fact Sjöström elaborated a method for the differential diagnosis between hepatitis and occlusion of the bile duct. For a discussion of this method reference may be made to the papers of Boothby and Adams (6), Buch (9), Hagelstam (19), Hammarsten (22) and Lövgren (37).

There are different opinions as to how the dissimilar behavior of the serum citrate in hepatitis and in bile duct occlusion may be interpreted. Sjöström (55) interprets the hypercitricemia of hepatitis as being dependent upon the liver, which under normal conditions is generally regarded as the principal organ of citric acid breakdown. If the liver is damaged and the citrate metabolism decreased, citric acid will accumulate in the blood and the citrate content will therefore rise. According to another view, originated by Mårtensson (45), which is based on better experimental evidence, the relationship is more complex. In his opinion liver damage may result inter alia in an increased concentration of amino acids in the blood, and these acids or other metabolites inhibit the breakdown of citrate in the kidneys, and thus a hypercitricemia develops.

The citric acid occurs in the plasma (serum).

Serum from animals other than man gives higher values, up to 100 μg. The horse, perhaps the best investigated animal in this respect, has a citric acid value of 50 μg. (68), the rabbit 100 μg. (34). The total amount of citric acid in serum, the 'circulating citric acid,' can be estimated approximately at 70 mg. for a man weighing 70 kg. The volume of serum is taken to be 2.8 l. and the citrate content 25 mg/l. The citric acid content of the red blood corpuscles has been neglected in these calculations.

Thunberg and Zetterström have investigated the effects of antigen injections for immunization on about 20 horses, and have studied the citric acid values before and after injections. As antigens they used *Clostridium histolyticum*, *C. oedematiens*, *C. Welchii*, *Corynebacterium diphtheriae*, *Bacillus tetanus* and *Meningococcus*. The injections, regardless of which antigen was used, regularly produced a marked decrease in the serum citrate level. The value often fell to 50 per cent of the original serum citrate values. After some days or weeks the original level was regained (68).

**Milk.** The citric acid content of human milk was found to be 0.50 per cent by Kunz (29) and 1.0 to 1.5 by Bleyer and Schwaibold (5a). Using a better method, Jerlov (26) found this value to be between 0.35 and 1.25 per cent. He was not however able to find any citric acid in milk until some days after delivery. Lennér (33), who examined 50 specimens of human milk, using a sensitive method, found only a minimal amount in colostrum.

**Urine.** On the basis of 300 determinations referring to 200 healthy persons, Östberg (72) has found the limits of the normal daily excretion of citric acid to be 0.20 to 1.0 gm. An oral intake of citric acid produces no increased excretion in the urine, even if the dose is 40 gm/day. The greatest excretion obtained in this way was 1.0 gm. On certain occasions after such a great intake not an increased but decreased excretion was observed.

Acids, other acid substances and acidosis produced by lack of carbohydrates cause a lessened excretion of citric acid, but alkaline substances always increase excretion. Östberg (72) attributes a certain importance to the excretion of citric acid in keeping the sparingly soluble calcium salts in solution. A decreased citric acid excretion is a regular symptom of diabetic acidosis and uremic conditions.

The citric acid excretion of dogs and sheep has been studied: the different values obtained seem to depend on the quality of their food (46).

A marked effect on the citric acid content of rats' urine is exerted by uranium salts, injected intraperitoneally in suitable amounts. A single dose of 2.5 mg. uranium nitrate/kg. raises the citric acid excretion to three or four times the normal amount for 3 days (23a).

**Semen.** The occurrence of citric acid in semen was discovered by Scherstén (53), who was also the first to point out that the acid is an extracellular component of semen and originates chiefly in the accessory organs of reproduction. These findings have since been confirmed and extended by several other investigators. In man, citric acid is mainly confined to the prostate, but in other mammals such as the bull, ram, boar and stallion most of it is found in the seminal vesicles (25). In the rabbit, citric acid is chiefly confined to the secretion of the glandula vesicularis, while in the rat it is located in the ventral prostate and the seminal vesicle proper.

There is a close relationship between citric acid formation and the level of testosterone. It should be possible to adopt citric acid assay as an indicator test for the androgenic hormones (39).

An interesting illustration of this relationship was found by Price, Lutwak-Mann and Mann (51) during a study of the 'female prostate' of the rat. This organ, which is sometimes found in the female rat and is morphologically homologous with the male ventral prostate, produces large amounts of citric acid in response to testosterone injections (39a).

When fresh semen is incubated *in vitro*, citric acid is utilized both aerobically and anaerobically by the spermatozoa. Citric acid can easily be removed by washing the spermatozoa with Ringer solution. It has been shown, however, that spermatozoa contain enzymes associated with the reactions of the tricarboxylic acid cycle. This metabolic pathway may be involved in normal sperm respiration (31).

It has been claimed that citric acid is connected with sperm motility, but its function in semen is still obscure. Possibly it has something to do with such phenomena as the coagulation and liquefaction of semen, hyaluronidase activity, or the calcium binding capacity of seminal plasma.

The distribution of citric acid in male reproductive organs has been determined as follows (39).

| | WT. OF ORGAN, MG. | CITRIC ACID, MG/100 GM. |
|---|---|---|
| *Rabbit* | | |
| Glandula seminalis | 120 | 50 |
| Glandula vesicularis | 820 | 180 |
| Prostate | 810 | 55 |
| Cowper's gland | 950 | 25 |
| *Rat* | | |
| Seminal vesicles | 780 | 39 |
| Coagulating glands | 130 | 0 |
| Median prostate | 20 | 60 |
| Ampullae | 70 | 0 |
| Dorsal prostate | 250 | 20 |
| Ventral prostate | 320 | 122 |

**Cerebrospinal Fluid.** Cerebrospinal fluid, C.S.F. obtained by lumbar puncture, contains about 50 p.p.m. of citric acid. The cisternal fluid contains much less citric acid, about the same amount as blood (4, 44). The fact that the citric acid content of the C.S.F. is much higher than that of serum (e.g. 40 p.p.m. in C.S.F. as compared with 25 p.p.m. in serum) provides evidence for the conclusion that the C.S.F. is formed by an active process. This justifies the assumption that the C.S.F. should be considered as a secretion and the choroid plexus as a gland, and strengthens the most widely held theory on this point (45a).

Hastings and Lees produced a typical tetanic syndrome in dogs by reinjecting calcium-depleted cerebrospinal fluid, or by injecting small amounts of sodium citrate into the cisterna magna of anesthetized dogs. The seizure could be overcome by restoring the calcium ion concentration to normal (23).

**Amniotic Fluid.** Amniotic fluid has a varying content of citric acid. According to Genell (16) it varies from 17 to 100 μg/ml., while according to Lennér (33) the mean value is 39 μg/ml. The citric acid content shows no clear correspondence with the serum citrate levels in mother or child even during labor.

**Sweat.** Scherstén (54) found sweat to have the low citric acid value of 1 to 2 p.p.m., a figure which lies at the lower limit for the application of his method.

**Tears.** The citric acid content of tears was determined by Grönvall (18), who found 5 to 7 p.p.m. citric acid.

**Aqueous and Vitreous Humors.** Grönvall (18) also found citric acid in the aqueous and vitreous humors. The values for aqueous humor vary very much in different animals, the citric acid value for certain birds being particularly high, up to 600 p.p.m.

**Muscle, Liver, Kidney, Brain.** Different tissues and organs show great differences in their citric acid content. Values from about 2 to 100 p.p.m. are found in skeletal muscle, liver, kidney and brain (15). However, one must take into consideration the fact that the breakdown of citric acid can take place more rapidly in the tissues than in serum, owing to the presence in the tissues of enzyme systems capable of metabolizing citric acid.

**Thyroid Gland.** Working with a large amount of material Haliman (20), Brolin and Thunberg (8) found the mean citric acid content of the human thyroid to be 900 p.p.m. The citric acid content of the thyroid is then remarkably high in comparison with that of the blood and most other tissues. The citrate concentration varies with the colloid content. It is low in the follicular epithelium. Age is also a factor which affects the citrate concentration. The citric acid concentration rises with increasing age, at first rapidly and then more slowly until 25 to 30 years of age. At this period

the content falls sharply but later rises again. The variations in citric acid content of the thyroid correspond particularly well with changes in structure. During sexual maturity, from 15 to 45 years of age, there is no difference in thyroid citrate content between men and women. In the next 20 years the thyroid citrate level in women falls considerably.

**Bone, Skin, Hair.** Most surprising is the high citric acid value of bone substance which Dickens discovered in 1940 (13). It can amount to 1 per cent of the fresh, undried material. Thunberg later published corresponding figures, based on a larger bulk of material, which also included bird and fish bones. The bone substance is the factor which above all others is responsible for the citric acid percentage of the human body (65).

With a body weight of 70 kg. there are about 11 kg. of bone substance, and 59 kg. of soft tissues (inclusive of fluids). If the percentage of citric acid in the bone substance is taken to be 0.75 per cent, then the total citric acid will be 82.5 gm. Taking 15 mg. of citric acid/kg. soft tissues (including fluids), then the total weight of citric acid is increased by 885 mg. The total citric acid content of the human body then comes to 83.385 gm. As a great approximation therefore, the total citric acid can be taken to be 100 gm.

Kuyper has studied the quantitative precipitation of citrates *in vitro*. While the precipitation of citrate as the calcium salt from aqueous solution was incomplete, its precipitation from urine was almost complete. This difference was attributed to the phosphate present in urine since citrate, calcium and phosphate apparently formed an insoluble complex. Further work showed that the amount of citrate precipitated depends on the concentrations of calcium and phosphate present in the solution. Almost complete precipitation of citrate is obtained from a solution containing 0.13 mg. citric acid/100 ml. if suitable proportions of calcium and phosphate are present (30).

Citric acid probably occurs chiefly in the compact part of the bone substance. The richer the bone in substantia compacta the higher the citric acid content under similar conditions. The presence of spongy substance however decreases the value. The difficulty in separating the two parts preparatively creates a corresponding difficulty in determining their citric acid content.

The citric acid content of the bone substance appears partly in a soluble and partly in an insoluble form. This is confirmed by experiments to extract finely divided bone meal with water. Part of the citric acid is immediately dissolved but further dissolution occurs very slowly. The most natural explanation is that the citric acid occurs in a weakly dissociated state extremely difficult to dissolve in water. The salts of the skeleton are now assumed to occur in a complex combination (apatite). The occurrence and condition of citric acid in the bone substance do not disagree with the apatite theory (65).

Dickens (13) has made the interesting observation that skin and hair from the mouse and the horse are rich in citric acid. He tends to the view that the citric acid in hair comes from the secretion of the skin glands. He bases this supposition on the fact that the greater part of the citric acid in hair can be extracted with boiling water (58 per cent of the total citric acid from horse hair, 73 per cent from mouse hair). From our own investigations it seems that citric acid occurs with keratin in cases where no impurities or impregnations from skin secretions could exist. Citric acid is found for example in the shell membrane from hens' eggs and the horny substance from horses' hooves. It is difficult to say what part citric acid plays in the horny sub-

stance. The content is so small, 250 μg/gm. horse hair, that one is disposed to think that any citric acid in the horny substance is merely an inclusion from the fluids from which keratin is built up, and that here citric acid is without biological importance.

**Concrements.** A survey of the citric acid content of different concrements drew attention to the concrements in the crayfish, which are known as crayfish stones (the pharmacists' *Lapilli cancri*). They are formed during seasons when the need for calcium is small, and are utilized in the animal during the periods of greatest calcium need (67). The citric acid content in crayfish stones was 0.8 per cent in average.

In the eggshells of different birds 0.1 to 0.8 per cent citric acid was found (66). A considerable amount of citric acid was found by Mårtensson (45) in pathological lithiases. Morris and Steenbock (42) produced urinary lithiasis in young rats by feeding them low phosphorus rations of approximately normal calcium content. The frequency of lithiasis was increased by giving the rats sodium bicarbonate or potentially alkaline salts, whereas adequate amounts of phosphate completely prevented its occurrence. The calculi produced on the basal low phosphorus-adequate calcium ration consisted mainly of calcium citrate, but when additional alkali was given their citrate content was reduced, their calcium content was increased, and carbonate and phosphate appeared as additional constituents.

Citric acid in lesser amounts has also been found in the endoskeleton of the octopus, 'cuttlebone,' and in the shell of the garden snail, *Helix pomatia*. No citric acid was found in the small amounts of real pearls available for investigation.

Wherever citric acid was found, phophate was also found to be present, in concentrations about 10 times as great as those of the citric acid. (Steinhardt, personal communication).

## Exogenous Citric Acid

Of the citric acid of the organism, part originates from the food, 'exogenous' citric acid, and part is synthesized by the organism, 'endogenous' citric acid.

Generally speaking, all our food contains citric acid. This substance occurs not only in fruits and berries, but also in vegetables. In animal foodstuffs citric acid represents a factor that must be taken into account. Milk and potatoes are the most important sources of citric acid in the food of the large majority of the Swedish people. Milk contains one or a few grams per liter. Potatoes contain up to 10 gm/kg., depending on the type and the season (57). Autumn potatoes have a high content, winter stored ones a lower. If these figures are calculated from the dry weight the citric acid content is some tens of grams per kilogram.

Cereals, meal and meal products are very poor in citric acid, but the seeds of leguminous plants are often quite rich (43).

Grass and green vegetables contain considerable quantities of citric acid. It has been calculated from studies of the citrate balance of calves that when a calf changes over from milk to grass there is no appreciable change in its daily intake of citric acid (70).

Perhaps the citric acid in feces ought to be considered as exogenous citric acid, since it is probably synthesized by the intestinal flora (33, 44). Certainly very little is known as to its quantity, but nonetheless this is a factor to be considered.

## Endogenous Citric Acid

**Condensing Enzyme.** As early as 1891 Claisen and Hori (12) published their synthesis of citric acid by an aldol condensation of oxaloacetic and acetic acids. It

was not until almost 40 years later that Breusch (7) suggested that the synthesis of citric acid in the tissues could take place from a *4C* and a *2C* compound under enzymatic influence. He worked with an impure extract from liver tissue, which contained the necessary ingredients for an enzymatic condensation to citric acid of oxaloacetate and acetate. Breusch called this substance citricogenase. It is now known by the name 'condensing enzyme.'

This substance was later obtained in purer form by Lipmann (27). It is made up of a apo-enzyme, Lipmann's enzyme A, and Coenzyme A. Coenzyme A appears to have the properties of a *2C* radical, which makes it possible for it to combine with oxaloacetic acid (*4C*) to form citric acid (*6C*).

Other factors necessary to fulfil the requirements of the enzyme system are $Mg^{++}$ and $Mn^{++}$ ions and adenosinetriphosphate.

Recent work has gradually elucidated the parts played by these different components. Novelli and Lipmann (48) demonstrated the catalytic effect of Coenzyme A on citrate synthesis in cell-free yeast extract. Studies on the velocity with which acetate is utilized by animal tissues had long suggested that acetic acid was activated in some way in metabolic reactions. Using acetate labelled with C-14 at the carboxyl group Buchanan, Hastings and Nesbett (10, 11) showed that 50 per cent and more of the acetate was resorbed and oxidized in under 2 hours. An activation of the acetate seemed likely, and since ATP was needed for enzymatic citrate synthesis, activation through some form of phosphorylation was proposed by several workers.

The nature of 'active acetate' has been obscure for some years, but the most recent work of Lipmann and his collaborators (36) shows that 'active acetate' is most likely to be acetyl-CoA. The mechanism of its formation is suggested to be a reaction between ATP and the coenzyme to form CoApyrophosphate, which then combines with acetate to give the active acetyl-CoA. *a*) ATP + CoA = AMP + CoApyrophosphate. *b*) CoApyrophosphate + acetate = acetyl-CoA + pyrophosphate. This view of Lipmann's agrees with the discovery of Ochoa and Stern (50) that two stages are necessary for the enzymatic synthesis of citric acid, catalyzed by two different enzymes. The first stage consists of the formation of active acetate (acetyl-CoA) from ATP and acetate, in the presence of an activating enzyme, found by Stern (58) in *Escherichia coli*. The second stage is the condensation of acetyl-CoA with oxaloacetate to form citrate. This is catalyzed by the condensing enzyme.

According to an older view, one of the two partners in the condensation reaction is pyruvic acid, the other being oxaloacetic acid. This does not seem probable, for such a combination would result in the formation of a *7C* compound, which in spite of the efforts of many investigators was never isolated from the reaction mixture (28).

Another opinion held that citric acid is formed from the combination of oxaloacetic acid with acetoacetic acid, but probably the acetoacetic acid is split into two molecules of acetic acid. Then the definitive reaction will be the same in both cases, and the acetoacetic can be regarded as merely the precursor of the acetate.

## Citric Acid or Isocitric Acid

At this stage one must consider whether the substance here referred to as 'citric acid' is really citric acid or its isomer, isocitric acid. These two substances are interconvertible under the influence of aconitase, the isomerizing enzyme discovered by Breusch (7) in 1937.

Originally it was thought that ordinary citric acid was the condensation product

of oxaloacetate with acetate, but work on the enzymes involved in the metabolism of citric acid casts doubts on this proposed identity. Since isocitrate appeared to be the more reactive substance, the question of which of the three tricarboxylic acids, citric, isocitric or cis-aconitic, was the actual product of condensation was debatable, and for some time citric acid was regarded only as a side product from cis-aconitic acid. Definite proof of the synthesis of citrate has now been obtained by Ochoa (27a, 58a), who used a condensing enzyme preparation which was aconitase free. As a result of this, the previous opinion has been reaccepted and citric acid is again considered to be the primary condensation product and the key to the citric acid cycle.

The controversy over the relative order of formation of cis-aconitic and citric acids was naturally reflected in the somewhat confused topic of the relationship of citric acid to the metabolism of the other tricarboxylic acids.

The tricarboxylic acid cycle was first postulated in a paper by Krebs and Johnson (28). Krebs here used the term 'citric acid' to mean one of the three possible acids, and did not define the exact form. His colleagues, however, took the view that although a tricarboxylic acid cycle existed, citrate as such was not present on it. Modern opinion has now reinstated ordinary citric acid as the key to the 'cycle.'

This paper by Krebs and Johnson on the role of citric acid in intermediary metabolism is of great importance. But the present author wishes to make a reservation regarding the foundation of the theory. It seems to him to be an inversion of the facts when Krebs looks upon citric acid as a catalyst in the oxidation of carbohydrates. In reality carbohydrate and other metabolites are 'donors' in this system (61). Krebs here seems to be following the old views of Szent Györgyi's which now seem generally abandoned (64).

## Biological Significance of Citric Acid

The position of citric acid as an metabolite is to a large extent the same as that of a simple sugar. It is completely consumed in the animal organism, its combustion value being 2.47 Cal/gm. A considerable amount of citric acid from food is utilized in man. According to Östberg's investigations, this amount can reach as much as 30 to 40 gm/day. Citric acid is of general importance in metabolism owing to its connection with the complicated enzyme system, the 'citric acid cycle,' which forms a connecting link between the three main metabolic pathways for complete oxidation of carbohydrate, fat and protein. The rich amount of citric acid in bone raises the question of whether citric acid has any mechanical function, for example as a cement substance. Citric acid's substantial occurrence in semen indicates that it has some as yet not fully understood importance in reproduction. The chemical properties of citric acid, particularly its affinity for calcium (and magnesium) and its widespread occurrence in tissues and fluids, are such that it affects cell permeability and the irritability of the nervous system.

## Addendum

At the time when this manuscript was being prepared, the reports of the second International Congress of Biochemistry in Paris were published. A great deal of attention was given to citric acid, particularly in the symposium organized by Michel Polonovski.

The titles of these papers, and their authors are:

Green, D. E. The Cyclophorase-Mitochondrial System.

Martius, G. Die ersten Reaktionen des Citronensäurecyclus.

BREUSCH, F. L. Fettsäureabbau und Citronensäure-cyclus.
KREBS, H. A. The Place of the Tricarboxylic Acid Cycle in Cell Metabolism.
LIPMAN, F., MARY ELLEN JONES AND S. BLACK, Studies on the Mechanism of the ATP-CoA-Acetate Reaction Including a Survey of Functions of CoA.
PETERS, R. A. Inhibitors of the Tricarboxylic Acid Cycle.
OCHOA, S. Enzymatic Synthesis of Citric Acid and Other Reactions of the Tricarboxylic Acid Cycle.
LEUTHARDT, F. Urégénèse, Cycle Tricarboxylique et Mitochondries.
GUZMAN BARRON, E. S. The Tricarboxylic Acid Cycle. Alternative Pathways.

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# Spectrophotometric Determination of Tartaric Acid and Citric Acid in Milligram Quantities

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Iron(III) is known to complex with a number of organic acids in both acid *(1)* and alkaline medium *(2)*. Das and Mohapatra *(1)* made a comprehensive study of the complexes of iron(III) with organic acids in acid medium. It was observed that tartaric and citric acids form stable, yellow complexes with iron(III). Based on the formation of this yellow iron–acid complex, a spectrophotometric method for the determination of tartaric and citric acids in acid medium has been developed.

When an acidic solution of ferric sulfate is added to the aqueous solution of citric or tartaric acid, a yellow water soluble complex is immediately obtained which shows maximum absorbance at 380 mμ. The absorbance of colored solutions containing varying concentrations of tartaric or citric acids were measured at 380 mμ. The complex obeyed Beer's law and hence was found suitable for the determination of milligram amounts of tartaric and citric acids.

## MATERIALS AND METHODS

### *Reagents*

*Sample solutions.* A 50-mg sample of tartaric or citric acid (A.R. B.D.H.) was accurately weighed, dissolved in distilled water and the volume was made up to 100 ml. Different aliquots of this solution were used.

*Ferric ammonium sulfate solution.* About 5 g of ferrous ammonium sulfate (A.R. B.D.H.) were dissolved in distilled water and 2–3 ml of concentrated sulfuric acid (A.R.) added to it. The solution was oxidized by the addition of 30% (w/v) hydrogen peroxide (B.D.H.) with constant stirring. The excess of hydrogen peroxide was boiled off and solution was made up to 250 ml with distilled water. Ten ml of this solution

were diluted to 100 ml and strength of this solution was found to be about 0.005 M. A 1-ml aliquot of this solution was employed as complexing reagent in each case.

*Apparatus*

Absorbance measurements were done with a Beckman model Du spectrophotometer equipped with a multiplier photo tube. The measurements at 380 mμ were done with a slit width of 0.18 mm. Beckman 1.005-cm silica cells were used throughout these studies.

*Procedure*

Two ml of an aliquot from the stock solution were delivered into a 10-ml volumetric flask and 1 ml of ferric sulfate solution was added to it. Immediately a yellow color appeared. The solution was made up to the mark with distilled water, shaken well, and allowed to stand for about 5 minutes. The absorbance of the solution was measured against a reagent blank in the region of 340–400 mμ.

*Preparation of Calibration Curve*

Aliquots of 1, 2, 3, 4, 5, 6, and 7 ml were delivered separately in 10-ml volumetric flasks. 1 ml of ferric sulfate solution was added in each flask. The contents were shaken well and finally made up to the mark. The absorbance of each solution was measured at 380 mμ against a reagent blank.

## RESULTS AND DISCUSSION

The method was applied for the determination of tartaric acid (Table 1) and citric acid (Table 2). The method was also applied for the determination of sodium salts of these acids. The complex formed between salts of citric and tartaric acids and Fe(III) is yellow and has maximum absorbance at 380 mμ. The determinations can be carried out with the same calibration curve both for the acid and sodium salt.

The extinction of ferric ammonium sulfate solution was also measured against water from 340–400 mμ. It was observed that absorbance of solution of ferric ammonium sulfate at 380 mμ was negligible (less than 0.05).



TABLE 1

DETERMINATION OF TARTARIC ACID

| Amount of tartaric acid (mg) | Absorbance* | Recovery (mg) | Error (%) |
|---|---|---|---|
| 0.685 | 0.160 | 0.700 | +2.1 |
| 1.143 | 0.250 | 1.150 | +0.6 |
| 1.550 | 0.340 | 1.550 | |
| 2.515 | 0.550 | 2.500 | −0.6 |
| 2.970 | 0.640 | 2.950 | −0.68 |

TABLE 2

DETERMINATION OF CITRIC ACID

| Amount of citric acid (mg) | Absorbance | Recovery (mg) | Error (%) |
|---|---|---|---|
| 0.545 | 0.160 | 0.550 | +0.92 |
| 0.875 | 0.260 | 0.900 | +2.80 |
| 1.575 | 0.440 | 1.600 | +1.50 |
| 1.925 | 0.540 | 1.950 | +1.30 |
| 2.275 | 0.630 | 2.300 | +1.10 |

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# Citric Acid in Saliva

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Positive evidence for the occurrence of citric acid in human saliva appears to be limited to a report by Pucher, Sherman, and Vickery (*5*), who found 0.04–1.30 mg. per cent citric acid present in 7 specimens. Previously, Kuyper (*1*) and Leake (*2*) were unable to detect citric acid in saliva. However, they both used methods which at the time were unsuited to detect less than 2.0 mg. per cent. With slight modifications the method proposed by Perlman, Lardy, and Johnson (*4*) has been adapted to saliva analysis using 10-cc. quantities. Some 180 saliva specimens obtained from 15 adult men have been analyzed, and the results (Table 1) afford considerably more evidence of the presence of citric acid in saliva than has been available heretofore. Paraffin-stimulated saliva was collected on three days at four different times, *i.e.* 9 A.M., 11 A.M., 1 P.M., and 3 P.M. The figures in the table are averages for three days according to time of day for each individual.

TABLE 1

CITRIC ACID* IN STIMULATED SALIVA

(Mg./100 cc.)

| Case | 9 A.M. | 11 A.M. | 1 P.M. | 3 P.M. | Average |
|---|---|---|---|---|---|
| 1 | 0.44 | 1.24 | 1.21 | 1.37 | 1.07 |
| 2 | 0.73 | 0.55 | 0.85 | 0.51 | 0.66 |
| 3 | 0.60 | 0.57 | 0.83 | 0.66 | 0.67 |
| 4 | 0.79 | 0.89 | 0.87 | 0.72 | 0.82 |
| 5 | 0.56 | 0.60 | 1.04 | 0.75 | 0.74 |
| 6 | 0.56 | 0.56 | 0.64 | 0.68 | 0.61 |
| 7 | 0.86 | 0.70 | 1.37 | 1.02 | 0.99 |
| 8 | 1.42 | 1.94 | 1.95 | 1.61 | 1.73 |
| 9 | 1.74 | 2.14 | 2.40 | 1.86 | 2.04 |
| 10 | 1.19 | 1.13 | 1.81 | 1.51 | 1.41 |
| 11 | 0.98 | 1.00 | 1.42 | 1.25 | 1.16 |
| 12 | 0.88 | 0.92 | 1.31 | 1.11 | 1.06 |
| 13 | 0.67 | 0.55 | 0.81 | 0.52 | 0.64 |
| 14 | 1.48 | 1.49 | 1.56 | 1.61 | 1.54 |
| 15 | 1.00 | 1.40 | 1.13 | 1.31 | 1.21 |

* Expressed as the monohydrate.

These results support the data of Pucher, *et al.* (*5*) and indicate that the average male adult's saliva may contain 0.50–2.00 mg. per cent citric acid. In nearly every case, results of triplicate analyses were quite consistent. In addition, samples taken every two hours throughout the day were essentially consistent, except perhaps for the slightly higher values for the 1 P.M. specimens.

Further studies are contemplated, particularly on the relation of salivary citric acid to dental erosion and dental caries. Decalcification of dental tissues by citrate ion is suggested by the observation that calcium and citrate form a soluble, slightly ionized complex (*6*). Also, it has been observed in previous studies from this laboratory that citrate in practically neutral drinking fluids has a pronounced destructive action on dental tissues *in vivo* (*3*).

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