Invitation for Nominations for 1968
American Institute of Nutrition Awards

Nominations are requested for the 1968 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed. (2) *A brief convincing statement setting forth the basis for the nomination and, where appropriate, a selected bibliography which supports the nomination. Seconding or supporting letters are not to be submitted.* (3) Five copies of the nominating letter must be sent to the chairman of the appropriate nominating committee *before October 1, 1967,* to be considered for the 1968 awards.

General regulations for A.I.N. awards. Membership in the American Institute of Nutrition is not a requirement for eligibility for an award and there is no limitation as to age except as specified for the Mead Johnson Award. An individual who has received one Institute award is ineligible to receive another Institute award unless it is for outstanding research subsequent to or not covered by the first award. A Jury of Award composed of A.I.N. members, which makes final selection and remains anonymous, may recommend that an award be omitted in any given year if in its opinion the work of the candidates nominated does not warrant the award. An award is usually given to one person, but, if circumstances and justice so dictate, a Jury of Award may recommend that any particular award be divided between two or more collaborators in a given research.

Presentation of awards will be made during the banquet at the annual meeting.

1968 Borden Award in Nutrition

The Borden Award in Nutrition, consisting of $1000 and a gold medal, is made available by the Borden Company Foundation, Inc. The award is given in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of milk or its components. The award will be made primarily for the publication of specific papers during the previous calendar year, but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time not necessarily including the previous calendar year. Employees of the Borden Company are not eligible for this award nor are individuals who have received a Borden Award from another administering association unless the new award be for outstanding research on a different subject or for specific accomplishment subsequent to the first award.

Former recipients of this award are:

1944 – E. V. McCollum	1955 – A. G. Hogan
1945 – H. H. Mitchell	1956 – F. M. Strong
1946 – P. C. Jeans and	1957 – no award
Genevieve Stearns	1958 – L. D. Wright
1947 – L. A. Maynard	1959 – H. Steenbock
1948 – C. A. Cary	1960 – R. G. Hansen
1949 – H. J. Deuel, Jr.	1961 – K. Schwarz
1950 – H. C. Sherman	1962 – H. A. Barker
1951 – P. György	1963 – Arthur L. Black
1952 – M. Kleiber	1964 – G. K. Davis
1953 – H. H. Williams	1965 – A. E. Harper
1954 – A. F. Morgan and	1966 – R. T. Holman
A. H. Smith	1967 – R. H. Barnes

Nominating Committee:

P. H. Weswig, *Chairman*
K. E. Harshbarger
R. T. Holman

Send nominations to:

Dr. P. H. Weswig
Department of Agricultural Chemistry
Oregon State University
Corvallis, Oregon 97331

1968 Osborne and Mendel Award

The Osborne and Mendel Award of $1000 and an inscribed scroll has been established by the Nutrition Foundation, Inc., for the recognition of outstanding recent basic research accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the

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most significant published contribution in approximately the calendar year preceding the annual meeting of the Institute, or who has published recently a series of papers of outstanding significance. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration.

Former recipients of this award are:

1949 – W. C. Rose	1959 – Grace A. Goldsmith
1950 – C. A. Elvehjem	1960 – N. S. Scrimshaw
1951 – E. E. Snell	1961 – Max K. Horwitt
1952 – Icie Macy Hoobler	1962 – William J. Darby
1953 – V. du Vigneaud	1963 – James B. Allison
1954 – L. A. Maynard	1964 – L. Emmett Holt, Jr.
1955 – E. V. McCollum	1965 – D. M. Hegsted
1956 – A. G. Hogan	1966 – H. H. Mitchell
1957 – G. R. Cowgill	1967 – Samuel Lepkovsky
1958 – P. György	

NOMINATING COMMITTEE:

ALEX BLACK, *Chairman*
L. E. HOLT, JR.
M. K. HORWITT

Send nominations to:

DR. ALEX BLACK
Pennsylvania State University
Agricultural Experiment Station
University Park, Pennsylvania 16802

1968 Mead Johnson Award for Research in Nutrition

The Mead Johnson Award of $1000 and an inscribed scroll is made available by Mead Johnson and Company to an investigator who has not reached his 46th birthday during the calendar year in which the Award is given. Selection by the Jury of Award will be based primarily on a single outstanding piece of research in nutrition published in the year preceding the annual meeting or on a series of papers on the same subject published within not more than the three years preceding the annual meeting.

Former recipients of this award are:

1939 – C. A. Elvehjem	1947 – W. J. Darby
1940 – W. H. Sebrell, Jr.	P. L. Day
J. C. Keresztesy	E. L. R. Stokstad
J. R. Stevens	1948 – F. Lipmann
S. A. Harris	1949 – Mary S. Shorb
E. T. Stiller	K. Folkers
K. Folkers	1950 – W. B. Castle
1941 – R. J. Williams	1951 – no award
1942 – G. R. Cowgill	1952 – H. E. Sauberlich
1943 – V. du Vigneaud	1964 – J. S. Dinning
1944 – A. G. Hogan	1965 – J. G. Bieri
1945 – D. W. Woolley	1966 – M. Daniel Lane
1946 – E. E. Snell	1967 – W. N. Pearson

NOMINATING COMMITTEE:

A. R. KEMMERER, *Chairman*
L. M. HENDERSON
H. E. SAUBERLICH

Send nominations to:

DR. A. R. KEMMERER
University of Arizona
Agricultural Sciences Building
Tucson, Arizona 85721

1968 Conrad A. Elvehjem Award for Public Service in Nutrition

The Conrad A. Elvehjem Award for Public Service in Nutrition, consisting of $1000 and an inscribed scroll, is made available by the Wisconsin Alumni Research Foundation. The award is bestowed in recognition of distinguished service to the public through the science of nutrition. Such service, primarily, would be through distinctive activities in the public interest in governmental, industrial, private, or international institutions but would not exclude, necessarily, contributions of an investigative character.

Former recipients of this award are:

1966 – C. Glen King	
1967 – J. B. Youmans	

NOMINATING COMMITTEE:

F. W. QUACKENBUSH, *Chairman*
R. E. SHANK
OLAF MICKELSEN

Send nominations to:

DR. FORREST W. QUACKENBUSH
Department of Biochemistry
Purdue University
Lafayette, Indiana 47907

Invitation for Nominations for 1968 American Institute of Nutrition Fellows

The Fellows Committee of the American Institute of Nutrition invites nominations for Fellows in the Society. Eligible candidates are active or retired members of the Society who have passed their sixty-fifth birthday (by the time of the annual meeting) and who have had distinguished careers in nutrition. Up to three Fellows may be chosen each year.

Nominations may be made to the Chairman of the Fellows Committee by any member of the Society, including members of the Committee.

Nominations (in 5 copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable.

Final selection will be made by the Fellows Committee and a suitable citation will be presented at the Annual Dinner in April.

Fellows Committee:

W. H. GRIFFITH, *Chairman*
AGNES F. MORGAN
RICHARD M. FORBES
T. H. JUKES
L. A. MAYNARD

Send nominations to:

DR. W. H. GRIFFITH
*Federation of American Societies for
    Experimental Biology*
9650 Rockville Pike
Bethesda, Maryland 20014

The following persons have been elected previously as Fellows of the Society:

Georgian Adams (1967)
J. B. Brown (1964)
Thorne M. Carpenter (1958)
George R. Cowgill (1958)
Earle W. Crampton (1967)
Henrik Dam (1964)
Eugene F. DuBois (1958)
R. Adams Dutcher (1961)
Ernest B. Forbes (1958)
Casimir Funk (1958)
Wendell H. Griffith (1963)
Paul György (1965)
Albert G. Hogan (1959)
L. Emmett Holt, Jr. (1967)
Icie Macy Hoobler (1960)
Paul E. Howe (1960)
J. S. Hughes (1962)
C. Glen King (1963)
Max Kleiber (1966)

Samuel Lepkovsky (1966)
Leonard A. Maynard (1960)
Elmer V. McCollum (1958)
Harold H. Mitchell (1958)
Agnes Fay Morgan (1959)
John R. Murlin (1958)
Leo C. Norris (1963)
Helen T. Parsons (1961)
Lydia J. Roberts (1962)
William C. Rose (1959)
W. D. Salmon (1962)
Arthur H. Smith (1961)
Genevieve Stearns (1965)
Harry Steenbock (1958)
Hazel K. Stiebeling (1964)
Raymond W. Swift (1965)
Robert R. Williams (1958)
John B. Youmans (1966)

Invitation for
Nominations for Honorary Membership in the American Institute of Nutrition

The Committee on Honorary Memberships of the American Institute of Nutrition invites nominations for Honorary Members.

Distinguished individuals of any country who are not members of the American Institute of Nutrition and who have contributed to the advance of the science of nutrition shall be eligible for proposal as Honorary Members of the Society.

Nominations may be made to the Chairman of the Committee on Honorary Memberships by two members of the Society.

Nominations (in three copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable but not necessary.

Final selection of nominees will be made by the Council of the American Institute of Nutrition and such nominations submitted to the Society at the spring meeting. Election requires a two-thirds majority of the ballots cast.

Honorary members pay no membership fees but are eligible to subscribe to the official journal(s) at member's rates.

Committee on Honorary Memberships:

> GRACE GOLDSMITH, *Chairman*
> R. W. ENGEL
> L. C. NORRIS

Send nominations to:

> DR. GRACE GOLDSMITH
> *Tulane University School of Medicine*
> *New Orleans, Louisiana 70112*

The following persons have been elected previously as Honorary Members of the Society:

Kunitaro Arimoto	Toshio Oiso
W. R. Aykroyd	H. A. P. C. Oomen
Frank B. Berry	Lord John Boyd Orr
Edward Jean Bigwood	Conrado R. Pascual
Frank G. Boudreau	V. N. Patwardhan
Robert C. Burgess	Sir Rudolph A. Peters
Dame Harriette Chick	B. S. Platt
F. W. A. Clements	Emile F. Terroine
Sir David P. Cuthbertson	Jean Tremolieres
Herbert M. Evans	Eric John Underwood
Joachim Kühnau	Artturi I. Virtanen
Joseph Masek	

Invitation for Nominations for the 1968 Hoblitzelle National Award in the *Agricultural Sciences*

The Hoblitzelle Award Committee of the American Institute of Nutrition invites nominations for the 1968 Hoblitzelle National Award in the Agricultural Sciences.

The Hoblitzelle Award, consisting of $10,000, a gold medal and an attesting certificate, is presented in recognition of the outstanding contribution in the agricultural sciences which has been published during the preceding four-year period. The donor of the Award is the Hoblitzelle Foundation of Dallas, Texas. The Award is administered by the Texas Research Foundation, Renner, Texas.

All American scientists working in the United States and its territories, irrespective of creed, color, nationality, sex, age, branch of science, or affiliation with scientific or scholastic organizations, are eligible. Either an individual or team of scientists may be nominated for the Award, but a team must include only those scientists who have contributed the basic ideas. Preferably no more than two scientists should be nominated as a team.

This Award covers research in agronomy, animal science, bacteriology, biochemistry, botany, entomology, genetics, horticulture, nutrition, soil science, veterinary science, zoology, and such other sciences as may be deemed to serve agriculture in their broadest aspects.

The various professional societies related to the agricultural sciences, the Agricultural Research Service and the Agricultural Experiment Stations have been requested to receive and screen nominations for the Award. Each may forward three nominations to the Texas Research Foundation from which the Final Awards Committee will select three candidates. The Hoblitzelle Foundation will select the recipient, or recipients, from these candidates.

The nomination for the Award must be accompanied by a complete set of publications by the scientist covering his scientific accomplishment, and a comprehensive evaluation of the potential significance of the work. The dates of publications for the 1968 Award must fall within the period January 1, 1963 through December 31, 1966. Final date for submission of nominations is November 15, 1967.

AIN Hoblitzelle Award Committee:

> ORVILLE G. BENTLEY, *Chairman*
> R. W. ENGEL
> R. G. HANSEN
> H. O. KUNKEL

Send nominations to:

> DR. ORVILLE G. BENTLEY
> *Dean of Agriculture*
> *University of Illinois*
> *Urbana, Illinois 61803*

Fatty Acid Composition and Weights of Organs from Essential Fatty Acid-deficient and Non-deficient Hens

HENRY MENGE

United States Department of Agriculture,[1] Beltsville, Maryland

ABSTRACT   Fatty acid analyses and relative weights of organs from hens severely depleted of linoleic acid were made and compared with those obtained from corresponding organs from non-deficient hens. Forty essential fatty acid (EFA)-deficient Leghorn pullets were selected at 23 weeks of age and placed into 2 groups of 20 pullets each. Group 1 was fed a purified EFA-deficient diet, and group 2 was given the same diet supplemented with corn oil calculated to supply 4% linoleic acid (18:2). The control group (group 3) was composed of 20 birds selected at random from pullets fed a practical-type diet since hatching. At 23 weeks group 3 was given a practical-type diet containing sufficient corn oil to supply 4% 18:2. The hens were killed after a 32-week experimental period (55 weeks of age). Group 1 exhibited every indication of a severe EFA deficiency, namely, significantly smaller body weight, repressed egg weight and production, zero hatchability, depressed feed consumption, high mortality, and a fatty acid profile indicative of an EFA deficiency in the lipids of all organs analyzed with the exception of the pituitary. The spleen, pituitary, pineal, thyroid, and adrenals of the EFA-deficient hens weighed significantly more than corresponding organs from non-deficient hens (groups 2 and 3). This increase in weight was regarded as a reflection of the severe dietary stress imposed on the hens. The fatty acid composition of the tissue lipids from group 2 differed only slightly from that of group 3, even though egg production, egg weight, and hatchability of fertile eggs were significantly lower in group 2 as compared with that of group 3. These results demonstrated that the purified diet lacked a factor(s) other than 18:2 that was necessary for optimal reproduction.

The deficiency of essential fatty acids (EFA) has been demonstrated to have a pronounced effect on reproduction in the female chicken resulting in zero hatchability of fertile eggs and significantly reduced egg size and production (1–4). The same studies have also shown that hens receiving an EFA-deficient diet exhibit marked changes in the fatty acid composition of plasma (1, 3, 4), heart (1, 4), liver (1), and yolk (3) lipids. The present study was designed to extend the fatty acid analyses to include the gallbladder, pancreas, kidney, spleen, ovary, brain, pituitary, pineal, thyroid, and adrenal lipids from EFA-deficient hens, together with the weights of these organs, and to compare these data with those obtained from corresponding organs from non-deficient hens.

EXPERIMENTAL PROCEDURE

Seventy-five White Leghorn pullets were reared to 2 weeks with a corn-soy diet. At this time, 50 of the pullets were fed an EFA-deficient casein-gelatin diet (5), and the remaining 25 were fed a practical-type diet until the pullets were 23 weeks old. At 23 weeks, the EFA-deficient pullets were placed at random into 2 groups of 20 birds each and fed the experimental diets for 32 weeks. Group 1 received a purified EFA-deficient casein-gelatin layer diet (5). Group 2 received the purified diet with corn oil[2] (6.92%) added to supply 4% linoleic acid (18:2). Group 3, which was composed of 20 pullets that had received the practical-type diet throughout the growing period was continued with this diet to which corn oil (5.0%) was added to supply 4% 18:2.

Six hens selected at random from each group were killed after an experimental period of 32 weeks, or when the birds were 55 weeks of age. Blood samples were collected and the following organs were excised from each hen: heart, liver, gall-

TABLE 1

Unsaturated fatty acid composition of plasma and organ lipids of essential fatty acid-deficient and non-deficient hens [1]

Groups	1	2	3
Diet	Purified	Purified	Corn-soy
Linoleic acid. %	0.0	4.0	4.0
	%	%	%

Plasma

	1	2	3
18:1	54.4	33.4	37.5
18:2	tr [2]	25.4	21.2
18:3	0.9	0.1	0.4
20:3 [3]	6.8	0.0	0.0
20:4	tr [2]	2.8	2.3

Pancreas

	1	2	3
18:1	51.7	34.4	21.5
18:2	0.2	23.6	27.3
18:3	0.7	tr [2]	0.2
20:3 [3]	7.2	0.0	0.0
20:4	0.9	4.0	7.3

Brain

	1	2	3
18:1	27.4	21.4	21.5
18:2	tr [2]	1.0	1.0
18:3	tr [2]	0.5	0.6
20:3 [3]	13.0	0.0	0.0
20:4	2.4	10.6	9.4
20:5ω3 [4]	2.1	4.2	3.8
22:5ω3 [4]	tr [2]	2.7	1.5
22:6ω3 [4]	tr [2]	4.2	6.4

Heart

	1	2	3
18:1	40.5	22.6	22.5
18:2	0.4	29.5	29.6
18:3	0.6	0.3	0.5
20:3 [3]	12.2	0.0	0.0
20:4	1.6	9.8	5.8

Kidney

	1	2	3
18:1	46.2	25.5	24.4
18:2	0.4	24.3	26.0
18:3	0.8	0.1	0.2
20:3 [3]	8.6	0.0	0.0
20:4	1.0	6.7	6.8

Pituitary

	1	2	3
18:1	30.6	44.6	34.6
18:2	13.1	14.2	17.2
18:3	0.0	0.0	0.0
20:3 [3]	tr [2]	0.0	0.0
20:4	6.2	4.9	4.1
20:5ω3 [4]	0.0	0.0	0.0
22:5ω3 [4]	0.0	0.0	0.0
22:6ω3 [4]	0.0	0.0	0.0

Liver

	1	2	3
18:1	45.2	27.7	34.6
18:2	0.2	23.0	20.8
18:3	0.8	0.2	0.2
20:3 [3]	9.4	0.0	0.0
20:4	0.9	7.3	2.3

Spleen

	1	2	3
18:1	34.9	21.9	25.5
18:2	0.4	16.5	15.6
18:3	1.1	0.1	tr [2]
20:3 [3]	14.0	0.0	0.0
20:4	4.4	11.9	10.8

Pineal

	1	2	3
18:1	34.6	26.4	30.0
18:2	1.8	16.8	12.5
18:3	0.0	1.3	0.0
20:3 [3]	9.6	0.0	0.0
20:4	0.8	2.5	3.7
20:5ω3 [4]	0.0	0.0	0.0
22:5ω3 [4]	0.0	0.0	0.0
22:6ω3 [4]	0.0	0.0	0.0

Bile

	1	2	3
18:1	48.4	30.0	24.3
18:2	0.2	19.7	21.4
18:3	0.9	0.2	0.2
20:3 [3]	10.3	0.0	0.0
20:4	0.4	7.2	7.2

Ovary

	1	2	3
18:1	47.4	31.4	29.4
18:2	tr [2]	22.3	19.4
18:3	1.7	0.5	0.5
20:3 [3]	10.7	0.0	0.0
20:4	0.8	6.1	8.3

Thyroid

	1	2	3
18:1	58.8	42.1	31.0
18:2	tr [2]	17.7	21.0
18:3	0.8	0.0	tr [2]
20:3 [3]	2.1	0.0	0.0
20:4	tr [2]	1.3	1.4
20:5ω3 [4]	tr [2]	0.0	0.0
22:5ω3 [4]	0.0	0.0	0.0
22:6ω3 [4]	0.0	0.0	0.0

Adrenal

	1	2	3
18:1	51.6	35.8	32.2
18:2	tr [2]	16.8	18.9
18:3	tr [2]	0.2	0.2
20:3 [3]	6.2	0.0	0.0
20:4	0.2	10.3	8.0
20:5ω3 [4]	tr [2]	0.0	0.0
22:5ω3 [4]	0.0	0.0	0.0
22:6ω3 [4]	0.0	0.0	0.0

[1] Each value represents average of 6 determinations. Hens killed after experimental period of 32 weeks (55 weeks of age).
[2] Trace amount present less than sensitivity of apparatus.
[3] The 20:3 acid is tentatively identified as the 5,8,11-isomer of eicosatrienoic acid.
[4] Tentative identification, see Experimental Procedure.

bladder, pancreas, kidney, spleen, ovary, brain, pituitary, pineal, thyroid, and adrenal. The larger organs were weighed and recorded as grams of tissue per 100 g body weight. The remainder were dissected carefully, weighed, and recorded as milligrams of tissue per 100 g body weight. Fatty acid analyses of plasma and tissue lipids from each hen were made individually. Averages of these individual samples are given in table 1. Methods for determination of plasma and tissue lipid fatty acids have been described in a previous paper (6). When no standards were available, tentative identification was based on carbon numbers. All data were subjected to statistical analysis.

RESULTS AND DISCUSSION

The data summarized in table 1 show the percentage of total fatty acids present in the lipids of the tissues from groups 1, 2, and 3 after a 32-week experimental period. All of the tissues from group 1 (18:2-deficient), with the exception of the pituitary, exhibited a fatty acid profile indicative of a severe EFA deficiency. This deficiency was also responsible for the significant repression of reproductive characteristics (table 2).

A deficiency of dietary EFA results in the synthesis of substantial amounts of eicosatrienoic (20:3) which is incorporated into most of the tissues of the experimental animal. Witting et al. (7) were unable to show any accumulation of 20:3 in the brain lipid of rats fed an EFA-deficient diet. Later, Mohrhauer and Holman (8) observed that severe dietary restrictions of EFA were necessary to show any detectable changes in the brain lipids of rats. The results of the present study show, however, that the pituitary of the chicken is more resistant to changes in 18:2, 20:3 and arachidonic (20:4) content than brain lipid under a severe EFA-deficient regimen (table 1). Eicosapentaenoic (20:5ω3), docosapentaenoic (22:5ω3), and docosahexanenoic (22:6ω3) were observed only in the brain lipids. The brain lipid from group 1 (18:2-deficient) contained less 20:5ω3 than that from groups 2 and 3. It also contained only traces of 22:5ω3 and 22:6ω3 in comparison with higher percentages of these acids in the brain lipid from groups 2 and 3. The significance of these observations cannot be explained at present, but it is a reflection of the severe EFA-deficient regimen.

The fatty acid composition of the tissue lipids from group 2 (purified diet plus 4% 18:2) differed only slightly from that of group 3 (practical-type diet containing 4% 18:2). Differences were noted in the oleic (18:1) content of the liver, bile, pancreas, pituitary, and thyroid lipids, and in the percentage of 20:4 in the liver and pancreas lipids (table 1). The significant differences in egg production, egg weights, and hatchability observed in group 2 in comparison with group 3 (table 2) cannot be explained on the basis of the fatty acid composition of the tissues since a deficiency of EFA did not exist in these groups. Jensen and Shutze (9) reported a decrease in production, egg weights, and percentage hatchability of fertile eggs from hens receiving a purified diet containing 5% safflower oil in comparison with hens receiving a corn-soy diet. They interpreted their results as indicative of the operation of factor(s) other than EFA. The results of the present study show that the purified

TABLE 2

Egg production, egg weights and hatchability of fertile eggs from EFA-deficient and non-deficient hens [1]

	Groups	1	2	3
	Diet	Purified	Purified	Corn-soy
	Linoleic acid, %	0.0	4.0	4.0
Production, %		19.2 [a]	68.4 [b]	74.4 [c]
Egg weight, g		37.0 [a]	50.4 [b]	55.7 [c]
Hatchability, %		0.0 [d]	78.0 [a]	93.4 [b]

[1] Means with different superscripts are significantly different at the 1% level according to Duncan's multiple range test (11).

diet plus 4% 18:2 lacks a factor(s) necessary for optimal reproductive performance of the hen (table 2). Further evidence for the existence of this factor(s) in the practical-type diet and its absence from the purified diet is presented in table 3. The daily average intake of protein, calories, and 18:2 per hen was almost identical for groups 2 and 3, yet, as demonstrated in table 2, egg production, egg weight, and hatchability of fertile eggs were significantly greater with the practical-type diet than with the purified diet.

The effect of the deficiency of 18:2 on feed consumption was very pronounced in group 1 (table 3). Feed records indicated that average daily consumption per hen decreased slowly and steadily throughout the 32-week experimental period. The deficiency of 18:2 also had a dramatic effect on percentage mortality in group 1 (table 3). This depressed feed intake and increased mortality was the result of the severe dietary stress imposed on the hens, and can be considered as part of the EFA-deficiency syndrome. The average body weights and the weights of organs excised from hens in groups 1, 2, and 3 are summarized in table 4. Significant differences in average body weights existed between all groups. The difference in body weights and reproductive traits (table 2) between groups 2 and 3 may be attributed to a dietary deficiency hitherto unknown (9). The significantly smaller body weights, egg production, egg weight, and zero hatch-

TABLE 3

Nutrient intake and mortality of EFA-deficient and non-deficient hens

Groups	1	2	3
Diet	Purified	Purified	Corn-soy
Linoleic acid, %	0.0	4.0	4.0
Feed intake, g [1]	63.3	101.2	102.1
Protein, g	12.7	20.3	20.6
Calories, kcal/100 g	190.0	304.0	306.0
Linoleic acid, g	0.0	4.1	4.1
Mortality, %	55.0(9) [2]	5.0(19)	5.0(19)

[1] Daily average for experimental period (32 weeks).
[2] Numbers in parentheses indicate number of survivors.

TABLE 4

Weights of organs from EFA-deficient and non-deficient hens

Groups	1	2	3	LSD [1]	
Diet	Purified	Purified	Corn-soy	$P = 0.05$	$P = 0.10$
Linoleic acid, %	0.0	4.0	4.0		
	g	*g*	*g*	*g*	*g*
Carcass	1328(9) [2]	1782(19)	2170(19)	230	190
	g/100 g body weight				
Heart	0.289	0.285	0.223	0.085	0.069
Liver	2.19	1.96	2.14	0.75	0.61
Bile	0.04	0.06	0.06	0.03	0.02
Pancreas	0.13	0.12	0.14	0.034	0.028
Kidney	0.42	0.75	0.60	0.24	0.20
Spleen	0.24	0.08	0.08	0.19	0.15
Ovary	0.12	0.33	0.20	0.10	0.08
Brain	0.22	0.19	0.15	0.07	0.06
	mg/100 g body weight			*mg*	*mg*
Pituitary	1.71	0.99	0.84	0.30	0.24
Pineal	0.45	0.18	0.29	0.16	0.13
Thyroid	12.46	8.38	9.00	3.88	3.15
Adrenal	12.50	6.79	5.41	4.12	3.35

[1] Least significant difference values.
[2] Numbers in parentheses indicate number of survivors.

ability of group 1 may be the result of 1) an unknown dietary deficiency, 2) an EFA deficiency, or 3) an irreparable impairment of some biological mechanism as a result of an EFA deficiency.

The spleen, pituitary, pineal, thyroid, and adrenals of group 1 were significantly greater in relative weight than corresponding organs from groups 2 and 3. The hypertrophy of these organs is probably the result of the long-term exposure of the hens to the "stress" of the EFA-deficient diet. Selye (10) has designated these morphologic and functional changes as the "general-adaptation-syndrome," the most prominent change being an enlargement of the adrenal cortex.

The ovaries from group 1, as expected, were significantly smaller compared with the ovaries from groups 2 and 3. The ovaries of three of the six birds from group 1 were regressed. The ova were in various stages of reabsorption.

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In vivo Interference of Zinc with Ferritin Iron in the Rat [1,2]

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ABSTRACT   A series of experiments was conducted to determine the nature of the effect of high zinc diets on iron metabolism. The rats used in all studies were fed a basal diet or the basal + 0.75% zinc. In the initial study radioisotope experiments with ^{59}Fe were conducted to determine whether zinc was interfering with iron absorption. Results indicated that orally administered radioactive iron was absorbed equally by the 2 groups. Subsequent studies showed that the high zinc intake reduced both the amount of ferritin and the percentage of iron content of the ferritin present in the liver. In the next study, the livers of the experimental animals were partitioned and zinc analyses made on the liver fractions. Livers from animals receiving zinc showed that fraction III, the ferritin-containing fraction, was highest in zinc. Iron-induced ferritin synthesis studies showed that ferritin synthesis was not impaired by high dietary zinc. The hypothesis formulated from these studies was that zinc interferes with iron incorporation and release from ferritin.

High levels of dietary zinc (0.4 to 1.0%) have been reported to result in decreased concentrations of liver iron and the development of iron deficiency anemia (1–3), but the means by which zinc brings about these effects is still obscure. In the present study, an attempt was made to gain further insight into the mechanism by which a high zinc intake affects iron metabolism. The effect of zinc on iron concentration in several tissues, on tissue uptake of ^{59}Fe administered orally and intraperitoneally, and on the distribution of iron and zinc in the different iron-containing fractions of the liver was investigated.

EXPERIMENTAL

Male rats of the Holtzman strain from 4 to 6 weeks old were used in all studies. They were maintained on experiment for 5 weeks, according to the experimental regimen of Magee and Matrone (1). The effects of high zinc intake were measured by use of diets supplemented with 0.75% zinc in the carbonate form. Hemoglobin values were determined by the method of Shenk et al. (4) on samples taken at the end of the experimental period. To study the concentration of iron and zinc in the different tissues of the rat, the animals were decapitated, the tissues to be studied were excised, and dry-weight measure-

ments were made on all tissues. All iron and zinc analyses were conducted on material which had been wet-ashed with nitric and perchloric acids, and analyses were made with an atomic absorption spectrophotometer.[5]

The effect of a 0.75% zinc intake on the uptake of iron from the digestive tract was measured by administration of 19 μCi ^{59}Fe using a stomach tube. Eight hours after the administration of the isotope, a blood sample was removed by cardiac puncture followed by the decapitation of the rats and the removal of several tissues. A group of rats was also used to study the distribution of intraperitoneally injected ^{59}Fe. The same procedure was followed as outlined for the oral ^{59}Fe study except that the samples were collected 4 hours after administration of the iso-

TABLE 1

Effect of 0.75% zinc intake on tissue iron levels

	Liver	Kidney	Spleen	Heart	Femur	Testes	Wt gain	Hemoglobin
	μg Fe/g dry tissue			*μg Fe/g dry tissue*			*g*	*g/100 ml blood*
Control[1]	403.7±25.5[2]	174.9±7.2[2]	609.0±43.6[3]	357.0±29.0[2]	83.5±7.1[3]	95.5±4.3[4]	172±10.1[2]	13.04±0.25[2]
0.75% Zn[1]	151.4±30.9	102.1±9.5	502.0±34.6	229.2±21.0	106.4±16.7	67.1±7.5	74±3.6	5.18±0.38

[1] Mean ± SE of 12 observations.
[2] Significant at the 1% level of probability.
[3] Statistically nonsignificant between control and zinc-fed rats.
[4] Statistically significant at the 5% level of probability.

tope. The amount of ^{59}Fe present in the various tissues was determined by use of a gamma scintillation counter.[6]

Heme, hemosiderin and ferritin fractions of the liver were prepared by the procedure of Mazur et al. (5) and of Gabrio et al. (6) with the exception that the ferritin was determined by the antibody technique. The ferritin antibody was prepared and the ferritin-antibody precipitate obtained by the procedure of Judd.[7]

The analysis of variance method was used for the statistical analysis of the data (7) and expression of differences are based on odds of at least 19 to 1.

RESULTS AND DISCUSSION

The addition of 0.75% zinc to the diet of rats caused significant decreases in the iron concentration of the liver, kidney, testes, and heart (table 1). Some indication was obtained that decreased iron levels were also present in the spleen, whereas an increase in concentration was observed in the femur, but these differences were not statistically different. The concentrations of zinc present in these same tissues are shown in table 2. Significant increases were observed in the liver, kidney, spleen, heart, and femur of the rats fed the 0.75% zinc diet. Thus, it appears that a decrease in iron and an increase in zinc occur not only in the liver, as reported previously (1, 3), but also in other tissues of the rat.

One possible means by which a high zinc intake could reduce body iron levels would be by a reduction in iron absorption from the digestive tract. In an attempt to determine whether intestinal absorption was involved, ^{59}Fe was administered by stomach tube, and the amount of ^{59}Fe present in several of the tissues was determined. The results are presented in table 3. The radioactivity present was assumed to represent iron absorbed through the digestive tract. Josephs (8) has reported that when the elemental iron is less than 5 mg, an indication of absorption can be obtained in a study of this

TABLE 2

Effect of 0.75% zinc intake on tissue zinc levels

	Liver	Kidney	Spleen	Heart	Femur	Testes
	μg Zn/g dry tissue			*μg Zn/g dry tissue*		
Control [1]	57.7± 2.6 [2]	94.9± 4.8 [2]	95.3± 3.9 [3]	88.4± 3.5 [2]	153.6±12.3 [2]	180.0± 6.0 [4]
0.75% Zn	513.0±49.4	389.2±41.6	226.7±24.8	133.9±10.4	3378±148	219.8±13.4

[1] Mean ± SE of 12 observations.
[2] Statistically significant at the 1% level of probability.
[3] Statistically significant at the 5% level of probability.
[4] Statistically nonsignificant difference between control and 0.75% zinc-fed rats.

TABLE 3

Distribution of ^{59}Fe given orally and intraperitoneally to control rats and rats fed 0.75% zinc

	Liver	Kidney	Spleen	Heart	Femur	Testes	Blood
Oral [1]	*total ^{59}Fe count/min of tissue*						*^{59}Fe count/min/ml*
Control	217,263	14,840	153,027	3,949	48,078	7,654	27,796
0.75% Zn	176,737	9,411	137,279	5,935	27,325	4,464	48,395
Intraperitoneal [1]							
Control	191,470	11,234	51,940	7,628	17,433	9,026	18,803
0.75% Zn	124,475	19,086	111,184	10,996	38,361	7,180	82,733

[1] Average of 3 observations.

TABLE 4

Distribution of iron in the liver fractions of control rats and rats fed 0.75% zinc

Replication	Heme	Hemo-siderin	Ferritin	Total Fe in liver
	μg Fe/g dry liver (per rat basis)			
Control rats				
1C	46.6	86.7	209.7	343
2C	28.6	68.4	287.9	385
3C	35.2	56.7	166.8	259
4C	25.4	38.4	192.4	256
Mean	34.0	62.6	214.2	311
0.75% Zn rats				
1Z	47.6	47.6	23.8	119
2Z	37.0	41.7	2.3	81
3Z	45.0	55.0	7.0	107
4Z	42.2	18.2	15.6	75
Mean	43.0	40.6	12.2	96

nature. In this study, only 1.5 μg of elemental iron was contained in the administered ^{59}Fe.

The incorporation of ^{59}Fe into the several tissues appeared to follow more or less the same pattern, indicating that absorption from the digestive tract of both groups of experimental animals was approximately the same. Intestinal absorption normally follows the body needs (9) and has been reported to increase as much as 15- to 25-fold as iron needs increase (10).[8] Since the zinc rats had an increased iron need,

as indicated by anemia (5.18 g/100 ml blood), and decreased body iron concentrations, an increase in absorption would be expected. However, no evidence was obtained to indicate that the expected compensatory increase in iron absorption occurred, offering the possibility that excess zinc may in some manner limit the absorption of iron. The distribution of intraperitoneally administered ^{59}Fe to experimental and control rats is also shown in table 3. The relative pattern of distribution with treatment groups was similar except for the femur. Femurs of rats fed the high zinc diet showed a higher relative proportion of radioactivity than those of the controls. Since the zinc-fed rats were anemic, it is presumed that the higher radioactivity in the femurs of these animals was associated with a greater level of hemopoietic activity.

In the next study, the effect of feeding this high zinc diet on the concentration of iron in the different "iron fractions" of the liver was determined. The results are presented in table 4. A highly significant amount (94%) of the decrease in liver iron in the rats fed the high zinc diet is attributable to the ferritin fraction. A sig-

[8] Yu, Chuan-Tao 1955 Utilization of different levels of dietary iron for the synthesis of blood hemoglobin by anemic sheep. M.S. Thesis North Carolina State University at Raleigh.

nificant decrease was also observed in the hemosiderin fraction, but no significant differences were observed in the level of iron in the heme fraction. This agrees with the report of Cox and Harris (11), who observed that when rats were fed a diet containing 0.4% zinc, 77% of the decrease in liver iron was from the ferritin fraction.

The level of ferritin and its iron content present in the livers of control rats and zinc rats are presented in table 5. The iron values obtained for the control rats closely approximate the 20 to 25% normally in ferritin (10) if corrected for the molecular weight (150,000) of the antibody portion (12). These results indicate that high zinc intake reduces both the amount of ferritin and the percentage of iron content of the ferritin present in the liver. Normally, as the iron content decreases, the protein moiety of ferritin breaks down (13, 14), thus maintaining the relatively constant 20 to 25% iron content. This decrease in the percentage of iron of ferritin from the rats fed high zinc may be an indication that a high zinc intake is affecting ferritin, possibly by inhibiting the incorporation of iron into ferritin or by limiting the synthesis of the protein fraction of ferritin. Either or both of these effects would result in a decrease in the level of liver iron stored as ferritin.

In an attempt to gain more insight into the effect of high zinc intake on ferritin, it was decided to test the capability of the rat liver to synthesize in vivo apoferritin using the procedure of Fineberg and Greenberg (13). Essentially the procedure consists of the intraperitoneal injection of 1.5 mg of ferric ammonium citrate for three consecutive days. At the end of 3 days, the livers were removed and ferritin was isolated by the antibody technique. The results are presented in table 6. In the high zinc-fed fed rats the amount of ferritin precipitate per gram of liver was more than doubled compared with levels reported in table 5, whereas the controls showed about a 60% increase in ferritin precipitate. From these results, it appears that the synthesis of apoferritin is not impaired by a high zinc intake.

In the next study, the livers of the experimental animals were fractionated and

TABLE 5

Level of ferritin in the liver of control rats and rats fed 0.75% zinc

Replication	Ferritin-antibody ppt	Iron content of ferritin-antibody ppt
	mg/g liver (per rat basis)	%
Control		
1C	1.06	16.6
2C	1.34	17.7
3C	0.82	16.9
4C	0.93	17.2
Mean	1.02	17.0
0.75% Zn rats		
1Z	0.81	2.87
2Z	0.50	0.46
3Z	0.72	0.97
4Z	0.72	2.11
Mean	0.69	1.74

TABLE 6

Effect of stimulated ferritin synthesis in control rats and rats fed 0.75% zinc

Replication	Ferritin-antibody ppt
	mg/g liver (per rat basis)
Control	
1C	0.93
2C	1.57
3C	2.14
4C	1.85
Mean	1.63
0.75% Zn	
1Z	1.94
2Z	1.40
3Z	2.00
4Z	1.13
Mean	1.60

zinc analyses made to determine the amount of zinc present in the different fractions of the liver and to determine which fraction was most active in accumulation of zinc. The liver fractions used to differentiate the iron compounds were analyzed for zinc. The results are shown in table 7. Fraction I corresponds to the hemosiderin fraction, fraction II contains the heme, and fraction III is the ferritin-containing fraction. There was an increase in zinc concentrations in each of the liver fractions obtained from the zinc-fed animals; however, the greatest increase was

TABLE 7

Distribution of zinc in the liver fractions of control rats and rats fed 0.75% zinc

Rat	Fraction I	Fraction II	Fraction III	Total Zn in liver
	μg Zn/g dry tissue (per rat basis)			
Control rats				
1C	20.8	58.3	0	79.2
2C	14.4	32.6	15.0	62.0
3C	16.3	30.8	0	48.0
4C	17.2	30.8	5.1	58.0
Mean	17.2	38.1	5.0	61.0
0.75% Zn rats				
1Z	47.6	123.8	212.6	384
2Z	56.6	82.6	148.8	288
3Z	35.0	86.8	93.2	214
4Z	49.0	109.6	47.4	206
Mean	47.1	100.7	125.5	373

in fraction III. Only 8.2% of the total liver zinc was present in this fraction from control rats, whereas 46% was present in fraction III (the crude ferritin fraction) for the zinc-fed rats.

Thus, two of the results obtained in these studies support the hypothesis that zinc interferes with iron incorporation and release from ferritin. This hypothesis is based on observations from these studies indicating that the percentage of iron content of isolated ferritin from high zinc-fed animals is reduced to approximately one-tenth that of the controls and that a major portion of the increase in liver zinc in the high zinc-fed rats was in the crude "ferritin fraction." Support for the hypothesis that zinc interferes with iron incorporation into ferritin is found in reports from the in vitro studies of Mazur (15). This investigator indicated that the sulfhydryl groups of ferritin are active in iron accumulation and that binding of these groups with sulfhydryl-reacting reagents (iodoacetamide, o-iodobenzoate and p-chlormercuribenzoate) either inhibit or completely prevent iron uptake by ferritin. In more recent work, Mazur et al. (5) have reported that cadmium, which also has a high affinity for protein sulfhydryl groups, inhibits incorporation of iron into ferritin. It is proposed that since zinc also has a high affinity for sulfhydryl groups (16) and has chemical properties corresponding to cadmium (17), zinc inhibits iron uptake in a manner similar to that of cadmium.

The consequences of an interference of zinc with the normal function of ferritin could be twofold: (a) a decrease in the absorption of iron from the intestinal tract, since ferritin is known to be involved in this process (10, 14); and (b) a decrease in the storage of iron as ferritin. Observations from this study indicate that both of these possibilities occur.

Data in the literature tend to further support these concepts. In studies with high zinc diets, Cox and Harris (3) reported that 77.2% of the decrease in liver iron was in the iron of the ferritin fraction and suggested that this was the result of a greater mobilization of iron from this fraction. However, Shoden et al. (18) and Morgan (19) have reported that normally iron is utilized as readily from either of the two iron storage compounds, ferritin or hemosiderin. The results of the present study indicate that the decreased tissue iron levels are the result of an inhibition of iron incorporation into ferritin or a release from ferritin, or both, rather than of an increased mobilization of ferritin iron. In other studies where zinc diets have been supplemented with iron (1, 20), hemoglobin levels have increased, but increases in tissue levels of iron have never been obtained, providing further evidence that the mechanism of iron storage has been impaired.

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In vivo Effect of Zinc on Iron Turnover in Rats and Life Span of the Erythrocyte [1,2,3]

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ABSTRACT   A series of experiments was conducted with rats to characterize further the effects of high dietary zinc (0.75%) on iron metabolism. It was found that the red blood cells from animals fed a diet high in zinc were irregularly shaped and microcytic and hypochromic. The osmotic fragility of the red blood cells from the zinc-fed rats was greater than that of the control rats. The zinc content of the blood of the former group was more than twice as high as that of the controls. Blood cells of rats fed the diet high in zinc had a life span (utilizing glycine-2-^{14}C) one-third of that of the controls. By means of ^{55}Fe studies, it was shown that iron incorporation into heme was not affected by zinc toxicity. ^{59}Fe studies showed that the greater turnover of red blood cells for the animals fed the zinc-supplemented diet brought about a greater loss of red blood cell iron via the feces. From the results of this study and those of a previous report, a hypothesis was developed rationalizing the effects of zinc toxicity on iron metabolism.

High zinc intakes have been reported to result in the development of anemia (1) which has been suggested, in part at least, to be the result of an effect of zinc on ferritin (2).

In vitro incubations with zinc have been shown to alter the properties of normal red blood cells (3), and other metals have been shown to affect their synthesis (4, 5). The present study was initiated to determine whether the anemia which develops in rats fed high zinc diets was associated with changes in other properties of the red blood cell.

EXPERIMENTAL

Male rats were maintained with the experimental diets as outlined in a previous publication (6).

Red blood cell stains were prepared according to the procedure of Cook (7). Osmotic fragility of the red blood cells was determined by the procedure of Siverd (8). Samples of whole blood, removed from the tail vein of rats were prepared for iron and zinc analyses by wet-ashing in nitric and perchloric acids. All metal analyses conducted in this study were made with an atomic absorption spectrophotometer.[5] Life span of the red blood cells was followed by using the in vivo labeling procedure of Shemin and Rittenberg (9). Nine rats fed the control diet and 8 rats fed a 0.75% zinc diet were injected with 15 µCi of glycine labeled in the methylene carbon. A 0.15-ml sample of blood was removed from the tail vein of a rat at alternate sampling periods to minimize the reduction in activity due to the sampling procedure. Hemin was isolated from the blood (9), and the amount of radioactivity present determined by use of a proportional counter.[6] After counting, the hemin was wet-ashed and an iron analysis made.

The incorporation of radioactive iron into hemin was followed by injecting 10 µCi ^{55}Fe intraperitoneally. Blood samples were collected daily from each rat, the hemin isolated and radioactivity present
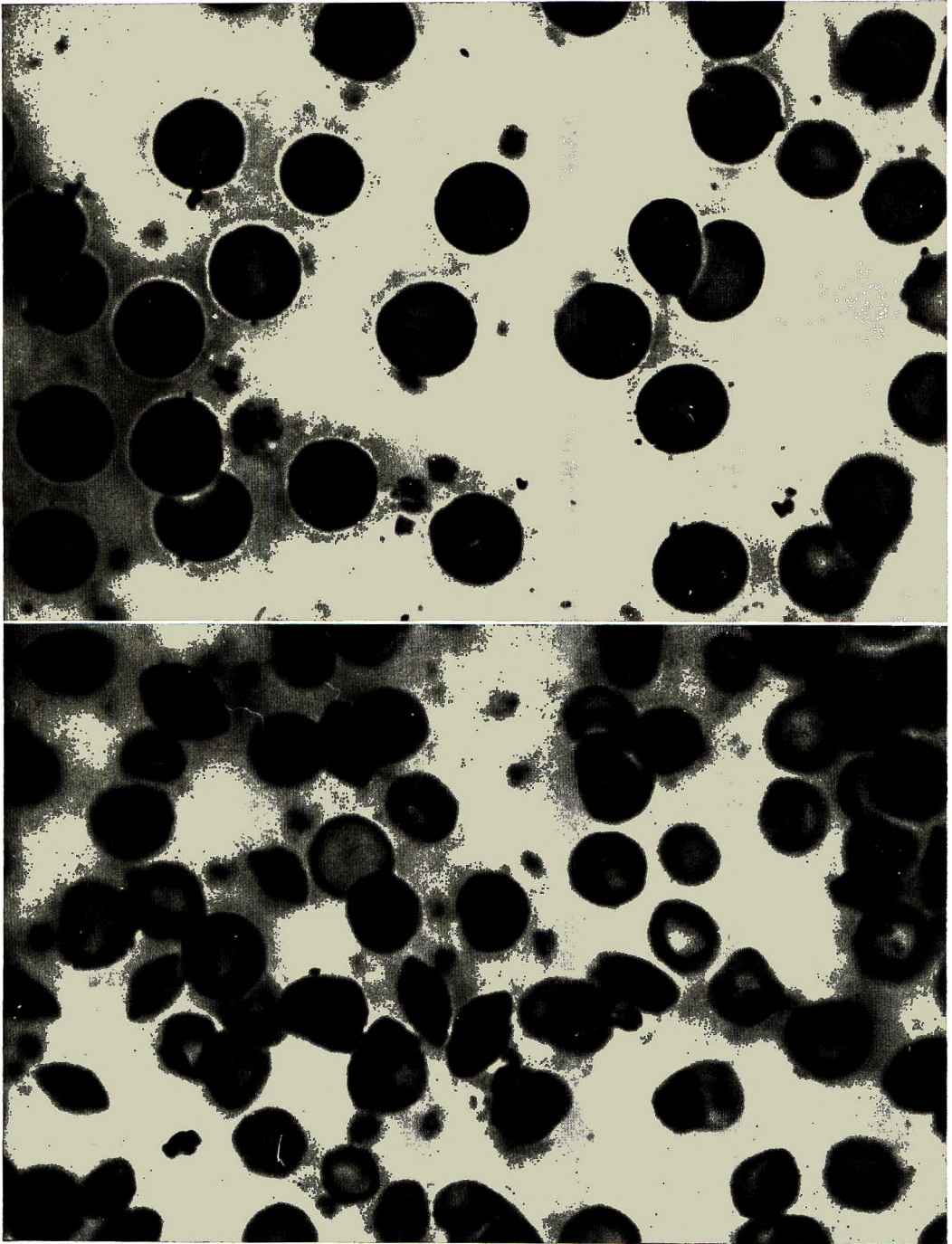
Fig. 1   Blood stains from a control rat (upper) and a rat high zinc (lower).

was determined by use of a proportional counter.[7] The iron content of the hemin was determined as in the life span study.

Measurement of excretion of iron was carried out by intraperitoneal injection of the experimental animals with 10 μCi of ^{59}Fe and counting the radioactivity excreted daily in the feces and urine. The amount of radioactivity persent was determined by use of a gamma scintillation counter.[8]

RESULTS AND DISCUSSION

The effect of a high zinc intake on appearance of red blood cells is illustrated in figure 1. The cells of the control rats were uniform in shape and show the even staining of hemoglobin typical of a normal cell. However, the red blood cells of rats fed a 0.75% zinc diet were irregular in shape and are typical of a microcytic-hypochromic cell.

The osmotic fragility of the red blood cells was determined in the next study. With blood from the control rats, hemolysis first appeared in a salt concentration of 0.40% NaCl, whereas with blood from the zinc-fed rats, hemolysis first appeared in the 0.32% solution. This change in the hemolysis pattern indicates a very definite decrease in osmotic fragility of the red cells. The cause of such a decrease is not known; however, it has been reported that when erythrocytes are incubated in vitro with solutions containing 65 μg of zinc/ml, an increase in osmotic fragility is obtained (3, 10) and is attributed to a large irreversible leakage of potassium from the cell.

The zinc concentration of whole blood was determined on blood removed from the tail vein of the rat. The results of this study are shown in table 1. The amount of zinc in whole blood of the zinc-fed rats increased approximately 90 μg/ml above that of controls, offering the possibility that the in vitro effect reported previously (3) may occur under the in vivo conditions of this study.

Since the red blood cells of the rats fed the zinc diet were shown to be abnormal in appearance and exhibited changes in osmotic fragility, another study was undertaken investigating the effect of high zinc intakes on the life span of the red blood cells. The results of this study are presented in figure 2. The red blood cell life span of the rats fed a high zinc diet was much shorter than that of the controls, as indicated by the sharp decline in specific activity of the hemin between 10 and 20 days, whereas the control rats followed a normal cycle (11). The high zinc intake shortened the life span of the red blood cell approximately one-fifth to one-fourth that of the control animals.

In the next study the effect of high zinc intakes on the rate of incorporation of radioactive iron into heme was investigated since the other metals have been shown to result in a decreased rate of iron incorporation into heme (4, 5). The results are shown in figure 3. The specific activity increased in both the control and high

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[7] See footnote 6.
[8] Gamma Scintillation Counter, Nuclear-Chicago Corporation, Des Plains, Illinois.

TABLE 1

Concentration of iron and zinc in the blood of control and high zinc-fed rats

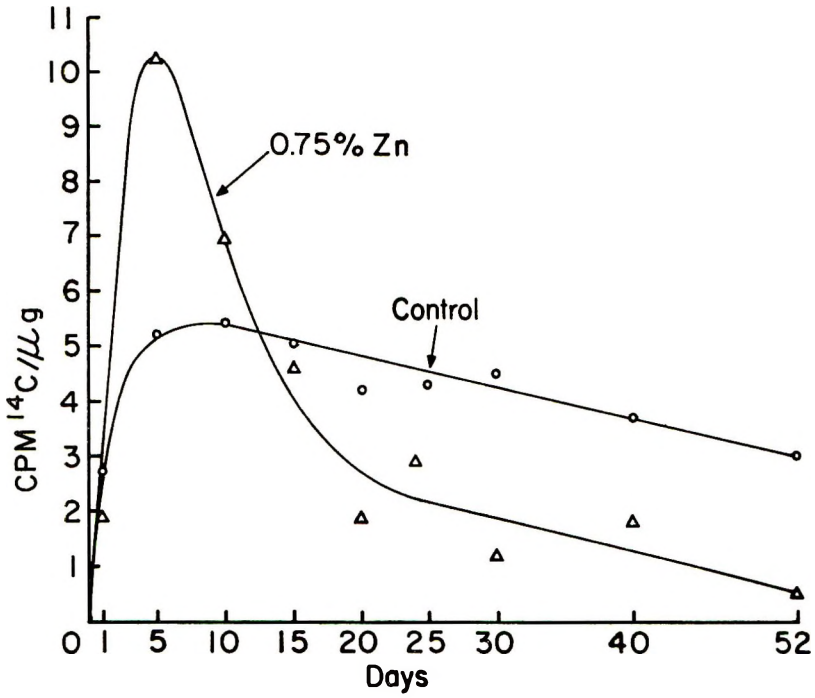| Rat	Hemoglobin	Weight	Blood Zn conc	Blood Fe conc
	g/100 ml blood	*g*	*μg/ml*	*μg/ml*
Control				
C-12	14.03	289	67.5	1,550.0
C-16	13.95	356	52.5	1,312.5
C-17	14.55	303	60.0	1,575.0
Mean	14.18	316	60.0	1,479.2
0.75% Zn				
Z-19	6.43	143	155.0	675.0
Z-20	7.03	110	152.0	975.0
Z-21	6.34	150	155.0	825.0
Mean	6.60	134	154.0	825.0

Fig. 2 Carbon-14 activity in hemin following injection of 15 μCi of methylene-labeled glycine ^{14}C; $\bigcirc$ = control (each point is mean of 5 rats); $\triangle$ = rats fed high zinc (each point is mean of 4 rats).
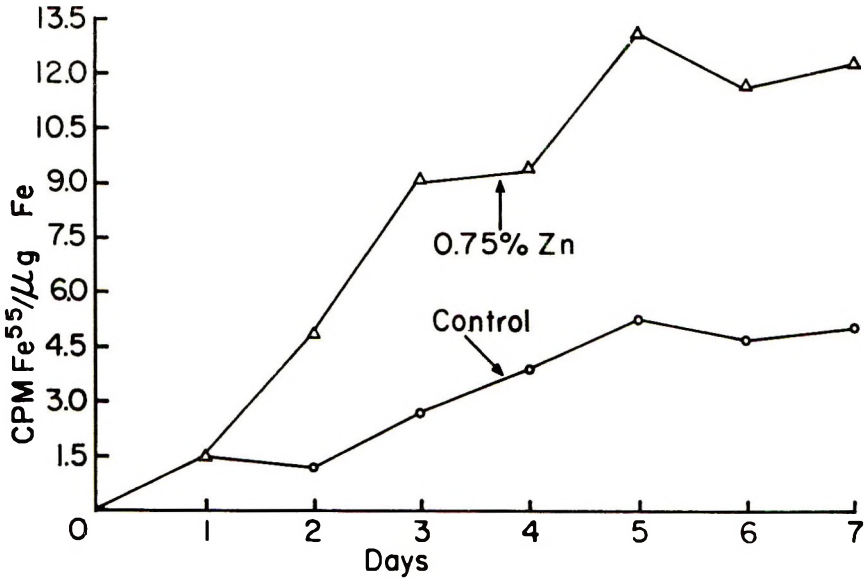
Fig. 3 Incorporation of ^{55}Fe into hemin.

zinc-fed rats during the first 5 days. This follows the pattern reported by Hahn et al. (12). The rate of incorporation was considerably higher for the zinc-fed rats. This greater rate is attributed to the fact that the rats were anemic (13). In any case these results suggest that incorporation of iron into heme was not impaired.

As previously described, the rats fed a high zinc diet have a shortened red blood cell life span or, in other words, a faster turnover of red blood cell iron. Since a portion of the red blood cell iron is excreted with each turnover (14), the decrease in tissue iron, which is observed in the high zinc-fed (2) may in part be a result of this faster turnover rate. To determine whether, in fact, iron excretion is affected, ^{59}Fe was injected (intraperitoneally) into control rats and rats fed the high zinc diet, and feces and urine were collected. The urine was found to contain no radioactivity. The average excretion of ^{59}Fe in the feces is shown in figure 4.

The pattern of ^{59}Fe excretion in the feces obtained from the control rats is similar to that reported by Dubach et al. (15) and by Thirayothin and Crosby (16). The average excretion of ^{59}Fe by the zinc-fed rats followed closely the pattern observed for control rats during the first 8 days after injection. Thereafter, a significant increase in ^{59}Fe excretion was observed in contrast with the decrease observed in the feces of the control animals.

This increase in ^{59}Fe excretion by the zinc-fed rats occurs 10 to 12 days after injection; this period of time corresponds to the length of the life span of the red blood cells of these rats. Thus, these results support the hypothesis that the decrease in body iron levels observed when rats are fed high zinc diets is in part associated with a more rapid turnover of the red blood cells.

The mechanism by which a high zinc intake reduces the life span of the red blood cell is not known. Copper is known to be involved in some manner in red blood cell formation (17), and zinc at high dietary levels has been shown to interfere with normal copper metabolism resulting in an abnormal red blood cell synthesis (18). The red blood cell may also be affected by the increased zinc content of the blood of the rats fed high zinc resulting in an abnormal red blood cell, as shown by the in vitro studies of Vincent and Blackburn (3).

The combination of the concepts developed in a previous paper (2) concerning the effect of a high zinc intake on ferritin and the results reported in this study indicate that these levels of zinc affect iron
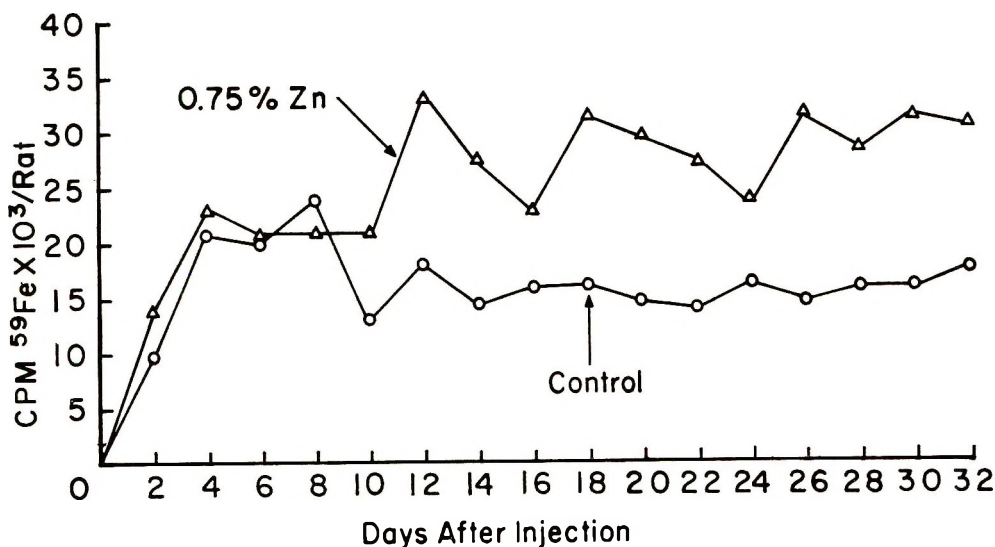

Fig. 4 Excretion of ^{59}Fe in feces of control and high zinc-fed rats (each point is mean of 3 rats).
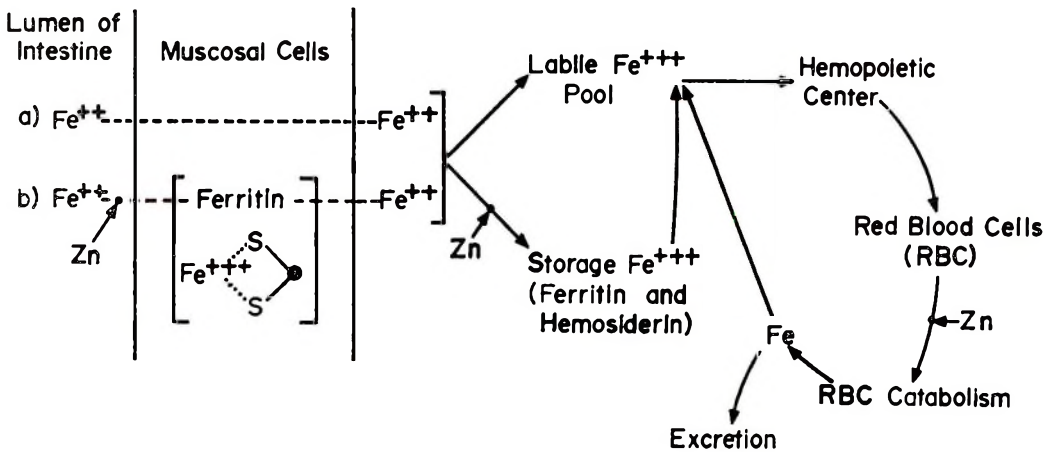
Fig. 5   Pathway of iron through the body with proposed sites of interference by zinc.

metabolism basically in 2 ways. These include (a) an effect of zinc on ferritin such that the incorporation of iron into or release from ferritin is impaired. This would, as discussed in the previous paper (2), impair iron absorption and limit the tissue storage of iron as ferritin. Evidence to support these concepts was presented. And (b) as presented in this paper, the feeding of high zinc diets was observed to result in a shortened life span of the red blood cells resulting in a faster turnover of iron. The points at which zinc is postulated to interfere with iron metabolism are summarized in the scheme shown in figure 5.

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A Growth Factor in Corpus Luteum Powder for Goitrogen-fed Rats [1]

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ABSTRACT   When a commercial preparation of corpus luteum powder was included in the diet, it reinstated growth of growth-arrested, sulfaguanidine-fed rats. A study was made, therefore, to determine whether the growth response to corpus luteum powder was due to thyroid hormones. The corpus luteum powder was hydrolyzed with acid. The acid-insoluble residue was extracted with alcohol and the addition of $AgNO_3$ produced a precipitate which contained a growth factor capable of reinstating growth of growth-arrested, sulfaguanidine-fed and of thyroidectomized rats. One microgram of 3,5,3'-triiodo-L-thyronine produced the same growth response as 3.3 mg of the growth factor preparation. No iodine could be detected on paper chromatograms of the growth factor and the growth factor differed from triiodothyronine in its ability to prevent goiter. Two enzymes were used to compare the growth factor with triiodothyronine. Inosine monophosphate dehydrogenase was inhibited by 10^{-9} M triiodo-L-thyronine but the growth factor had no effect on this enzyme. Adenylosuccinate synthetase was stimulated by 10^{-9} M triiodo-L-thyronine, whereas the growth factor inhibited this enzyme. These results are taken as evidence that corpus luteum contains a growth factor which is capable of substituting for thyroid hormones insofar as growth is concerned but the growth factor is not an iodinated thyronine.

Previous reports (1–5) have presented evidence that certain animal tissues contain growth factors which are capable of reinstating growth of growth-arrested, goitrogen-fed rats. These growth factors appear to be able to substitute for the thyroid hormones insofar as growth is concerned. However, thyroidectomized and goitrogen-fed rats do not appear to require any substance other than thyroid hormones. Therefore, the significance of the growth factors remains obscure. The growth factors appear in such tissues as thymus, duodenum, and pancreas, but not muscle or liver (1). In addition, no tissue tested thus far has been consistently active. This suggested that the growth factors have a specific function and that this function is limited to sex, time in the life of the animal, or to other phenomena.

Our attention was then directed to the possibility that the growth factors appear in certain tissues in conjunction with a particular biological event — possibly pregnancy. This would account for the inconsistency in the activity of various tissues since many of the commercial preparations tested could have been prepared from male animals or from non-pregnant female animals. It would also suggest a function for these growth factors.

When considered from this point of view, it was observed that a commercial preparation of corpus luteum powder [2] was 4 times more active than the duodenal powder used in previously reported experiments (5) and a growth factor preparation could be prepared from this powder in a manner similar to that used for preparing the growth factor from duodenal powder (4, 5). However, it was necessary to establish that the growth factor in corpus luteum powder was not one of the known thyroid hormones and it is the purpose of this report to show that a growth factor is present in corpus luteum powder and that it is not an iodinated thyronine.

MATERIALS AND METHODS

Materials. The sulfaguanidine-containing diet has been described (2). When sulfaguanidine was omitted from the diet, it is referred to in this report as the "nor-

mal" diet. All rats were of the Sprague-Dawley strain.[3] The rats that were fed the sulfaguanidine-containing diet were housed in groups of 6 in screen-bottom cages for 4 weeks. They were then housed individually and weighed every 2 days until growth arrest was established. On day 35 they were used for growth assays. Such rats were 8 weeks of age and weighed 110 to 135 g. No rat that had gained more than 3 g between day 28 and 35 of sulfaguanidine feeding was used for growth assays.

Twelve thyroidectomized-parathyroidectomized male rats (90 to 120 g) were maintained with the normal diet with 1% calcium lactate in the drinking water until growth arrest was established (less than 4 g gain in body weight/week) in six of the rats. This required about 28 days. In all experiments, food and water were supplied ad libitum.

Thyroxine (T_4) was used without further purification, and 3,5,3′-Triiodo-L-thyronine (T_3) [4] was recrystallized from boiling 2 N HCl (6) and dried over $CaSO_4$ (mp 201–203°).

Preparation of the growth factor. Corpus luteum powder [5] (300 g) was suspended in hot (80°) n-butanol and filtered while hot. This was repeated twice and then twice with each of the following boiling solvents: benzene, methanol, water, acetone, and chloroform. The residual powder was suspended in 1200 ml of 6 N HCl containing one gram of Na_2SO_3 and then refluxed for 22 hours during which time N_2 was bubbled through the mixture.

After 2 days at room temperature, the hydrolysate was filtered by gravity. The acid-insoluble residue was suspended in water which was then made approximately 1 M with NaOH. After standing for 3 hours at room temperature, the solution (approx. 250 ml) was adjusted to pH 10 with acetic acid and then filtered to remove an inactive precipitate.

The pH 10 filtrate was adjusted to pH 1 with HCl and after standing at 5° overnight, it was filtered by gravity. The acid-insoluble residue was washed twice by dissolving it in NaOH and precipitating it from acid solution. The acid-insoluble residue was again dissolved in NaOH and after adjusting to pH 4 with acetic acid, cooling

at 5° overnight, it was filtered by gravity. The precipitate on the filter paper was air-dried and then extracted with ethanol for 8 hours in a Soxhlet extractor.

The ethanol extract was evaporated to dryness at room temperature and the residue was dissolved in 100 ml of 0.2 N NaOH. This was brought to pH 1 with HCl and the insoluble precipitate was recovered by filtration. The precipitate was again washed twice by dissolving it in NaOH and precipitating it from acid solution. The washed precipitate was air-dried on filter paper and then extracted with benzene for 2 hours in a Soxhlet extractor.

The benzene-insoluble residue on the filter paper was dissolved in 40 ml of 2 N NaOH and this was extracted 5 times by shaking with 40, 40, 20, 20 and 10 ml of n-butanol. The combined butanol extracts were adjusted to pH 4 with acetic acid to which was added 26 ml of 2% (w/v) of aqueous $AgNO_3$ and 26 ml of methanol. After standing overnight at 5°, a precipitate was collected by centrifugation and washed 3 times by suspension in methanol and centrifugation. To remove the silver and dissolve the growth factor, the precipitate was washed 5 times with 10 ml of methanol:2.0 N HCl (9:1). The combined washings were made to 100 ml with methanol and this solution will be referred to as the growth factor. The solution was bright yellow in color and an air-dried aliquot contained 10.1 mg of dry residue/ml.

Recovery of triiodothyronine-iodine-125 [6] *(T_3-^{125}I) from corpus luteum powder.* This experiment was run to determine how far T_3-^{125}I would be carried through the procedure described above. Corpus luteum powder (300 g) was extracted with organic solvents as described above and just before acid hydrolysis, 200 µCi of T_3-^{125}I were added. After acid hydrolysis, another 20 µCi of T_3-^{125}I were added to the acid insoluble residue that had been dissolved in NaOH. The growth factor solution was prepared as described above and aliquots were removed at various steps for radioactive measurements.

[3] Purchased from Dublin Animal Laboratories, Dublin, Virginia.
[4] T_3 and T_4 were obtained from the Sigma Chemical Company, St. Louis.
[5] See footnote 2.
[6] T_3-^{125}I, 10 mCi/mg, Volk Radiochemical Company, Chicago.

An aliquot of the original T_3-^{125}I and aliquots of the fractions obtained during purification were measured for radioactivity when the purification procedure was completed. Radioactivity was measured in a Nuclear-Chicago Model DS-5, well-type scintillation counter.

Standardization of the growth factor. The growth-promoting potency of the growth factor was compared with T_3. Thirty-six male, growth-arrested sulfaguanidine-fed rats were divided into 9 groups of 4 rats each. Each rat in 6 groups was fed ad libitum 30 g of the sulfaguanidine-containing diet supplemented with 0, 0.5, 1.0, 2.0, 3.0 or 4.0 µg of T_3. Each rat of the other 3 groups received 0.30, 0.40 or 0.50 ml of the growth factor in 30 g of diet. When the rats had consumed the supplemented diet, the sulfaguanidine-containing diet was fed for the remainder of a 14-day period. The 2-week gain in body weight was plotted against the dose of T_3 and the equivalent quantity of the growth factor was estimated from the graph. That quantity of the growth factor or T_3 which produced a body weight gain of 30 g in 2 weeks was defined as "one unit."

Growth response of thyroidectomized rats. To establish that the growth factors would also elicit a growth response in thyroidectomized rats, 6 growth-arrested, thyroidectomized rats were divided into 3 groups of 2 rats each. Each rat was fed 30 g of the normal diet containing: one unit of the growth factor, or 2 units of the growth factor or, normal diet only. The rats were weighed every other day for a 2-week period.

Paper chromatography. Onto separate strips of Whatman 3 MM paper, 2.5 cm wide, were applied: 1) one µg of T_3; 2) 2 µg of T_4; 3) one unit of growth factor containing 1 µg of T_3; and 4) one unit of growth factor. These were developed with methanol:H_2O (1:1) by descending chromatography in the dark at room temperature. When dried, the strips were tested for iodine by the ceric sulfate-arsenious acid procedure of Kono et al. (7).

Goiter prevention assay. The procedure of Dempsey and Astwood (8) was used to test the ability of the growth factor to prevent goitrogen-induced hyperplasia of the thyroid gland. Forty-eight female rats

(110–130 g) were divided into 8 groups of 6 rats each. They were housed individually and fed the normal diet for 3 days. On the fourth day, 7 groups were fed the normal diet containing 0.5% of 2-thiouracil. The last group was continued with the normal diet and served as the normal control group. On the fifth day and every day for 10 days, each rat of 4 groups of thiouracil-fed rats was injected intraperitoneally with 0.0, 0.10, 0.25 or 0.4 µg of T_3/100 g body weight. The other 3 groups of thiouracil-fed rats received 0.2, 0.3 or 0.4 units of the growth factor/100 g body weight/day by intraperitoneal injection. Both T_3 and the growth factor were administered in a volume of 0.3 ml/100 g of body weight.

To avoid the possibility of decomposition, neither the T_3 nor the growth factor were sterilized. However, they were diluted to suitable volumes with sterile saline in sterile containers.

On the eleventh day after the first injection, the rats were killed. The thyroid glands were removed, cleaned of adventitious tissue, and weighed on a Roller-Smith torsion balance to the nearest 0.2 mg.

Effect of the growth factor on inosine-5'-monophosphate dehydrogenase. This enzyme catalyzes the oxidation of inosine-5'-monophosphate (IMP) to xanthine 5'-monophosphate, and it is inhibited by thyroid hormones.[7] This reaction was used to compare the growth factor with T_3. The IMP-dehydrogenase was partially purified from the liver of normal rats [8] and then incubated with IMP, nicotinamide adenine dinucleotide (NAD) and, T_3 or growth factor. The reduction of NAD was followed for 15 minutes at 340 mµ in 1-cm cuvettes of a Beckman model DU spectrophotometer. The reaction is inhibited maximally by 10^{-9} M T_3 (or 0.00065 units/ml). The growth factor was tested at concentrations of 0.000065, 0.00065 and 0.0065 units/ml which would be equivalent to 10^{-10}, 10^{-9} and 10^{-8} M T_3.

Effect of the growth factor on adenylosuccinate synthetase. Thyroid hormones stimulate the synthesis of adenosine-5'-monophosphate (AMP) (9, 10) by stimulating the enzyme adenylosuccinate syn-

[7] Unpublished experiments.
[8] Unpublished experiments.

thetase.[9] This enzyme converts IMP to adenylosuccinate and the effect of the growth factor on this reaction was determined. The enzyme was partially purified from the soluble fraction of livers obtained from normal rats by treatment with $Ca_3(PO_4)_2$ gel, precipitation with $(NH_4)_2SO_4$ (50 to 75% of saturation) and passage through a Sephadex A-50 column. Details of the purification procedure will be described elsewhere. The enzyme and necessary cofactors were incubated with 0.0016 units/ml of T_3 (2.5×10^{-9} M) or 0.00016, 0.0016 or 0.016 units/ml of growth factor. The synthesis of adenylosuccinate was followed at 280 mμ (11) in 1-cm cuvettes of a Beckman Model DU spectrophotometer.

Ashing and treatment with ninhydrin. Four grams of the crude corpus luteum powder were heated at 600° for 3 hours. The residue was taken up in 4 ml of 0.1 N HCl and this was assayed for growth-promoting activity with 2 rats.

To 4 units of the growth factor solution were added 4 ml of methanol containing 60 mg of ninhydrin. This was allowed to stand for 4 hours at room temperature and then assayed for growth promoting activity with 3 rats.

RESULTS AND DISCUSSION

Recovery of T_3-^{125}I. Of the initial 200 μCi (141,216,075 cpm) added to the corpus luteum powder just before hydrolysis, only 7.0% (10,468,775 cpm) were recovered in the acid-insoluble residue after hydrolysis. Of the total T_3-^{125}I added (220 μCi), only 4.6% (7,040,000 cpm) were recovered in the final methanol:HCl solution of the growth factor. The bulk of the radioactivity was in the initial acid filtrate as would be expected because T_3 is relatively soluble in HCl and it is unstable to acid hydrolysis (4). Small quantities of radioactive material (less than 2% of the total added) were recovered in the other fractions. Thyroxine is less soluble than T_3, but it also would be destroyed by acid hydrolysis (12). Haga (12) has reported that 86% of thyroid hormones are destroyed by acid hydrolysis in 3 N HCl for 12 hours.

The radioactivity in the final product probably represents iodinated artifacts produced during acid hydrolysis (13). These data do not provide conclusive evidence that thyroid hormones are absent from the growth factor solution, but serve to indicate that the probability of T_3 appearing in the final product is low.

Standardization of the growth factor. Figure 1 illustrates the growth response of
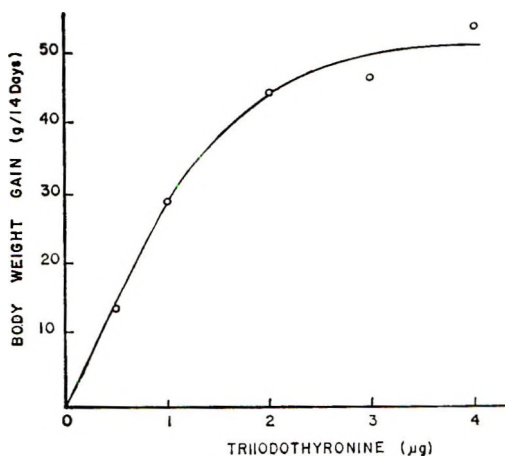

Fig. 1   Growth response of growth-arrested, sulfaguanidine-fed rats to dietary 3,5,3′-triiodo-L-thyronine (T_3). The T_3 was incorporated into 30 g of sulfaguanidine-containing diet and fed ad libitum until it was consumed. Each point is the average growth response of 4 rats. The maximal standard error for any point was ±1.06 g. The growth response to 0.3, 0.4 or 0.5 ml of the growth factor solution was 30, 34, 39 g/2 weeks, respectively. From this, it was calculated that 0.33 ml of the growth factor would elicit a body weight gain of 30 g in 2 weeks and thus, this quantity would be equivalent to 1 μg of T_3.

TABLE 1

Growth response of growth-arrested,[1] thyroidectomized rats to the growth factor

Supplement to diet	Initial body wt [2]	Body wt gain [2]
	g	*g/2 weeks*
None	153, 159	4, 1
Growth factor: [3]		
1 unit [4]	145, 136	28, 31
2 units	132, 157	30, 39

[1] No rat had gained more than 4 g in the 7 days before this experiment.
[2] Two male rats/group. The data for each rat are shown.
[3] The growth factor was incorporated into 30 g of normal diet and fed ad libitum until it was consumed. The normal diet was then fed for the remainder of the 14-day period.
[4] One unit of growth factor is that amount which will elicit a body weight gain of 30 g in 14 days in growth-arrested, sulfaguanidine-fed rats. This is equivalent to 1 μg of 3,5,3′-triiodo-L-thyronine (T_3).

[9] Unpublished experiments.

growth-arrested rats to varying quantities of T_3. One microgram of T_3 produced a gain of 29.8 ± 1.1 g in 2 weeks. Based on the growth responses to the growth factor, it was calculated that 0.33 ml (or 3.29 mg of dry matter) was equivalent to 1 μg of T_3. This quantity was defined as "one unit."

During these investigations, it became evident that the sensitivity of rats to T_3 decreased as the rats grew older. This is evident by comparing the results of previous experiments with the results reported here. In experiments with rats 13 weeks of age, 2.7 μg of T_3 were required to produce a gain of 30 g in 2 weeks (5). For this reason, a unit is defined more explicitly as that quantity of growth factor (or hormone) which will produce a gain of 30 g in 2 weeks in growth-arrested, sulfaguanidine-fed rats, 8 weeks of age.

Growth response of thyroidectomized rats. As summarized in table 1, growth-arrested, thyroidectomized rats also respond to the growth factor. Although the number of rats used in this experiment was limited to conserve the growth factor, the growth response was approximately the same as that of growth-arrested, sulfaguanidine-fed rats, for example, one unit elicited a gain of 29 g in 2 weeks.

These data suggest to us that the growth factor is not an intermediate in the synthesis of thyroid hormone by the thyroid gland, but the growth factor can substitute for the thyroid hormones insofar as growth is concerned.

Paper chromatography. Figure 2 demonstrates that no iodine could be detected in the growth factor preparation and, therefore, the growth factor is not an iodinated compound.

Goiter prevention assay. As shown in figure 3, the ability of T_3 to prevent hyperplasia of the thyroid gland increased linearly with the dose. The projected intercept with the weight of thyroid glands of normal rats was 0.77 μg of T_3/100 g body weight/day, which is somewhat higher than the value of 0.65 μg/100 g body weight/day observed by Gross and Pitt-Rivers (6). However, the growth factor did not produce the same response pattern as the T_3. It tended to prevent hyperplasia but not to the same degree as T_3. The pattern is similar to the response observed

previously with a growth factor obtained from duodenal tissue (3). These results support the conclusion that the growth factor is not one of the known thyroid hormones.

Fig. 2 Paper chromatography of thyroid hormones and the growth factor. Strip 1, 1 μg of T_3 (triiodothyronine). Strip 2, 2 μg of thyroxine. Strip 3, 1 unit of the growth factor plus 1 μg of T_3. Strip 4, 1 unit of the growth factor. Solvent: methanol:water (1:1). The papers were treated with ceric sulfate-arsenious acid as described by Kono et al. (7) to detect the iodinated compounds.
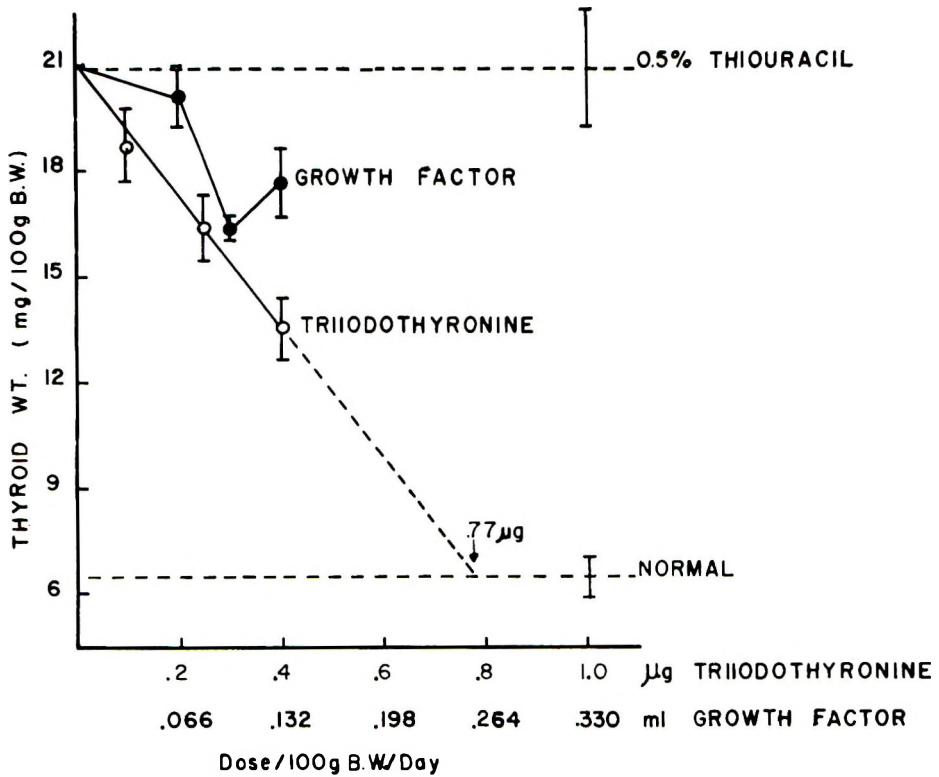
Fig. 3 Comparison of the growth factor with T₃ (triiodothyronine) by the goiter-creven-tion assay. Female rats (110–130 g) were fed a diet containing 0.5% thiouracil for 11 days. The growth factor or T₃ was administered intraperitoneally for 10 days. Each point is the average of 6 rats ± SD. One microgram of T₃ was equivalent to 0.33 ml of the growth factor (see fig. 1). "Normal" represents the relative weight of thyroid glands of rats fed the diet without thiouracil.

Effect of the growth factor on IMP-dehydrogenase. Figure 4 illustrates that the reduction of NAD by this enzyme was markedly inhibited by 10^{-9} M (0.00065 units/ml) T₃. The growth factor had no effect on this reaction over a 100-fold concentration range. We conclude from these results that the growth factor is not T₃.

Effect of the growth factor on adenylo-succinate synthetase. Although 2.5×10^{-9} M (0.0016 units/ml) T₃ stimulated this reaction as expected, the effect of the growth factor was less clear cut (table 2). All 3 concentrations of the growth factor inhibited the reaction but the degree of inhibition was not correlated with the concentration of the growth factor. Maximal inhibition occurred with 0.00016 units/ml but increasing the concentration 100-fold

had no greater inhibitory effect. When T₃ and the growth factor were incubated simultaneously with the enzyme, the maximal stimulatory effect of T₃ was not realized.

This inhibition by the growth factor may be the result of toxic contaminants in the preparation but the possibility that inhibition is due to the growth factor must be considered until a more purified preparation of the growth factor is obtained.

Effect of ashing and of ninhydrin on the growth factor. When the acid extract of the ashed sample of corpus luteum powder was fed to 2 rats, the growth response was 4 and 6 g in 2 weeks. Therefore, the growth response to corpus luteum powder was not due to some inorganic compound.
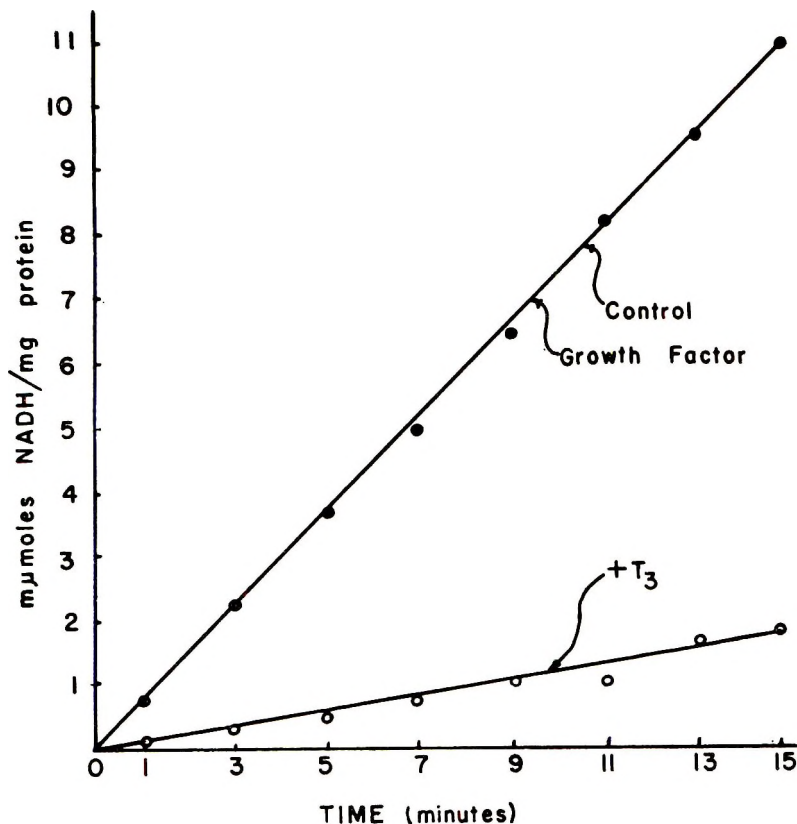
Fig. 4 Response of inosine monophosphate-dehydrogenase to the growth factor and to T_3 (triiodothyronine). Incubation media: (in μmoles) nicotinamide adenine dinucleotide (NAD), 14.7; inosine-5'-monophosphate, 19.8; Tris(hydroxymethyl)amino methane-phosphate (pH 7.6), 20; 0.03 mg of enzyme protein in a total volume of 1.5 ml. T_3 was 10^{-9} M (or 0.00065 units/ml) and the growth factor was tested at 3 levels: 0.000065, 0.00065, and 0.0065 units/ml (equivalent to 10^{-10}, 10^{-9}, 10^{-8} M of T_3, see fig. 1). The reduction of NAD was followed at 340 mμ for 15 minutes in spectrophotometer cuvettes using a blank which contained enzyme, NAD and buffer. All 3 concentrations of the growth factor gave a response identical to that of the control.

Three units of the growth factor treated with ninhydrin produced gains of 2, 6, and 10 g/2 weeks in 3 rats. This suggests that the growth factor may be an amine. It is of interest to note that ninhydrin-stable growth factors have been obtained from duodenal powder (4) and from thyroid powder.[10]

These results lead us to conclude that corpus luteum powder contains a growth factor which is capable of reinstating growth of growth-arrested, thyroid hormone-deficient rats and that it is not one of the known thyroid hormones.

Because of the presence of the growth factor in corpus luteum, we suggest that the growth factor may be necessary or important in the development of the fetus. The corpora lutea exist only during gestation or pseudo-pregnancy, and it is conceivable that the presence of growth factors in certain other tissues (1) is dependent on the existence of the corpora lutea. Experiments are now underway to test this hypothesis. It seems reasonable to suppose also, that the growth factor is related in some way to thyroid hormone function. We have previously reported [11]

[10] E. Ruark and C. J. Ackerman. Growth factors in corpus luteum and thyroid tissue. VII International Congress on Nutrition, August, 1966, Hamburg, Germany.
[11] See footnote 10.

TABLE 2

Response of adenylosuccinate synthetase to the growth factor and to triiodothyronine

Incubation media	Absorbancy (A) at 280 mμ
	$\Delta A/3\ min$
Complete [1]	0.625
Complete + T$_3$, 0.0016 units/ml [2]	1.223
Complete + growth factor, 0.00016 units/ml [2]	0.494
Complete + growth factor, 0.0016 units/ml [2]	0.490
Complete + growth factor, 0.016 units/ml [2]	0.550
Complete + growth factor, 0.0016 units/ml + T$_3$, 0.0016 units/ml [2]	0.720

[1] Complete media: (in μmoles) Tris (hydroxymethyl) aminomethane·HCl (pH 7.2), 20; inosine-5'-monophosphate, 0.7; guanosine-5'-triphosphate, 0.5; MgCl$_2$, 2.0; aspartate, 2.5; and 0.102 mg of enzyme protein in a total volume of 3.0 ml incubated in air at 37° for 30 minutes. The reaction was stopped by the addition of 0.2 ml of 6 N HClO$_4$. After centrifugation, the absorbancy of the supernatant fractions was measured against a blank which was obtained from a reaction vessel that had been incubated with all ingredients except aspartate.

[2] One unit of T$_3$ (triiodothyronine) equals one μg of T$_3$ (or 0.0016 units/ml equals 2.5 × 10^{-9} M). One unit of growth factor is defined in table 1, footnote 4.

briefly the preparation of a ninhydrin-stable growth factor from thyroid powder by a procedure very similar to that described here.

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Zinc, Copper and Iron Concentrations in Hair and Other Tissues: Effects of low zinc and low protein intakes in rats [1,2]

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ABSTRACT   The effects of purified diets containing 2 to 4 ppm of zinc upon concentrations of zinc in liver, kidney, pancreas, spleen, small intestine and in hair were studied in rats. Pair-fed controls received 20 or 30 ppm of zinc. Casein and gelatin served as sources of protein. Among the visceral organs studied, only the small intestine showed consistent and significantly decreased concentrations of zinc compared with the controls. The zinc concentration in hair was found to decrease substantially within 40 days after the low zinc diets started to be fed, and decreased progressively thereafter. During the same intervals, the concentration of zinc in the hair of control rats increased considerably. The combined effects of low zinc with low protein (7.4%) intakes were studied. No effect upon tissue zinc concentrations which could be attributed to the low protein intake was demonstrated. However, low intakes of protein, either with or without zinc supplementation, were associated with significantly elevated concentrations of copper in liver. In contrast, low intakes of zinc had no demonstrable effect upon the concentration of copper in the tissues studied. Iron concentration in the hair of weanling rats was uniform within litters but differed markedly between litters. The differences disappeared with time. No relationship of iron in hair to zinc nutrition was detected.

Consumption of diets containing less than 12 ppm of zinc causes impairment of growth and well-being in the rat when casein is the principal source of protein (1). However, attempts to relate the effects of low zinc intake to changes in zinc concentration in the tissues have yielded conflicting results. Most examinations of tissue zinc concentrations have been made using the fowl. Turk (2) observed no lowering of tissue zinc concentration and Kienholz et al. (3) reported none in 14 of 15 tissues examined. The exception was liver which some (4–5) but not all investigators noted to be affected. The same is true of pancreas (6) and duodenum (5). A study of zinc deficiency in calves showed a striking decrease in zinc in pancreas with much smaller changes in bone, liver, kidney and spleen, in that order. By contrast no change in zinc concentration occurred in parakeratotic pigs in pancreas nor in muscle, skin, intestines or erythrocytes, with variable results for liver, kidney, bones and plasma (8). In studies of the zinc content of zinc-depleted rats considerable change occurred in the carcass (9) but these results reflect primarily changes in zinc-rich tissues such as bone and hair. Macapinlac et al. (10) observed zinc concentrations in bone to be decreased by two-thirds in rats fed diets low in zinc for 7 weeks. Zinc concentration in the testis also decreased significantly. In contrast, a tendency toward high values occurred in the liver and muscle. It is evident that additional information concerning changes in tissue zinc concentration in rats and the factors governing them is needed to obtain insight concerning the disturbances associated with zinc deficiency.

Our interest in the relationship of zinc intake to the zinc content of tissues arose in part from the need to correlate measurements of enzyme activity in tissues (to be reported later) with the tissue zinc con-

centrations. A further objective was a search for aids to the diagnosis of zinc deficiency to decrease the dependence upon the measurement of zinc concentration in blood serum for this purpose. The latter has serious shortcomings. The analysis of hair to establish its concentration of zinc offers promise as such an aid (11–13). Experimental support for such use has been obtained and is described.

The metabolism of zinc is believed to be closely related to that of calcium, copper, iron and other trace metals (14). Zinc deficiency in man (15) is accompanied by a low concentration of iron and an elevated concentration of copper in blood serum. Moses and Parker [4] observed copper and iron to accumulate in tissues of rats on low zinc intake. The coincidence of hepatomegaly in youths with zinc deficiency (15, 16) and the prevalance of unexplained liver involvement in young boys in the Shiraz region (17) where human zinc deficiency was first described, suggested that copper might be an etiological agent. Moreover, Ritchie et al. (18) reported that zinc protected against copper poisoning. We have, therefore, included studies of copper in tissues of zinc-deficient and zinc-supplemented rats to test this possibility. Measurements of iron concentration were also made.

EXPERIMENTAL

Rats of a local Sprague-Dawley strain were housed in stainless steel cages. The diets used were based on those described by Forbes and Yohe (1) except that a protein mixture consisting of casein, 4 parts, and gelatin, 2 parts, was fed at levels of 14.8% unless otherwise specified. For removal of zinc, casein was suspended in 0.15% solutions of disodium ethylenediaminetetraacetate (EDTA) which were renewed two to five times after decantation or filtration. The casein was then resuspended in water repeatedly to remove the EDTA and air-dried on paper with the aid of an electric heater. Gelatin and sucrose contained no measurable zinc and were used as purchased. The diet contained 1% calcium monohydrogen phosphate [5] which was also used without purification. This was the major source of the zinc which remained in the diet. The

salt and vitamin mixtures were based on those described by Forbes and Yohe (1); however cellulose, tetracycline and vitamin B_{12} were omitted. Diets were mixed mechanically and stored in a refrigerator.

The zinc content of the diets was measured following digestion with sulfuric, nitric and perchloric acids with the aid of dithizone according to Wolff (19).

The series of experiments described was conducted over a period of 30 months. Two to four groups of 4 to 8 rats of both sexes, ranging in weight from 40 to 94 g were used to investigate factors such as low protein intake which may affect zinc metabolism. Each such experiment is designated as a series. Rats from the same litter were paired so as to provide a control of the same sex for each rat fed a zinc-deficient diet. When the effects of a factor such as protein level were evaluated, pair-fed controls were included at each protein level.

Food consumption which was equalized daily averaged 6 g/day during the first 4 weeks, increasing to 7, 8, 9 and 10 g by approximately weekly increments thereafter to a maximum of 15 g at 5 months in series 5. The concentration of zinc in the diets shown in table 1 multiplied by these figures enables calculation of the zinc intakes. These ranged during the first 4 weeks from 12 to 26 µg of zinc/day in the animals designated "deficient," and increased correspondingly during later stages of growth as food intakes increased.

The pair-fed control rats received the same diet except that 20 ppm of zinc was incorporated into the diet as carbonate in series 1 to 5 and 30 ppm in the later series. Deionized water was supplied ad libitum.

Rats were sheared with electric clippers over the entire ventral surface 7 to 10 days after being fed the diets. Hair so removed was washed with a sulfonated fatty acid detergent, rinsed repeatedly with water until no evidence of foaming was detected, washed twice with 95% ethanol and finally with ether. The washing consistently caused small losses of zinc, never

TABLE 1

Effect of zinc-deficient diets on zinc concentration of various rat tissues

Series	No. of rats	Days fed diet	Zn in diet	Body weight				Zinc concentration Hair			
				Control		Deficient		Control		Deficient	
				Initial[1]	Final[1]	Initial	Final	Initial	Final	Initial	Final
			ppm	g	g	g	g	µg/g	µg/g	µg/g	µg/g
8	16	61	4.30	94	173	93	146	219±5.8[2]	202± 6.9	209±11.4	145±12.7
6	14	64	2.00	46	106	47	89	168±8.0	177± 6.7	173± 3.6	140± 5.4
7	16	73	2.97	60	187	61	182	193±6.6	204±10.9	177± 4.7	168±10.3
3NP	11	105	2.92	58	130	59	116	186±6.8	187± 6.7	187± 7.7	145±14.7
3LP	12	105	2.25	60	85	61	75	188±4.7	194± 3.9	194± 4.8	156± 4.2
5	9	150	1.78	49	151	48	141	165±3.5	190± 2.9	181± 6.9	121±11.8

Series	No. of rats	Days fed diet	Zn in diet	Zinc concentration					
				Liver		Duodenum		Jejunum	
				Control	Deficient	Control	Deficient	Control	Deficient
			ppm	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g
8	16	61	4.30	26.8±3.01	21.6±3.07	15.7±1.67	17.7±1.95	21.0±2.33	20.2±1.95
6	14	64	2.00						
7	16	73	2.97	29.1±2.43	27.3±2.93	25.0±2.95	19.5±0.95	23.2±0.91	20.7±1.10
3NP	11	105	2.92	17.9±2.01	18.2±1.50	22.2±5.20	22.2±1.64	23.8±1.85	20.7±0.95
3LP	12	105	2.25	21.6±1.83	18.7±1.63	18.3±1.37	18.4±2.18	21.8±2.53	20.3±2.24
5	9	150	1.78			24.2±3.05	18.8±1.59	26.3±1.55	19.4±2.83

[1] Initial and final refer to body weights on the first and last days, or the samples of hair sheared during the first or final 10 days of the experiment.
[2] Tissue concentrations are expressed as mean ± SE.

exceeding 5%. Doubling the number of washing operations caused no additional loss. Subsequent shearings were confined to the newly grown hair. Sufficient yield of hair for analysis could be obtained at intervals of 30 to 40 days, although unfavorable hair-growth cycles and impaired hair growth when diets with low zinc or protein contents were fed sometimes extended this period to 50 days.

Samples of tissues for analysis were removed within a few minutes after exsanguination of the rats. The animals were anesthetized lightly with ether, the thoracic cavity was exposed and 0.3 ml of heparin solution introduced. The vena cava was opened by a small cut made with scissors and blood aspirated from the thoracic cavity into a polypropylene syringe. The small intestine was removed. A catheter was introduced into the vena cava and the liver perfused with cold 0.85% NaCl solution until no further blood appeared in the returned perfusate and the liver was nearly colorless. (Perfusion was omitted in series 1 to 3). The intestine was flushed repeatedly with cold saline, placed on a glass plate supported on ice and the mucosa stripped with a microscope slide. Samples of liver, kidney, pancreas, spleen, and intestinal mucosa were weighed and reduced to a homogenate in a Potter-Elvehjem homogenizer after dilution with 10 or 20 volumes of 0.85% NaCl. A glass homogenizer and Teflon pestle were used. Samples were removed for nitrogen analysis by a micro-Kjeldahl method. Zinc analyses were performed after digestion as described elsewhere (11, 17) by means of Zincon (20) (series 1 to 6 inclusive) or later by atomic absorption spectrophotometry (series 7 and 8). The results obtained by the 2 methods applied to samples of the same tissue did not differ significantly. Copper was measured in series 5 and 6 by the method of Stoner and Dasler (21) and in later series by atomic absorption spectrophotometry. For analysis of iron in hair, organic matter was destroyed by digestion with equal parts of nitric and perchloric acids and water. The residue was taken up in water after addition of 0.2 ml of 0.1 N H_2SO_4. Iron was measured by use of sulfonated bathophenanthroline (22). Blood was centrifuged and plasma stored in polyethylene containers. Plasma zinc was measured by atomic absorption spectrophotometry after treatment with hydrochloric and perchloric acids as described elsewhere (17).

RESULTS

Effects of low zinc intake on growth and development. Rats receiving the diets with a low content of zinc gained less weight than their pair-fed controls. Impairment of growth became evident from the time the rats fed the zinc-depleted diets began to recover weight lost when they were originally fed the purified diets. Decreased food consumption was an early and conspicuous response to the zinc-deficient diets. The resulting limitation of food intake required by paired feeding of the controls often curtailed growth of the latter. The most marked difference in weight between deficient and controls was 18% at 60 days (series 8). The smallest was 2.7% at 73 days (series 7). The median difference was 12%.

The rats making up series 8 with a somewhat higher daily intake of zinc were more vulnerable in terms of weight differences and depletion of zinc in hair and liver than were others whose zinc intake was less. The rats making up series 5, however, showed little impairment of growth, but marked differences in tissue zinc. The longer duration of the feeding period may partially account for the latter.

Concentration of zinc in tissues. The effect of feeding diets from which nearly all zinc had been removed upon zinc concentration in tissues is shown in tables 1 and 2. The behavior of the zinc concentration of visceral organs was evaluated by comparing the mean concentrations in the depleted rats with those of their pair-fed controls. The frequency of a given response was tabulated as well. The difference between pairs of rats was considered significant when it exceeded 4 times the precision of zinc measurements for a given tissue.

The jejunum and duodenum showed the greatest and only significant effects, as shown in table 2. The behavior of the ileal portion of the intestine, for which results are not presented, was similar. A

TABLE 2

Summary of data on zinc concentration in tissues of rats[1]

	Liver		Kidney		Spleen		Pancreas		Duodenum		Jejunum	
	Control	Deficient	Control	Deficient	Control	Deficient	Control	Deficient	Control	Deficient	Control	Deficient
No. of rats	43	45	16	17	30	31	29	33	41	42	36	38
Mean conc, µg/g	23.90	21.56	19.39	19.35	16.15	16.23	17.45	17.45	22.26	19.61	22.44	18.83
SD	5.84	5.48	4.20	3.32	2.20	4.48	5.40	6.02	6.79	5.20	5.82	4.48
SE	0.91	0.83	1.23	0.83	0.42	0.83	1.04	1.09	1.07	0.81	0.98	0.74
Difference between means	> 0.05		ns		ns		ns		$P = 0.05$		$P < 0.01$	
Significantly decreased[2]	17		4		13		8		14		20	
No change	18		9		5		16		20		10	
Significant increases	8		3		12		5		7		5	
P value, (χ^2)	ns				0.02		ns		ns		$P < 0.02$	

[1] Includes the results of series 1 and 2 which are omitted from table 1 because measurements of zinc in hair were not made.

[2] Differences between pairs greater than 4 × precision, calculated as $SD_{diff} = \sqrt{\frac{(X_1 - X_2)^2}{2N}}$; 4 SD is 3.7 µg/g, where X_1 and X_2 are duplicates and N is the number of analyses.

trend toward lower concentrations occurred also in the liver but the difference between means fell short of significance. Moreover, the frequency of decreased concentrations did not differ from that of kidney which remained least affected among the organs examined.

Zinc concentration in pancreas and spleen varied more widely than that of other tissues. Although the mean zinc concentration in the pancreas decreased in rats fed the deficient diet, the difference was not significant statistically. The spleen showed a curious lack of consistency in behavior in that zinc concentration was higher in twelve of the deficient members of the 30 pairs of rats in which the spleen was examined. This was offset by the occurrence of a significant decrease in thirteen, so that the mean remained unchanged.

There was no significant change in nitrogen content of the tissues associated with intake of the low zinc diets having a low content of zinc. DNA and RNA measured in 2 experiments in liver, kidney, pancreas and intestine showed no difference which could be attributed to zinc intake and data have been omitted.

Concentration of zinc in hair. The largest and most consistent changes in tissue zinc concentration occurred in hair. The data are summarized in table 1. There is a reasonably close correlation between decrease in zinc concentration in hair and difference in weight gain between control and zinc-deficient rats, those in series 5 being the exception. In figures 1 and 2, the original zinc concentration is equated to zero and changes from this value is recorded. In contrast with the decreased concentration of zinc in the hair of deficient rats, its concentration in the pair-fed controls increased during the periods of study (fig. 2). For this reason, the effect of low zinc intake on zinc concentration in hair is most accurately demonstrated by the difference in zinc concentration between the rats fed the deficient diet and that in their pair-fed controls. However, the original concentration serves as an adequate base line for comparison. The response of hair composition to low zinc intake was somewhat more consistent in males than females.

Effect of lowered intake on Zn in hair

Male

Female

Δ Zn μg/g

50

0

-50

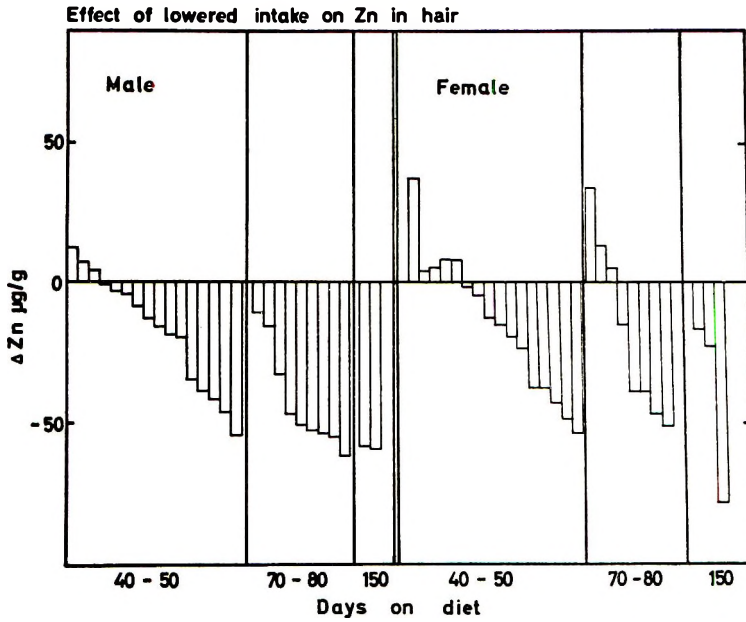40 - 50   70 - 80   150   40 - 50   70 -80   150

Days on diet

Fig. 1 Effects upon zinc concentration in hair of the consumption of diets containing 2 to 4 ppm of zinc. The change in zinc concentration is shown on the ordinate. This represents loss or gain in zinc during the interval between an initial shearing and subsequent shearings at the intervals shown. Each rectangle represents the data for a single rat during the period of days shown on the abscissa.

Zinc in hair of control rats

Males

Females

Δ Zn μg/g

50

0

-50

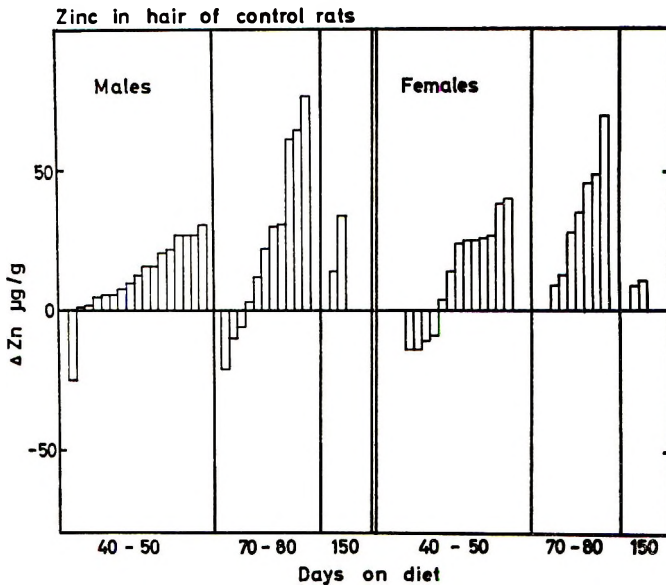40 - 50   70 - 80   150   40 - 50   70 - 80   150

Days on diet

Fig. 2 Effects upon zinc concentration in hair of the consumption of diets containing 30 ppm of zinc. These are pair-fed controls of the rats for which data are shown in figure 1, although the positions of the controls do not necessarily correspond to those of their pair-mates in figure 1.

Effects of low protein intake on zinc concentration in tissues. In 2 experiments, rats were fed diets containing 7.4% protein in combination with low zinc intake, the casein-gelatin mixture being maintained in the same proportions as in rats receiving the 14.8% protein diet. Calories were replaced by extra sucrose. The results of one of these experiments designated 3LP are shown in table 1. Comparison with those rats receiving the diet containing 14.8% protein, designated NP, fails to show any significant effect on tissue zinc concentration which could be attributed to the lowered protein intake. However, it is possible that effects on the rate of growth of low protein and low zinc intake are to some degree additive. Studies are being continued on this aspect of the problem.

Concentrations of zinc in plasma. A low yield of plasma in many experiments, particularly those on smaller rats, prevented dependable zinc analyses. Hemolysis also proved troublesome. Twenty-eight acceptable samples of plasma were obtained, 13 from the rats receiving low zinc intakes and 15 from random controls. The concentration of zinc in the former was 1.586 µg/ml, in the latter 1.685 µg/g, an insignificant difference.

Effect of low zinc intake on copper concentration in tissues. No effect of low zinc intake on the copper concentration in liver, kidney, duodenum, jejunum, ileum or pancreas was detected. Table 3 shows data selected from 3 series of rats in which tissue copper concentration was measured in those receiving low zinc and zinc-supplemented diets.

A significant effect of lowered protein intake on the copper concentration in liver occurred in series 3. The concentration of copper in the liver was doubled in the rats receiving the 7.4% protein intake. This difference is highly significant. A similar trend occurred in series 2; however, several of the liver copper analyses were lost in the control rats of this series and differences were not significant.

Copper concentrations in hair. No correlation between zinc intake and copper concentration in hair was observed in the course of these experiments. The copper concentration in the hair of 58 weanling

TABLE 3

Effects of variations in zinc and protein content of diets on concentration of copper in rat tissues

Series	No. of rats	Days fed diet	Protein in diet	Copper concentration					
				Liver		Kidney		Duodenum	
				Control	Low Zn	Control	Low Zn	Control	Low Zn
			%	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g
2NP	9	56	14.8	3.10 ± 0.37(3) [1,2]	3.36 ± 0.35	3.80 ± 0.75	3.43 ± 1.05		
2LP	10	56	7.4	4.20 ± 3.3 (2)	4.96 ± 0.75	4.75 ± 0.70	2.96 ± 0.60		
3NP	11	105	14.8	4.66 ± 0.60 [3]	4.84 ± 0.82 [3]	4.28 ± 0.65	3.64 ± 0.26	2.93 ± 0.93	1.97 ± 0.49
3LP	12	106	7.4	8.97 ± 1.32 [3]	9.15 ± 1.32 [3]	4.74 ± 0.73	3.48 ± 0.55	1.83 ± 0.28	1.88 ± 0.29

[1] Mean ± SE.
[2] Numbers in parentheses indicate number of rats on which analyses were based.
[3] Differences significant at $P < 0.01$ between rats receiving 14.8 and 7.4% protein.

TABLE 4

Iron concentration in hair of control and zinc-deficient rats

Series	Litter	No. of rats	Iron concentration, days		
			0	50–80	150
			$\mu g/g$	$\mu g/g$	$\mu g/g$
			Control		
6	A	4	28.5 ± 3.31 [1]	10.8 ± 2.45	—
	B	3	11.1 ± 0.39	13.1 ± 3.42	—
5		4	7.8 ± 5.46	13.1 ± 2.04	22.4 ± 5.84
			Zinc-deficient		
6	A	3	30.2 ± 6.90	13.1 ± 3.40	—
	B	4	13.9 ± 3.23	28.4 ± 8.00	—
5		5	9.2 ± 7.22	15.3 ± 3.18	16.3 ± 5.03

[1] Mean $\pm$ SD.

rats was 13.5 ± 0.42 µg/g. This agrees well with the values for hair of humans of various ages (11). A trend toward higher copper concentration in hair occurred in the rats. After 50 to 80 days it had increased to 15.6 ± 1.11 µg/g and at 150 days was double the original figure (series 5). The increase in the zinc-deficient rats was the same as in the control rats.

Concentration of iron in hair. Iron concentration was measured serially in the hair of 2 groups of rats. The results are shown in table 4. It was observed that the concentration of iron of young rats varied widely although the concentration within a given litter was relatively uniform. Typical results are illustrated by the 3 litters included in table 4. In 8 additional litters the mean iron concentrations ranged from 8.5 to 39.9 µg/g. Analysis of variance shows the differences between litters to be highly significant as compared with variations within the litters. The initial iron concentration also appeared to be related to subsequent changes in the iron concentration. When the initial values were high, the concentration decreased and vice versa. No relationship of iron in hair to zinc concentration or to zinc nutrition could be demonstrated.

DISCUSSION

One of the major objectives of our study was the evaluation of the responsiveness of the zinc concentration in hair to lowered content of zinc in the diet. Studies of hair in humans suffering from zinc deficiency have demonstrated a considerable decrease in zinc concentration (12, 13). Few studies have been made of the effects of controlled zinc intake upon the composition of hair. In contrast with our results, Macapinlac et al. (10) failed to demonstrate a decrease in concentration of zinc in dorsal hair after 50 days of a low zinc intake. However, the deficient rats of these workers differed from control rats in failing to exhibit the large increase in zinc concentration which occurred in the latter. In our experiments, the decrease in zinc concentration observed occurred only in hair grown during the experimental period. However, the ability of hair analysis to provide evidence concerning the state of zinc nutrition is supported.

The increase in zinc concentration in the hair of the controls with an intake of 30 ppm of zinc suggests that the optimal requirement for zinc may have been exceeded and that the surplus was being removed from the carcass, in part, by way of the hair. Further observations of the quantitative relationship between zinc in the diet and in hair, particularly at concentrations intermediate between those studied, will be of interest.

It is noteworthy that consumption of diets containing a substantially lowered concentration of zinc caused relatively little change in the zinc concentration of liver, kidney, pancreas, or spleen. However, zinc concentration in the small intestine was significantly decreased throughout its length. Studies to be described later show that the changes in zinc concentration were associated with markedly decreased alkaline phosphatase activities.

Hence the suggestion made by Hove et al. (23) that the intestine may be particularly vulnerable in zinc deficiency in the rat is upheld.

Since the quantity of zinc provided to the deficient rats was one-tenth or less of that ordinarily present in the diet and one-third to one-sixth of that stated to be a minimum requirement for the rat, a considerable ability to conserve zinc is demonstrated by visceral organs, other than the small intestine, over periods extending up to 5 months. Effects on growth were moderate but significant.

No evidence of an increase in tissue copper concentration in zinc-deficient animals such as that reported by Moses and Parker [6] was observed in our study. The significantly increased concentration of copper in livers of the rats fed diets low in protein is noteworthy. A similar response is suggested by a study made by McCall and Davis (24). However, low protein intakes apparently had no effect upon the zinc concentration in tissues. Platt and Frankel (25) observed a considerable change in zinc in serum and livers of pigs fed diets in which protein content was low and of poor quality.

Hair is one of few tissues in which measurement of iron concentration is not complicated by the presence of blood. A marked difference in iron concentration in hair between litters contrasts with the uniformity of results within the litter. Prenatal factors are undoubtedly responsible.

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[6] See footnote 4.

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Vanadium in Some Biological Specimens [1]

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ABSTRACT   The concentration of vanadium was studied in some common fruits and vegetables, animal foods, and in drinking water from various geographical areas by means of thermal neutron activation analysis. In general, the concentration of vanadium was found to be very low (10^{-4} ppm), although there was a pronounced difference among species. Lobster gelatin, mackerel, parsley, dill, lettuce, radishes, sardines, and liver (calves) were found to be relatively rich in vanadium. A commercial laboratory ration for rats was found to contain as much as 1.5 ppm vanadium (fresh weight). There were, however, some geographical variations of the vanadium concentration in the specimens analyzed. The uptake of radiovanadium (^{48}V) by vegetables from the soil varied from one vegetable to another. However, the ratio of the values obtained with neutron activation analysis and with the ^{48}V uptake study was constant throughout. Autoradiography showed that fish accumulated ^{48}V in bone, fins, skin, and liver.

Reviews concerning the possible physiological roles of vanadium have been published (1, 2). For example, inhibition of the biosynthesis of cholesterol has been ascribed to vanadium. It has also been stated, although contradictory, that the mineralization of teeth and bones during the period of development is promoted by vanadium and that a proper dietary intake of vanadium has an inhibiting effect on dental caries.

Vanadium has not been found to be particularly toxic to man (2). However, it has been observed to be very toxic to mice, rats, and chicks (3–6). Pam-Huu-Chanh (6) reported that sodium metavanadate is an extremely toxic agent with a very prompt action in rats; in mice the effect is much slower.

Vanadium is probably widely distributed in minute concentrations in the sea, soil, and in plants. Fukai and Meinke (7) studied the concentration of vanadium in seaweeds, mollusks, fishes, and sea water using neutron activation analysis. The soft tissues of the fishes contained about 2×10^{-7} g vanadium/g dried matter, and seaweeds and mollusks about 2×10^{-6}. The concentration of vanadium in sea water was found to be 3×10^{-10} g V/g dried water. Activation analysis has shown that random samples of milk powder from San Diego have a vanadium content of 15 ppb. The vanadium content of drinking water in the U.S.A. varies from zero to 220 ppb (8).

It is known that species of ascidians concentrate vanadium in their blood cells. Centrifuged blood cells of *Ascidia nigra* contain 1.45% vanadium (9). Rezayera (10) showed that vanadium in *A. aspersa* was in a dynamic equilibrium of V^{III} and V^{IV} in the blood cells. He concluded that the physiological action of vanadium is likely to play a role in an oxidation-reduction reaction in the blood cells. Neutron activation analysis showed vanadium in human erythrocytes in an extremely low concentration (11). The effects of sodium chromate, molybdate, tungstate, and metavanadate on the activity of xanthine dehydrase have been compared (12); it was found that sodium chromate and metavanadate inhibit the action of this enzyme, whereas the other two activate it.

The pentavalent metavanadate can inhibit the cellular division of *Chaos diffluens*, whereas the tetravalent vanadyl sulphate has no effect (13). An exceptionally high vanadium content, (about 100 ppm, dry weight) has been observed in toadstools (14).

The distribution of $^{48}V_2O_5$ has been studied in mice autoradiographically and by scintillation counting at various intervals after an intravenous injection of the labeled solution (15). The amount of radiovanadium was found to be highest in bones and teeth. It decreased in the following tissues in this order: visceral yolk sac epithelium, lactating mammary glands, renal cortex, liver, lung, skin, salivary glands, intestinal content, muscle, amniotic fluid, heart muscle, feces, cartilage, brain, blood, gastric content, gallbladder content, and spleen.

It has been stated recently that the "abnormal trace metal" vanadium is not present in the newborn (2), and the authors also claim that there is "a reasonably valid assumption that a metal not found in the newborn or in milk is probably not essential for life or growth." However, autoradiography has shown carrier-free ^{48}V does accumulate in fetuses, especially in the fetal skeleton. It also accumulates in lactating mammary glands of mice (15). It has been suggested that pulmonary accumulations of vanadium are the result of contamination by the presence of vanadium in the air (2). However, injection of ^{48}V in rats also results in a pronounced accumulation of the element in the lungs (15). It has also been stated that vanadium is concentrated in fat (2); another investigation failed to confirm this (15). In view of the possible physiological functions of vanadium (16–29), and since the concentration of vanadium in the human and animal body appears to be related to the vanadium content of the diet, further study of the concentration of this element in drinking water and various foods is indicated.

The analytical method used in this study, thermal neutron activation analysis, is more sensitive than any other technique available for vanadium. The radioactive isotope, ^{52}V, formed on thermal neutron irradiation, has a half-life of only 3.76 minutes. ^{52}V decays by a β-ray and a γ-ray of the energy of 1.43 Mev. This γ-ray can be counted with an efficiency of about 25% when using a 7.6 × 7.6 cm scintillation-well crystal.

MATERIALS AND METHODS

Activation analysis. Between 2 to 4 g fresh weight of the samples were used for the analysis; 250 ml were used for each analysis of water samples. Samples were always taken from different batches.

The water samples were boiled in a carefully cleaned Pyrex bottle until the volume had been reduced to about 6 ml. During the boiling, 2 ml (concentrated) distilled HNO_3 were added to each sample. Each water sample was transferred to a quartz ampule, in which it later was neutron-irradiated.

All other samples were weighed and ashed in a temperature slowly rising to 400°; this temperature was kept constant for 3 to 10 days. After ashing, the sample was weighed again and transferred to a polyethelene tube.

The samples together with vanadium standards ($NaVO_3 \cdot 4H_2O$; 6.4623×10^{-3} mg V per 0.2 ml) were irradiated for 10 minutes in a thermal neutron flux of 1.4×10^{12} neutrons $\times$ cm^{-2} $\times$ sec^{-1}. After irradiation, the samples were transported from the reactor to the laboratory by a pneumatic tube system. The sample was radiochemically processed as follows: It was transferred to a platinum crucible containing 3 g $K_2S_2O_7$ and 1 mg vanadium as carrier in the form of $NaVO_3 \cdot 4H_2O$. The crucible was heated until a clear melt was obtained and then cooled in boiling water. The melt was dissolved in 75 ml 7 M HCl and transferred to a beaker, which then was cooled for one minute during magnetic stirring. One milliliter of 0.2 M $KMnO_4$ was added to oxidize the vanadium to V^{5+}. The solution was transferred to a separatory funnel containing 10 ml n-benzoyl phenyl hydroxylamine (1 g/100 ml chloroform). After shaking for 30 seconds, the organic phase was poured in another separatory funnel containing 25 ml 7 M HCl. After shaking, the organic phase was collected in a tube taken for measuring in a 7.6 × 7.6 cm scintillation-well crystal combined with a 512 channel analyzer.

The γ-spectra were followed continuously to observe the characteristic half-life of the γ-ray peak. The amount of ^{52}V present was determined by measuring the area of the photopeak. The amount of

vanadium initially present in the sample was determined by comparing the area of the photopeak obtained with the standards. The standards were chemically processed in the same way as the unknown samples. Because of the short half-life of ^{52}V, correction was made for decay during counting.

The yield of this radiochemical method was tested with ^{48}V and found to be 75.4 $\pm$ 1.7%. When adding radioactive sodium, copper, zinc, phosphorus, and manganese, no traces of these nuclides could be found in the radiochemically separated vanadium phase.

The instruments and equipment used before irradiation, such as glass knives, glass bottles, polyethelene bottles, and quartz tubes, did not contain measurable amounts of vanadium that could contaminate the samples. This was tested by neutron activation analysis of the types of materials used. There was no measurable loss of vanadium in the drying or ashing. This was checked by ^{48}V. The amount of vanadium remaining in glass bottles when reducing the water volume was tested by ^{48}V and found to be 14.5 $\pm$ 2.6%. No significant amounts of activity were left in the polyethelene tubes or quartz ampules in which the samples had been neutron-irradiated. Activation analysis of the chemicals used for cleaning glass beakers, etc. (HNO_3, H_2O_2, H_2SO_4, distilled water, and acetone) was also made. The results obtained are tabulated in table 1.

Uptake of radiovanadium in fruits and vegetables. The uptake of vanadium-radiovanadium, ^{48}V, was studied in some fruits and vegetables. The radioactive nuclide,

carrier free ^{48}V, was supplied by Phillips Duphar in the form of vanadium trichloride. Soil was poured into eternite boxes, 54 liters in each box, that is, a soil layer of 60 $\times$ 60 cm and 15 cm deep. Water was added to the soil in each box in 2 portions of 1 liter each; to the first portion were added 10 g NH_4NO_3, 4 g $Mg(NO_3)_2$, 50 mg $NaVO_3 \cdot 4H_2O$, and 4.27 mCi carrier-free ^{48}V. To the other portion of water 10 g KH_2PO_4 and 2 g K_2SO_4 were added. The soil was then homogenized.

Seeds of the vegetables to be studied were put into the soil. Ordinary tap water was added to the boxes every second day. The vegetables were grown in a constant temperature of 20°. After 40 to 70 days the vegetables were harvested. The samples were rinsed in water, weighed, and dried for 12 hours at 100°, weighed, and then ashed for 8 hours at 160° and finally for 12 hours at 210°. The ash was weighed. At the time of the harvesting the activity had decayed to not less than 0.1 mCi ^{48}V. The dried samples were measured on the amount of ^{48}V in a 7.6 $\times$ 7.6 cm scintillation detector. ^{48}V standards of various volumes, all containing 0.4 µCi, were also measured.

The amount of ^{48}V added to the soil was homogeneously distributed in the soil of each box when the experiment started. The distribution was studied in various layers of the soil in eight of the boxes immediately after the harvest and was found to be insignificantly uneven. The relative ^{48}V content of the various layers of the soil at the time the fruits and vegetables were harvested is tabulated in table 2.

Distribution of radiovanadium in fish. Four adult red tail swordfish (*Xiphophorus*

TABLE 1

Amount of vanadium in some chemicals studied by means of neutron activation analysis and γ-spectrometry [1]

Chemical	Vanadium
	ppm
HNO_3, pro analysi [2]	43
HNO_3, pro analysi, distilled	not detectable
H_2O_2, pro analysi [2]	1.1×10^{-3}
H_2SO_4, pro analysi	not detectable
Distilled water	not detectable
Acetone, distilled	not detectable

[1] Each value is the mean of 3 analyses.
[2] Not in contact with the biological samples to be analyzed before they were irradiated.

TABLE 2

Distribution of radiovanadium in the soil at the time of harvesting the fruits and vegetables [1]

Layer	Relative amount of ^{48}V	Layer	Relative amount of ^{48}V
	count/min/g soil		*count/min/g soil*
1	465 [2]	4	490
2	463	5	488
3	480	6	505

[1] Successive layers of the soil are indicated in the first column; each layer was about 2-cm thick; layer 1 was the surface layer.
[2] Mean from 8 boxes of soil.

TABLE 3

Vanadium in some animal specimens [1]

Specimen	Ash wt Mean	Wet wt Range	Wet wt Average
	ppm	*ppm*	*ppm*
Calf liver, Stockholm	0.51	0.83×10^{-2}–1.18×10^{-2}	1.0×10^{-2}
Calf liver, Boston	0.11	0.18×10^{-2}–0.30×10^{-2}	0.24×10^{-2}
Calf flesh, Stockholm	$< 10^{-4}$		
Calf teeth, Stockholm	$< 10^{-4}$		
Calf bone, Stockholm	$< 10^{-4}$		
Pork, Stockholm	$< 10^{-4}$		
Fresh trout, soft tissues	0.06	0.03×10^{-2}–0.07×10^{-2}	0.04×10^{-2}
Fresh mackerel, soft tissues (North Sea)	0.20	0.15×10^{-2}–0.36×10^{-2}	0.26×10^{-2}
Fresh mackerel, bone	2.9	1.1 –4.1	2.0
Sardines, Sweden	0.28	0.40×10^{-2}–1.32×10^{-2}	0.86×10^{-2}
Sardines, Norway	0.20	0.42×10^{-2}–1.0×10^{-2}	0.70×10^{-2}
Sardines, Portugal	0.46	0.81×10^{-2}–1.8×10^{-2}	1.3×10^{-2}
Fresh milk, Boston	0.24×10^{-3}	0.71×10^{-4}–0.94×10^{-4}	0.84×10^{-4}
Fresh milk, Chicago	0.16×10^{-3}	0.66×10^{-4}–0.88×10^{-4}	0.77×10^{-4}
Fresh milk, New York	0.13×10^{-3}	0.52×10^{-4}–0.96×10^{-4}	0.74×10^{-4}
Fresh milk, Stockholm	0.48×10^{-3}	0.76×10^{-4}–1.45×10^{-4}	1.1×10^{-4}
Fresh milk, Oslo (Norway)	0.20×10^{-3}	0.70×10^{-4}–0.92×10^{-4}	0.80×10^{-4}
Fresh milk, Göteborg (Sweden)	$< 10^{-4}$		
Dried skim milk:			
Carnation [2] (U.S.A.)	0.50×10^{-3}	1.8×10^{-4}–2.8×10^{-4}	2.3×10^{-4}
Starlac [3] (U.S.A.)	0.48×10^{-3}	1.3×10^{-4}–2.3×10^{-4}	1.9×10^{-4}
Famos [4] (Sweden)	$< 10^{-4}$		
Semper [4] (Sweden)	$< 10^{-4}$		
Lobster, meat (North Sea)	16.1	3.2×10^{-2}–5.3×10^{-2}	4.3×10^{-2}
Gelatin (Sweden)	2.5	3.9×10^{-2}–5.0×10^{-2}	4.4×10^{-2}

[1] Values represent 10 samples of each of the specimens listed.
[2] Carnation Food Company, Los Angeles.
[3] Borden's Food Products, New York.
[4] Semper Company, Stockholm, Sweden.

TABLE 4

Vanadium in some fruits and vegetables [1,2]

	Ash wt Mean	Dry wt Mean	Wet wt Range	Wet wt Average
	ppm	*ppm*	*ppm*	*ppm*
Dill	4.6	0.84	0.12 –0.15	0.14
Lettuce	2.8	0.58	1.9×10^{-2}–2.3×10^{-2}	2.1×10^{-2}
Parsley [3]	29.5	4.52	0.50 –1.11	0.79
Cucumbers	0.38	5.6×10^{-2}	1.6×10^{-3}–2.3×10^{-3}	2.1×10^{-3}
Radishes [3]	7.9	1.26	5.22×10^{-2}–5.21×10^{-2}	5.21×10^{-2}
Strawberries [3]	0.66	3.1×10^{-2}		
Wild strawberries	0.72	4.1×10^{-2}		
Red whortleberries	0.54	1.02×10^{-2}	1.5×10^{-3}–1.7×10^{-3}	1.6×10^{-3}
Apples [3]	0.33	0.86×10^{-2}	1.89×10^{-3}–1.25×10^{-3}	1.10×10^{-3}
Tomatoes [3]	0.041	0.53×10^{-3}	0.16×10^{-4}–0.38×10^{-4}	0.27×10^{-4}
Cauliflower	0.093	1.09×10^{-3}	0.72×10^{-4}–0.83×10^{-4}	0.77×10^{-4}
Potatoes [3]	0.93×10^{-2}	0.64×10^{-2}	0.75×10^{-3}–0.89×10^{-3}	0.82×10^{-3}
Pears	$< 10^{-4}$			
Carrots [3]	$< 10^{-4}$			
Common beets	$< 10^{-4}$			
Peas, frozen	$< 10^{-4}$			

[1] Values represent 10 samples of each item.
[2] All samples were taken from the Stockholm area.
[3] Of these species 5 samples of each grown in New Hampshire (U.S.A.) and 5 samples grown in Rhode Island (U.S.A.) were also analyzed. The results from these 70 samples were, in general, somewhat lower than those tabulated for the fruits and vegetables grown in Sweden.

helleri) were allowed to live for 3 days in 1000 ml H_2O to which about 1 mCi [48]V had been added. After this exposure to [48]V the fish were kept in 100 liters fresh water overnight. The fish were then killed by complete immersion in a cooling solution ($-76°$). After about 30 seconds, the fish were completely frozen. Sagittal sections, 10-μ thick, were taken through the entire frozen fish at various levels by a sledge microtome. Sectioning and drying of the sections were carried out at about $-12°$ in a freezing room. The exposure time on the Gevaert Dentus Rapid films was 14 days. The whole-animal autoradiographic technique used has been described previously (30).

RESULTS

Activation analysis. The thermal neutron activation analyses of the concentration of vanadium in various biological materials gave the results shown in tables 3, 4 and 5. Corrections have been made for known loss as mentioned above.

A commercial laboratory ration [2] showed an expectedly high vanadium concentration of 1.50 ppm fresh weight as a mean value of 10 samples.

Uptake of radiovanadium in some fruits and vegetables. The results of the study on the uptake of [48]V in some vegetables are tabulated in table 6.

Distribution of radiovanadium in fish. The autoradiographic study of the uptake of [48]V in fish showed an accumulation of radiovanadium in bones, fins, skin, and the liver. The concentration of vanadium was low in the central nervous system and

[2] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

TABLE 5

Vanadium in some water samples [1]

	Vanadium in water samples	
	Range	Average
	ppm	*ppm*
Water samples from:		
Atlantic ocean	5.0×10^{-3}–4.4×10^{-3}	4.6×10^{-3}
Baltic sea	3.5×10^{-3}–5.0×10^{-3}	4.0×10^{-3}
Lake Maelar (Sweden)	0.12×10^{-3}–0.24×10^{-3}	0.1×10^{-3}
Small lake (Stockholm, Sweden)	0.45×10^{-3}–0.48×10^{-3}	0.46×10^{-3}
Drinking water from:		
Boston	0.9×10^{-4}–1.3×10^{-4}	1.1×10^{-4}
New York	0.3×10^{-4}–0.5×10^{-4}	0.4×10^{-4}
Chicago	0.8×10^{-4}–1.9×10^{-4}	1.4×10^{-4}
Oslo (Norway)	0.4×10^{-4}–0.7×10^{-4}	0.6×10^{-4}
Stockholm (Sweden)	4.9×10^{-4}–5.0×10^{-4}	5.0×10^{-4}
Kiruna (Sweden)	0.7×10^{-4}–0.9×10^{-4}	0.8×10^{-4}
Trosa (Sweden)	8.3×10^{-4}–8.6×10^{-4}	8.5×10^{-4}

[1] Values represent 10 samples of each water specimen, fresh weight.

TABLE 6

Uptake of vanadium ([48]V) in some vegetables [1]

	Uptake of [48]V		
	Ash wt	Dry wt	Wet wt
	%	%	%
Lettuce	0.211×10^{-4}	1.01×10^{-5}	0.68×10^{-6}
Spinach	0.595×10^{-4}	2.09×10^{-5}	1.72×10^{-6}
Dill	1.05×10^{-4}	3.16×10^{-5}	2.98×10^{-6}
Parsley	5.66×10^{-3}	1.50×10^{-3}	1.45×10^{-4}
Carrots, peeled	6.55×10^{-4}	6.02×10^{-5}	0.91×10^{-5}
Carrots, leaves	3.57×10^{-4}	6.14×10^{-5}	0.79×10^{-5}
Radishes, peeled	1.58×10^{-3}	2.53×10^{-4}	0.21×10^{-4}
Radishes, leaves	6.28×10^{-4}	1.49×10^{-4}	0.14×10^{-4}
Potatoes, peeled	7.87×10^{-4}	2.27×10^{-4}	0.34×10^{-4}
Potatoes, leaves	2.29×10^{-4}	4.41×10^{-5}	0.30×10^{-5}

[1] The uptake is here indicated as % [48]V (of the entire amount of [48]V in the soil of the box where the vegetable grew) per gram of the vegetable.
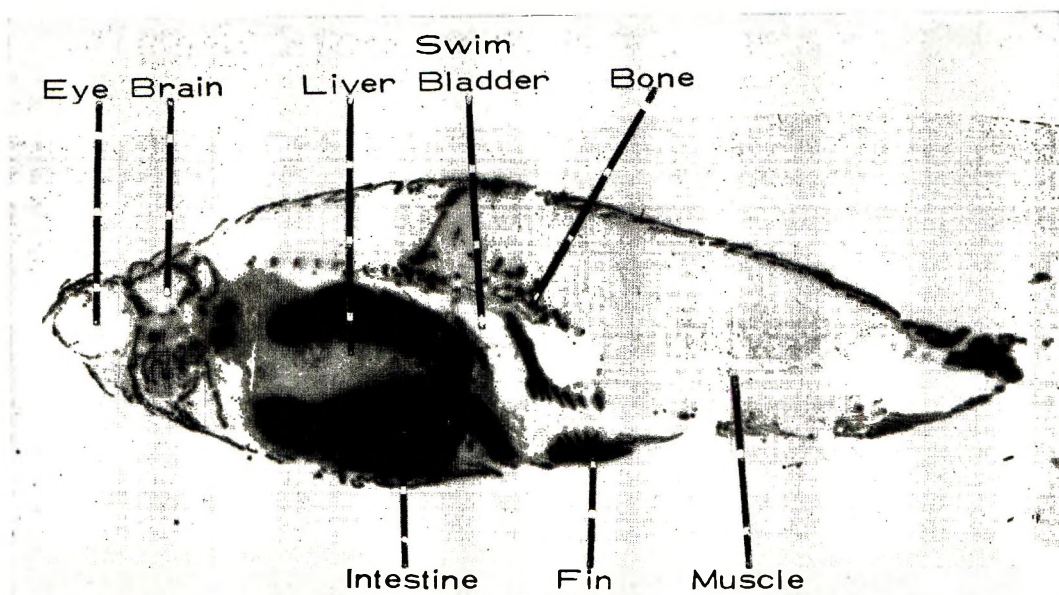
Fig. 1   Autoradiogram of a sagittal whole-body section of a fish (*Xiphophorus helleri*) killed after having been kept for 3 days in water containing ^{48}V. Black areas indicate the accumulation of ^{48}V in the fish.

muscles. An autoradiogram of a section of a fish is shown in figure 1. In the intestinal content a high amount of activity was visible. This was probably because the ^{48}V was absorbed by the food in the water before ingestion.

DISCUSSION

The values of the vanadium concentration in biological specimens obtained in this study are, in cases where comparisons can be made, generally somewhat lower than in previously reported data (2). The analytical method used in this study — thermal neutron activation analysis — is, for vanadium, more accurate and sensitive than any other method (31). In addition, this technique almost obviates risks of contamination, an important advantage when analyzing elements in concentrations in the parts per billion range (31, 32).

As in the previous studies (2, 8), there was a higher concentration of vanadium in sea water than in fresh water. Marine animals living in salt water and fresh water reflected this difference in the vanadium level. Thus, fishes from the Atlantic Ocean, (lobster, sardines, mackerel)

were found to have high concentrations of vanadium. The distribution pattern of vanadium in fish as obtained by means of autoradiography using ^{48}V was similar to that in mammals. The hard tissues, fins, and skin contained a very high concentration. In the mammalian samples studied, the vanadium level was very low. However, in gelatin and calf liver relatively high concentrations were noted.

In fruits and vegetables the concentrations varied from one species to another. The highest concentrations were observed in parsley, radishes, dill, and lettuce. The range of values obtained for the 10 samples of each species analyzed was extensive for most fruits and vegetables, especially when the values of those grown in U.S.A. were compared with those grown in Sweden. But there was a pronounced difference in the vanadium level among most of the vegetable species. A study was made to determine whether this difference in the vanadium level could be reproduced when growing the different fruits and vegetables in the same soil. Radioactive vanadium, ^{48}V, was mixed in the soil. The species still differed in the uptake of ^{48}V (table 6), although this

difference was not as pronounced as that in the neutron activation analysis study. Parsley and radishes again showed the highest accumulation of vanadium of the vegetables studied.

The presence of vanadium in foods is irregular, but there does appear to be a rather constant dietary intake. As noted before, the commercial laboratory ration tested [3] contained relatively large amounts of vanadium, which might invalidate some types of vanadium experiments on rats and mice where the effect of low levels of vanadium are studied. The vanadium levels in the laboratory ration and food in general are far from toxic (3–6, 33–35).

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Taste and Food Intake in Domesticated and Jungle Fowl [1]

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ABSTRACT   Domestic and jungle fowl were tested to determine their response to a sucrose solution when the caloric density of their diet was increased or decreased. Neither the domestic nor jungle fowl exhibited an avidity for sucrose solutions. The caloric intake of the birds fed an adequate diet was not significantly different with or without a sucrose solution choice. However, the nutritive state modified preference behavior and volume intake of sucrose solutions. In short-term trials where the caloric source was available in solution, the preference behavior of the jungle fowl was more complementary to nutritional need than that of the domesticated bird. The jungle fowl corrected for diet dilution more effectively and reduced food intake after diet enrichment. However, both groups failed to reject the sugar solution when eating the calorie-enriched diet. Domestication appears to have produced an animal with diminished sensitivity to caloric regulation.

Domestic chickens, herring gulls, laughing gulls, starlings, Japanese quail, robins, and other avian species do not select sucrose or glucose solutions, over a broad range of concentrations, in preference to water (1, 2). This indifference by the bird to several common sugars in solutions is in sharp contrast with the appetite displayed by a number of domesticated mammals for some compounds described as "sweet" by man (3). However, an absence of an avidity towards sugars has also been observed in a few mammals, for example, the cat and the armadillo (4, 5). Whether an indifference to common sugar solutions is of any physiological and evolutionary significance has not been determined.

Most of the research on taste has been carried out with domestic or laboratory animals. Since these animals have little or no choice of foodstuffs, possible function or survival value of this sense might not be detectable. Furthermore, selection in breeding programs has been vigorous for factors such as growth rate, disease resistance or efficiency of feed utilization, whereas, the sense of taste is ignored if not inadvertently eliminated.

Domestication has changed the anatomical, physiological, and behavioral characteristics of the rat and the fowl (6, 7). The possibility arises that the function of taste may have also been altered particularly as it pertains to the selection and ingestion of food. For example, laboratory rats have been reported to consume greater quantities of a variety of sugar solutions than the wild Norway rat (8).

The failure to evoke an approach response to sugar by some species of birds raises a number of questions. Is the bird able to detect sugar in solutions? Kare and Ficken (3) indicated that the fowl will respond to the calories in a sugar solution in regulating its food and fluid intake. That is, when food was limited to 75% of that consumed by the controls, the intake and selection of a sucrose solution was increased. Hill and Dansky (9) reported that over a long-term period the chicken will adjust its food intake in response to a reduction in the caloric density of the diet created by the addition of a nonnutritive material.

Experiments were carried out to determine whether the reaction to a sucrose solution differed between wild and highly domesticated fowl. Furthermore, the experiment sought to determine whether taste preference behavior would be modified by the nutritional state of the animal as a consequence of increasing as well as decreasing the caloric content of the diet.

METHODS

Thirty-six jungle fowl from stock that we are reasonably certain had been bred only in the United States for several generations were used. The thirty-six domesticated counterparts (white Vantress crossbreds) were 6 to 7 weeks old at the beginning of the experiment, but were larger and had grown faster (mean body weight, 858 g) than the jungle fowl that ranged from 7 to 11 weeks old (mean body weight, 345 g). The animals were of both sexes and were reared in the laboratory from one-day-old with the basal diet.[2]

The animals were housed in 4-tiered batteries and were assigned at random to individual wire-floor pens, which measured 60 × 60 × 35 cm. The animals were given continuous access to feed and fluid. The cages, as well as the feeding and watering equipment, were designed or modified for preference testing. Intake was measured daily for 18 days and body weight initially and subsequently every sixth day.

The basal diet, which the control animals received throughout the experiment, was a standard commercial ration. The calorically diluted diet contained 25% cellulose, that is, 3 parts by weight basal diet, one part cellulose.[3] The calorically enriched diet contained 25% corn oil.[4] The kilocalories were computed on the basis of gross energy using the Atwater factors. Distilled water was used throughout the experiment.

Six animals from both the wild and the domestic groups were assigned to each treatment and were tested individually. These included the 3 caloric levels, that is, basal, calorically diluted and a calorically enriched diet.

Two choices of fluids were available, and their position on the cages was alternated daily to attenuate the effect of position habits (10). The standard precautions for preference testing were observed. These included equal illumination, identical waterers equally distant from the feeder, and equal temperature of the choices.

Six animals fed every diet received a choice between water and a 10% sucrose solution. Six animals on every diet chose between two identical waterers containing only water. The 10% sucrose solution, that is, 10 g of reagent grade sucrose in 100 ml of solution, was selected because it evokes near maximal preference and intake in a number of domesticated animals (3) and contains sufficient calories to be of consequence to the intake of the animal. This concentration of sugar in solution has been shown to be selected indifferently by the fowl under an ad libitum feeding regimen (2), but is selected when food intake is restricted (3). The percentage preference refers to the amount of test solution consumed expressed as a percentage of the total fluid intake, that is,

$$\frac{\text{test solution consumed (ml)}}{\text{total fluid consumed (ml)}} \times 100.$$

In those instances where water constituted both choices, one container of water was designated as the control fluid. Theoretically, in a 2-choice situation a value of 50% preference should be obtained on the basis of chance selection alone.

RESULTS

Table 1 summarizes the preference response of the wild and domestic birds to a sucrose solution. The preference measures in those instances where only water was available ranged between 47 and 53%; this reflects the precision of the testing procedure.

The domestic fowl neither selected nor rejected sucrose with either a basal or calorically enriched diet. However, with the calorically diluted diet the sucrose solution was selected 2:1 over water (64%). In contrast, the jungle fowl selected the sugar solutions in preference to water (58, 63, and 56%) under all conditions.

Table 2 compares the ability of the jungle fowl and domestic fowl to regulate their caloric intake on the basal, calorically diluted, and calorically enriched diets. The jungle fowl adjusted to food intake under all three dietary conditions. When sugar was available with the diluted diet, the additional calories were obtained by both selections of the sugar

[2] Protein (min.) 20.0%; fat (min.) 3.5%; fiber (max.) 7.0%; and carbohydrates (min.) 55.0%.
[3] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[4] Mazola, Corn Products Company, Argo, Illinois.

TABLE 1

Preference [1] of domestic and jungle fowl for sucrose

Diet	Fluid choice	Domestic fowl [2]	Jungle fowl [2]
Standard	water-water	49 ±6 [3]	49 ±6
	sucrose-water	51 ±6	58 * ±6
Diluted	water-water	48 ±7	53 ±6
	sucrose-water	64 * ±6	63 * ±6
Enriched	water-water	47 ±6	51 ±6
	sucrose-water	53 ±7	56 ** ±6

[1] Mean preference (18 days) $= \dfrac{\text{test solution intake}}{\text{total fluid intake}} \times 100.$

[2] Thirty-six birds/group.

[3] Mean ± SD.

* $P < 0.01$ (difference from 50%).

** $P < 0.05$ (difference from 50%).

TABLE 2

Food and fluid intake of domestic and jungle fowl

Diet	Standard		Diluted		Enriched	
Fluid choice	H_2O/H_2O	$H_2O/$ sucrose [2]	H_2O/H_2O	$H_2O/$ sucrose [2]	H_2O/H_2O	$H_2O/$ sucrose [2]
Domestic fowl						
Total intake, kcal	268	303	207	262	359	347
Intake as sucrose, kcal	—	38	—	78	—	27
Adjusted,[3] total kcal	255	272	231	279	356	354
Feed intake, g	92	91	98	88	90	80
Fluid, ml/food, g	1.8	2.0	1.9	3.5	1.6	1.6
Wt increase, %	69	69	47	47	65	59
Jungle fowl						
Total intake, kcal	124	132	99	149	119	149
Intake as sucrose, kcal	—	35	—	66	—	40
Adjusted,[3] total kcal	123	123	93	124	128	136
Feed intake, g	45	33	47	36	33	28
Fluid, ml/food, g	1.9	3.6	2.1	5.8	2.0	5.5
Wt increase, %	52	52	35	43	43	43

[1] Mean for 18 days.

[2] 10% sucrose solution.

[3] Covariance adjustment for body weight.

(2:1) and also by a threefold increase in fluid intake (5.8 ml fluid/g food). The domestic fowl also corrected for dilution of the diet in a similar manner; however, the fluid increase was more limited (3.5 ml fluid/g food).

The caloric intake of the domestic fowl with the enriched diet was significantly greater ($P < 0.01$) than that of the control birds. The jungle fowl fed the enriched diet, even though it selected the sugar, restricted its food to maintain caloric intake.

There was a small numerical increase in grams of food consumed with the diluted diet by the birds receiving only water. However, this increase was not significant for either the jungle or domestic fowl.

Table 3 summarizes the sucrose intake in terms of total caloric intake for successive 6-day periods under all conditions of the experiment. The response of the domestic fowl to sucrose, with a single exception, showed little change in successive periods, whereas the response of the jungle fowl to sucrose increased progressively.

Large fluid intake by caged domestic birds (polydipsia) is occasionally encountered. In this experiment, two of the domestic birds fed the diluted diet could be classified as compulsive drinkers and

TABLE 3

Average sucrose intake of domestic and jungle fowl

Diet	Average sucrose intake					
	Domestic fowl, days			Jungle fowl, days		
	1–6	7–12	13–18	1–6	7–12	13–18
	% kcal of total food intake			% kcal of total food intake		
Standard	10.1	12.0	12.2	20.5	24.4	34.5
Diluted	15.5	15.6	34.1 [1]	45.4	40.8	54.0
Enriched	7.7	6.9	8.2	22.2	26.6	28.7

[1] High value attributable in part to polydipsia in 2 birds (median value, 29.9).

this fact has exaggerated the fluid intake data. None of the jungle fowl were observed to exhibit polydipsia.

DISCUSSION

The jungle fowl's intake behavior was more complementary to maintaining a steady caloric intake than was that of the domesticated bird. The appeal of sucrose which man and other mammals experience is largely or totally lacking in both the domestic and jungle fowl (2). The jungle fowl, fed an adequate diet, selected the sucrose solution ($P < 0.01$) in a choice situation, whereas the domesticated bird did not distinguish between distilled water and a sucrose solution. However, the jungle fowl's preference for sucrose over water was 1.4:1 which is of a modest magnitude compared with the 20:1 selection or greater than might be observed in the rat or ruminant (3).

The preference data become more meaningful in terms of total calories consumed. The domestic bird, fed an adequate diet, accepted the sucrose solution (13% of total caloric intake) equally as well as the distilled water. The jungle fowl fed an adequate diet, obtained twice that caloric intake from sugar (27% of total caloric intake). This caloric intake from sucrose was in large part the result of increased fluid intake rather than a consequence of a pronounced preference. However, the calories supplied by the drinking fluid did not effect an increased weight gain.

With calorically diluted diets, both the wild and domesticated birds selected sugar solution by a 2:1 preference. Also, both groups increased their fluid intake so that sucrose from solution constituted a sub-

stantial portion of their total caloric intake. In the correction for the needed calories, the increase in preference was similar for both groups of animals; however, the wild fowl appeared to make a better and more rapid adjustment by virtue of a greater increase in its fluid intake, that is, 5.8 ml fluid/g food vs. 3.5 ml fluid/g food.

With the enriched diet, it would have been nutritionally advantageous for the birds to reject the sucrose and obtain more of the calories from the balanced diet. However, neither the domestic nor jungle fowl avoided the sucrose solution. In fact, the jungle fowl marginally selected the sucrose solution. For the jungle fowl, there was no overall significant difference in caloric intake when covariance adjustments are made for differences in body weight, with all 3 diets. Apparently, when consuming the enriched diet, food intake by the jungle fowl receiving sucrose was reduced to 28 g as opposed to 45 g by the controls. The jungle fowl receiving only water reduced their intake to 33 g. In contrast, the domestic fowl overate with the enriched diet, (80 g for those receiving sugar and 90 g for those limited to water) in comparison to the controls (92 g) to the extent of consuming nearly the same weight of food ($P < 0.01$) and an increase in caloric intake of approximately 35%. This is similar to the situation observed with the laboratory rat which does not reduce its volume intake as rapidly as the wild counterpart when the energy content of the diet is increased by the addition of fat (8).

The relatively elastic volume of fluid intake observed with both groups of birds is an interesting phenomena. When su-

gar solution was available, the jungle fowl eating the diluted diet tripled its fluid intake; and even the domesticated birds doubled their intake. Although the data suggest that sucrose is a discriminable stimulus, it is not clear why the birds fed the diluted diet increased their intake of total fluid rather than primarily increasing their intake of the sucrose solution. One possibility is that the sucrose is not an easily discriminable taste stimulus for the bird and with the constant alteration of position, many errors in identification were made. The altered fluid intake is apparently unrelated to the composition of the diet since the caloric deficiency created by cellulose dilution of the diet did not alter fluid intake when sugar was not a choice. Also, the jungle fowl even with an adequate diet increased total fluid intake when sucrose was available. Furthermore, the domestic fowl, when fed an adequate or an enriched diet, consumed a considerable quantity of a sucrose without increasing water intake. Therefore, it is doubtful that the observed small increases in sugar intake caused diuresis, which stimulated water ingestion.

It is interesting that the wild rat, as opposed to the domestic rat (8), was more rigid in its intake of fluid. In contrast, the jungle fowl compensated for its caloric needs with considerable flexibility in its fluid intake. However, the question of physical capacity is raised by the observation that neither the jungle fowl nor its domestic counterpart responded to the calorically diluted diet by substantially increasing food intake. Although there is a small numerical increase in grams of food, and in volume, this does not suggest the rapid adjustment in volume intake encountered in rodents (11). Reddy et al. (12) suggest that the volume of a loose cellulose diluted diet, as opposed to pelleted feed, made greater demands on the birds' energy expenditure involved in eating more frequently. Our data indicate that cellulose dilution resulted in a reduced caloric intake, possibly related to palatability or to the mechanical problems of ingesting a powdery material of less density than the basal diet.

While the fowl does not share man's sensory reception for sucrose, it is reason-able to entertain the possibility that the selection of sucrose solutions under mild food deprivation represents a change in the preferential threshold for sucrose. Also, a sucrose solution could convey sensory information to the bird other than the taste quality man would recognize. Kitchell et al. (13) and Halpern (14) reported that the fowl has a "water sense," that is, pure water will evoke a taste response in recordings of electrical activity from the glossopharyngeal nerve. This was borne out in our extensive behavioral testing with a variety of taste stimulants where the fowl failed to evidence a significant preference for any solution over pure water, whereas many compounds reduced the acceptability of fluids by the bird. The fowl appears to be indifferent to viscosity as created by concentrated sugar solutions (2); therefore, it is doubtful that this quality was used as a guide for the intake.

The 18-day periods used might be described as short-term. Jacobs (15) has shown that caloric constancy with sugar solutions, under similar conditions, are maintained by rats in long-term studies. The data in table 3 suggest that the jungle fowl, fed a diluted diet, responded immediately to the sugar solutions (first 6 days), whereas the reaction of the domestic bird was delayed. Furthermore, the jungle fowl under all conditions increased its sugar intake with time, whereas the response of the domestic animals was more modest.

In preliminary experiments, the domestic fowl, fed a calorically deficient diet or restricted diet, was given an opportunity to increase its caloric intake from aqueous emulsions of corn oil (plus polysorbate 80) which was isocaloric with 10% sucrose. The solutions of fat were almost totally rejected in a 2-choice situation. In similar trials with animals acutely deficient of protein, a choice of supplemented 1% casein and water was presented to the birds. The animals totally rejected the casein solutions. While other protein or fat solutions may be more acceptable, the preference studies indicate substantial taste problems would be encountered in their use for experimental studies of dietary adjustments in the fowl. Since the

fowl does not select the other common sugars in solution there is no reason to suggest that the results presented here are uniquely related to sucrose.

The failure of the fowl to respond to fat and protein solutions cannot be attributed entirely to their possible offensive taste. Solutions of dimethyl anthranilate, which in preliminary experiments were rejected 3:1, were offered to calorically deficient chicks. When sucrose was added to the normally offensive solution, it was selected over pure water. This suggests that caloric needs took precedence over a moderately offensive taste.

There are some reports in the literature of preference for sugar solutions by the fowl (16), quail (17), and the great tit (18). The possibility that these data actually reflect a caloric need of the test animals, rather than a taste preference, might be considered.

The present study was limited to a single strain of domesticated and one of the jungle fowl. However, if the results are broadly confirmed, they indicate that with domestication has come a sluggishness in sensitivity to chemical stimuli which are related to caloric regulation. The wild animal fed a calorically diluted diet, more effectively corrected its needs from the sucrose solutions, and also with a calorically enriched diet made food intake adjustments to maintain a relatively constant caloric intake. The wild animal was apparently more responsive to the nutritional consequences of its food and fluid intake.

ACKNOWLEDGMENT

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Copper, Manganese, Cobalt, and Molybdenum Balance in Pre-adolescent Girls [1,2]

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ABSTRACT   Metabolic balance data were obtained on 36 pre-adolescent girls, aged 6 to 10 years, consuming measured intakes of diets varying in protein from predominantly animal to all-plant protein and varying in protein content so that intake per child ranged from 0.6 to 3.0 g/kg body weight. From food, fecal and urine analyses, estimates of daily dietary requirements for equilibrium for Cu, Mn, and Co were found to be 1.3 mg, 1.0 mg, and 7.7 μg, respectively. Suggested daily allowances for these elements are 2.5 mg, 1.25 mg, and 15 μg, respectively, based on estimates of growth needs, integumental losses, and reasonable margins of safety. Mo was found to be retained by all subjects when intakes approached 100 μg/child /day.

Few metabolic balance studies have been conducted on pre-adolescent children, and even less attention has been directed toward the minor elements. A cooperative study designed primarily to investigate nitrogen balance under varying protein intakes in 36 pre-adolescent girls in the southeastern United States provided opportunity for collecting balance data on Zn, Cu, Mn, Co, and Mo. The observations relating to Zn have been published elsewhere (1).

A detailed review of minor element balance studies will not be attempted here. Estimates of requirement for copper range from a low of 0.6 mg/day for adults (2) to 2 mg for adults and children (3, 4). Scoular (5) indicated the daily copper requirement was 1.0 to 1.6 mg for the average 3- to 6-year-old child weighing up to 20 kg.

Balance studies by Everson and Daniels (6) suggested that children should receive 0.2 to 0.3 mg of Mn/kg body weight. De (4) concluded from balance studies in adult Indians that the Mn requirement was 2.74 mg/day. Little is known of requirement for Mo. With respect to cobalt, whether there is a requirement for man other than in the form of vitamin B_{12} is not known.

PROCEDURE

The descriptive details of these studies, including objectives, general plan, organization, methodology of sample collection, control over subjects, and dietary regimens have been published (7, 8). The 3 studies were conducted during the summer months in 1956, 1958 and 1962. Minor element balance data were collected on 12 girls, 6 to 10 years of age, maintained with controlled dietaries in each study. The major dietary variables were level and source of protein. Diets were planned such that the energy intake fell within the range of 75 to 80 kcal/kg body weight (9) and protein intake ranged from 0.6 to approximately 3.0 g/kg body weight. Dinner menus illustrative of the foods and diet pattern used are given in table 1.

From these it can be seen that the 1956 and 1958 studies were based on foods commonly available. The 1956 study provided liberal animal protein intake, the 1958 study was aimed at more restricted animal protein intake. The 1962 menus used entirely plant protein, with nuts or legumes serving as protein staples. The 1956 study involved 12 girls maintained with a controlled diet pattern of 4 menus

Study	Item	
		g
1956	Baked ham	75
	Boiled potato	50
	Lima beans, green	100
	Gingerbread (dry mix)	40
	Bread	10
	Margarine	10
	Milk	200
1958	Baked ham	15
	Boiled potato	65
	Broccoli	25
	Pineapple tapioca, fortified	100
	Margarine	10
	Sweetened beverage [1]	5
	Sugar	23
1962	Lentil patty	50
	Ground Brazil nuts	20
	Boiled potato	65
	Green beans	40
	Margarine	10
	Applesauce	100
	Grape juice	100
	Almonds, ground	10
	Peanut butter fondant	20

[1] Kool Aid, General Foods Corporation, New York.

consumed during 14 consecutive 4-day periods. The minor element analyses reported here were conducted on food, feces and urine collected during the even-numbered periods (periods 2,4,6,8,10,12 and 14).

The 1958 study involved 12 girls maintained with a diet pattern of 6 menus consumed during 5 consecutive 6-day periods (periods 1–5) during which protein intake averaged 0.8 g/kg body weight. This was followed by three 6-day periods (periods 6–8) when protein intake was reduced to 0.6 g/kg.

The 1962 study involved 12 girls maintained with a diet pattern of 6-day periods during which protein intake was at 0.8 g (6 girls) and 1.4 (6 girls) g/kg body weight. A preliminary 6-day standardization period was included during which all children received controlled intakes of an all-plant protein diet composed of menu items common to the experimental diets.

The children serving as subjects were determined clinically to be normal, healthy individuals from families in a college community (Blacksburg, Virginia). Pre-experimental nutrient intakes, obtained by recall interviews with the mothers, indicated a satisfactory dietary history. The children were housed on the campus of the Virginia Polytechnic Institute and a summer camp atmosphere was maintained with scheduled recreational activities. Throughout the studies close supervision was maintained over the subjects to insure complete control over nutrient intake and collection of excreta.

Special precautions were used to safeguard against contamination of food or excreta with the minor elements under study. All glassware was cleaned with hot chromic acid. Pyrex glassware or stainless steel was used for food containers and for excreta collection. Ion-free water (distilled water passed through Amberlite MB-3) was used for sample dilutions and for rinsing utensils. The blades and bearing inserts in Waring Blendors were nickel- and gold-plated.[4]

Weighed aliquots of the blended food, feces, and urine composites, collected during each metabolic balance period, were wet-ashed in Pyrex beakers with redistilled nitric acid, 70% perchloric acid, and 30% hydrogen peroxide until a white ash was obtained. The ash was dissolved in hot 1:1 redistilled HCl:ion-free water, filtered, and made to volume. Aliquots of this solution were then used for the determination of Cu, Co, and Mn by AOAC methods (10). Mo was determined by the thiocyanate method of Evans et al. (11).

RESULTS AND DISCUSSION

The primary purpose of these studies was that of determining the influence of dietary protein intake level on the metabolic patterns of many other nutrients, many of which were maintained at constant intakes. With reference to the micronutrient elements under study here, however, no attempt was made to arrive at uniform intake. Rather, the amount of the respective elements furnished in the diet ingredients constituted the intake. These are shown in terms of concentration in the dry matter of the respective diets (table 2). The corresponding protein (nitrogen) intake is also shown as

TABLE 2

Subject weights and diet composition in dry matter, nitrogen, and micronutrients

Year	Diet [1] no.	No. of subjects	Avg wt of subjects	Nitrogen			Dry matter	Copper	Manganese	Cobalt	Molybdenum
				Animal	Plant	Total					
			kg	*g*	*g*	*g*	*g*	*ppm*	*ppm*	*ppb*	*ppb*
1956	2	3	24	5.4	2.3	7.7	410	2.63	5.10	32.4	177
	3	3	27	9.0	2.6	11.6	496	2.24	4.50	27.6	149
	4	3	31	6.7	2.6	9.4	397	3.03	6.99	36.5	189
	5	3	33	11.4	2.7	14.1	496	2.68	6.05	35.9	163
1958	8	12	28	1.8	1.7	3.5	480	2.23	6.02	17.9	99
	8'	12	29	1.1	1.8	2.9	460	2.20	6.30	17.4	94
1962	9 and 10	6	27	0	3.5	3.5	460	6.15	9.41	42.8	101
	11 and 12	6	29	0	6.3	6.2	480	8.06	10.66	42.5	148

[1] Dietary details given in (7, 8).

well as its distribution between plant and animal sources.

The diets used in 1958 and 1962 were developed to obtain nutrient metabolic patterns at protein intakes approaching estimated minimum requirements and with diets low in or devoid of animal protein sources. These diets were no more bulky (dry matter content) than diets relatively high in protein used in the diets in the 1956 study, diets not unlike those commonly consumed by children in the United States. The all-plant protein diets (1962 study) reveal a considerably higher content of Cu, Co, and Mn than those used in previous studies, a reflection of the relatively high concentration of these elements in such legumes as peanuts, soybeans, cashew and almond, protein sources common to the 1962 diets.

Table 3 summarizes the excretion pattern and balances for copper, manganese, cobalt and molybdenum. Only traces of copper were excreted via the kidneys. Manganese behaved similarly. Cobalt was partitioned approximately 90% in fecal and 10% in urine, whereas urinary molybdenum excretion exceeded that in feces by a factor of approximately two.

Some of the diets used represent rather drastic departures from common consumption patterns. It was therefore of interest to determine whether there were any significant changes in metabolic patterns for these minor elements during the course of these studies. Table 4 summarizes average retention patterns. In each case the average values are aligned from lowest to

highest. It is obvious from the scattering of period numbers that in most instances there was no indication of a trend toward either more efficient or less efficient utilization with time. The exception is evident with respect to copper in the 1962 study. Here a relatively high retention in early periods, one apparently unrealistic in terms of estimated accretion needs, is followed by significantly lower retentions during the final 3 periods of the study.

Individual subject variation also contributed significantly to total variation in the retentions observed for these minerals. Table 5 summarizes the average retentions for these minerals for each subject over the entire study, and the variations between subjects are evident.

Copper balance was generally not achieved by these children when intakes were as low as 1.0 to 1.3 mg/day (1956 and 1958 studies). Appreciable retention of this element was uniformly achieved at intakes of 3 to 4 mg/day in the 1962 subjects.

The average retention values shown in table 5 and the average intake data shown in table 3 suggest that for Cu, Co, and Mn there is a reasonable positive relationship between intake and retention (or excretion). Even though the intake variable for these respective elements may be confounded with year, it was deemed desirable to calculate regression of excretion on intake of these elements so as to arrive at estimates of intakes necessary for equilibrium. These are shown in table 6. The high correlation coefficients place consider-

TABLE 3

Average micronutrient (Cu, Mn, Co, Mo) balance (24-hour) in pre-adolescent girls

Year	Diet	No. of subjects	Copper				Manganese			Cobalt				Molybdenum			
			Intake	Fecal	Urine	Balance	Intake	Fecal	Balance	Intake	Fecal	Urine	Balance	Intake	Fecal	Urine	Balance
			mg	mg	mg	mg	mg	mg	mg	μg	μg	μg	μg	μg	μg	μg	μg
1956	2	3	1.08	1.12	0.02	−0.06	2.10	1.95	0.15	13.3	9.1	1.3	2.9	72.4	23.8	40.4	8.2
	3	3	1.10	1.12	0.01	−0.03	2.28	1.88	0.42	13.9	9.5	1.2	3.2	74.0	21.0	46.4	6.6
	4	3	1.20	1.22	0.01	−0.03	2.76	2.32	0.47	14.5	10.1	1.1	3.3	74.9	21.3	50.2	3.4
	5	3	1.33	1.31	0.02	0.00	3.00	2.56	0.49	17.8	11.6	1.9	4.3	80.8	21.4	56.5	2.9
1958	8	12	1.07	1.19	0.01	−0.13	2.89	2.07	0.82	8.6	7.2	0.5	0.9	47.7	8.1	28.3	11.3
	8'	12	1.06	1.08	0.01	−0.03	2.90	1.89	1.02	8.0	7.1	0.6	0.3	43.2	8.3	27.1	7.8
1962	9 and 10	6	2.83	2.16	0.01	0.66	4.33	2.58	1.75	19.7	12.2	0.3	7.2	46.5	11.7	22.7	12.1
	11 and 12	6	3.87	2.48	0.01	1.38	4.84	3.55	1.29	20.4	12.4	0.3	7.7	71.2	11.9	26.7	32.6

able reliability upon these estimates of dietary requirements for achieving equilibrium. It must be recognized, however, that such estimates do not include possible losses through integumental surfaces and do not allow for growth needs. No attempt at establishing an estimate for equilibrium for Mo was made in view of a relatively low correlation coefficient ($r = 0.46$). In contrast with the trend toward lower utilization of copper with time when intakes were between 3 to 4 mg/day, as already mentioned, there was no indication that rising intakes were influencing retention of Co or Mn over the range of intakes studied. Requirement estimates are difficult to achieve since only approximations can be made with respect to other factors, namely, losses of these elements through the skin and needs for growth.

With respect to Cu, if the body content for this element is accepted as approximately 3 mg/kg (12) the daily accretion necessary to meet growth needs is about 0.025 mg/day, a small value compared with the estimated daily copper loss via feces and urine of 0.6625 mg (extrapolation to zero intake, table 5). It appears to represent no more than 5% of the estimated endogenous loss via these routes. Thus the increment of Cu necessary for growth appears to be small. With respect to integumental losses, few reference data are available. Mitchell (13) observed sweat to contain 5.8 μg of copper/100 ml. If one assumes 350 ml insensible sweat loss for children (800 ml for adult men) in a comfortable environment, Cu loss via perspiration would be negligible. On the other hand, Cu losses via sweat have been reported to be as high as 1.9 mg/day in men with intakes of 3.5 g/day under conditions of high temperature (35–40°) and forced exercise (350–400 g sweat/hour).

The suggested Cu allowance of 2.5 mg (table 6) assumes that sweat losses may equal up to about 0.5 mg/day and allows an additional 0.7 mg as a margin of safety. The Cu balance trends noted in the 1962 study (intakes of 2.83 to 3.87 mg/day) suggest that intakes in excess of 2.5 mg/day would lead to increased fecal excretion.

TABLE 4

Period trends in micronutrient retention [1]

Copper — Period no. and avg balance, mg/24 hr

Study								
1956	6	8	12	10	14	2	4	
	−0.11	−0.08	−0.04	−0.03	−0.02	0.03	0.06	
1958	5	4	3	2	1	8	7	6
	−0.20	−0.14	−0.14	−0.13	−0.07	−0.04	−0.02	0.02
1962	5	7	6	2	4	3		
	0.28	0.34	0.51	0.87	1.78	2.35		

Manganese — Period no. and avg balance, mg/24 hr

Study								
1956	14	4	6	8	2	10	12	
	0.13	0.20	0.31	0.39	0.43	0.43	0.80	
1958	1	4	2	5	8	7	3	6
	0.6	0.7	0.8	0.8	0.9	0.9	1.1	1.2
1962	2	7	6	3	5	4		
	0.9	1.1	1.6	1.7	1.7	2.1		

Cobalt — Period no. and avg balance, μg/24 hr

Study								
1956	12	8	14	10	2	6	4	
	−0.2	2.1	2.3	2.9	4.3	5.6	7.0	
1958	7	6	2	3	4	5	1	8
	−1.5	−0.3	0.3	0.4	0.4	1.0	2.7	2.7
1962	7	5	2	6	4	3		
	3.5	5.1	7.3	7.3	10.0	11.9		

Molybdenum — Period no. and avg balance, μg/24 hr

Study								
1956	4	14	10	8	6	12	2	
	2.0	2.4	2.9	4.6	5.7	6.6	13.9	
1958	1	6	8	2	7	4	5	3
	−6.0	5.3	8.8	9.0	9.3	15.6	18.0	20.2
1962	3	6	4	7	5	2		
	17.5	19.9	21.5	21.6	22.0	31.2		

[1] Common underscore denotes nonsignificant difference.

TABLE 5

Average micronutrient retention by subjects

Year	Subject no.	Diet	Copper	Manganese	Cobalt	Molybdenum
			mg/24 hr		*µg/24 hr*	
1956	12	2	−0.06	0.09	2.3	6.3
	13	2	−0.09	0.13	3.4	11.9
	14	2	−0.03	0.20	3.1	6.4
	15	3	−0.04	0.43	3.3	7.1
	16	3	0	0.42	4.2	6.1
	17	3	−0.05	0.41	1.8	6.6
	18	4	−0.05	0.49	3.5	4.0
	19	4	−0.04	0.41	3.8	4.0
	20	4	0	0.49	2.6	2.1
	21	5	0.01	0.68	3.8	5.7
	22	5	−0.05	0.34	4.5	2.9
	23	5	0.06	0.44	5.0	0.3
1958	24	8	−0.12	0.92	−0.2	8.4
	25	8	−0.09	0.91	1.3	13.6
	26	8	−0.16	0.82	−1.1	13.0
	27	8	−0.14	0.88	0.8	8.2
	28	8	−0.20	0.90	1.5	13.6
	29	8	−0.13	0.74	0.4	13.2
	30	8	−0.20	0.77	−0.5	13.0
	31	8	−0.06	0.85	0.2	12.2
	32	8	−0.10	0.73	2.2	13.6
	33	8	−0.14	0.77	2.4	10.2
	34	8	−0.15	1.01	3.4	10.2
	35	8	−0.17	0.51	0.8	6.4

Year	Subject no.	Diet	Copper	Manganese	Cobalt	Molybdenum
			mg/24 hr		*µg/24 hr*	
1958	24	8′	−0.09	1.05	−0.9	6.7
	25	8′	−0.02	1.23	0.2	9.3
	26	8′	0.06	0.99	1.6	9.7
	27	8′	−0.13	1.03	−1.6	7.0
	28	8′	−0.04	1.02	0.4	8.7
	29	8′	−0.12	1.12	1.2	8.7
	30	8′	0.03	0.97	−0.6	12.7
	31	8′	0.01	0.98	0.1	6.3
	32	8′	−0.01	1.07	1.1	3.0
	33	8′	−0.04	0.82	0.5	4.7
	34	8′	0	0.93	0.8	10.0
	34	8′	0.01	1.03	1.0	8.3
1962	42	9	0.59	1.64	7.8	11.0
	44	9	0.79	1.95	7.2	10.2
	50	9	0.72	1.71	7.3	10.0
	40	10	0.68	1.98	7.0	16.7
	46	10	0.57	1.70	7.4	12.2
	49	10	0.66	1.54	6.6	12.0
	41	11	1.20	0.79	7.1	33.8
	43	11	1.24	1.53	6.3	28.5
	47	11	1.51	1.31	8.9	36.4
	39	12	1.57	1.53	7.7	29.7
	45	12	1.42	1.29	8.8	34.2
	48	12	1.31	1.25	7.7	32.9

TABLE 6

Regression analysis for deriving estimates of utilization, endogenous loss, equilibrium, and suggested allowances (excretion = y, intake = x)

	y intrecept,[1] x = 0	Slope [2]	Correlation coefficient	y = x [3]	Suggested [4] allowance
Copper	0.6625 mg	0.4914	0.984	1.3 mg	2.5 mg
Manganese	0.4162 mg	0.5976	0.942	1.0 mg	1.25 mg
Cobalt	4.3520 μg	0.4323	0.944	7.7 μg	15.0 μg

[1] Estimate of daily endogenous loss via feces and urine.
[2] Estimate of % utilization from the diet (1.0 − slope × 100).
[3] Estimate of daily intake required for equilibrium.
[4] See text.

The utilization value observed for Cu, 51% of intake (table 6), also the apparent absorption value since little Cu appeared in the urine, was as good from all-plant protein sources as from mixed plant-animal sources, assuming dietary protein level was without influence on the absorption of this element. The apparent absorption may constitute a considerable deviation from true absorption. The turnover studies of Cartwright and Wintrobe (15) indicate that three-forths or more of fecal copper represents biliary secretion.

The estimated manganese requirement for equilibrium was found to be 1.0 mg/child/day (table 6). Total body Mn is estimated to be only about one-tenth that of Cu (12) and hence the daily requirement for growth can be assumed to be even lower than that for Cu. Sweat losses can be estimated from the data of Mitchell (13) to be very similar to Cu under normal environmental conditions. In a hot environment and with exercise, daily Mn losses of 61 to 121 μg were observed by Consolazio and associates (14) when daily intake was 4.2 g. The suggested allowance of 1.25 mg Mn, half that for Cu, is consistent with its lower total body content and with its presence in lower concentration in sweat.

With respect to Co, the suggested allowance of 15 μg, or approximately twice the amount required for equilibrium, is consistent with the observation that hot climates may result in daily sweat losses of Co as high as 15 to 18 μg (14). Whether this element is of nutritional importance in its inorganic form is not known. It is of interest that of the total Co in the diets, less than 1% could be accounted for as vitamin B₁₂. Vitamin B₁₂ intake ranged between 0.65 and 4.4 μg/child/day, based on analyses of food composites and supplementary vitamin B₁₂ administered.

Although Mo is recognized as an essential element, no estimates have been made as to requirements. The balance data of table 3 show that there was considerable variation in retention of Mo from the various diets. All diets, however, resulted in positive balances, and hence the results support the view that storage of this element occurred when the intake approximated 100 μg/day.

The procedures used here in estimating requirements or allowances for these elements do not consider adaptive phenomena that may express themselves in the event that intakes of these elements should fall below the levels of intake characteristic of the diets used. There is lack of knowledge particularly concerning integumental losses with varying intakes. Also, it is appropriate to recognize that the values for daily losses of these elements under conditions of zero intake constitute extrapolations beyond the range of intakes studied (table 6) and their validity must await results of studies with diets free of these elements, a condition not easily achieved.

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Essential Fatty Acid Deficiency and Its Effects upon Reproductive Organs of Male Rabbits [1]

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ABSTRACT   To study the effects of essential fatty acid deficiency upon rabbits, especially upon spermatogenesis, five immature, male, New Zealand rabbits were fed a purified diet devoid of fat for 14 weeks. The fatty acids of the testes showed a marked increase of 5,8,11-eicosatrienoic acid and a decrease in the members of the linoleate family of fatty acids. Gross evidence of essential fatty acid (EFA) deficiency included diminished growth and feed efficiency, and loss of hair. Total lipids, phospholipids, and free cholesterol of testes were found to be decreased, whereas triglycerides followed the reverse pattern. No qualitative or quantitative differences in the fatty acid composition of seminal vesicles were found in the deficient and control groups. Testes of deficient animals showed an extensive degenerative change in the seminiferous tubules; no stage beyond secondary spermatocyte was evident. Glucose 6-phosphate dehydrogenase and Δ^5-3β-hydroxysteroid dehydrogenase activity of Leydig cells in both groups showed that these enzymes were present. Male accessory gland weights were significantly reduced in the deficient animals, indicating reduced androgen secretion. Histological examination of the anterior pituitary gland showed signs of degenerative changes in PAS-positive basophilic cells, which appeared to be shrunken and partially degranulated in EFA-deficient rabbits, suggesting that EFA deficiency is accompanied by diminished secretion of the anterior pituitary. There was also an increase in the number of chromophobes and a decrease in acidophil cells. These observations suggest that the degeneration of gonads observed during essential fatty acid deficiency may be due to primary impairment of anterior hypophyseal function.

The effects of essential fatty acid (EFA) deficiency in rabbits have not been previously reported in full. In studies on experimental atherosclerosis in rabbits, Wigand (1) fed a semipurified diet containing 8% of various fats. He observed that in those groups fed glyceryl trilaurate or hydrogenated coconut oil, the rabbits grew poorly and lost hair. When the animals were fed corn oil these symptoms did not occur. The loss of hair in rabbits fed trilaurin could be prevented by supplements of corn oil or linoleate to the trilaurin diet. These results were in agreement with those of Lambert et al. (2) who observed that rabbits fed a semipurified diet (3) containing 20% hydrogenated coconut oil developed a "ruffled" appearance, lost hair and had a poorer feed efficiency than did comparable groups fed safflower oil. It appears likely that these symptoms were the result of essential fatty acid deficiency. In experiments from this laboratory, EFA deficiency has been induced in rabbits fed a semipurified diet, but the results have been published only in abstract form.[4] The diet contained 2% hydrogenated coconut oil as the sole source of fat, plus 1% of cholesterol, and within 3 months the rabbits developed skin eruptions on the feet, and lost hair within an additional month. They became emaciated, had excessive loss of hair and some died. On the contrary, deficient rabbits given 2% corn oil for the fourth month showed new hair growth and the skin lesions disappeared. One purpose of the present study was to describe physical and biochemical changes in the EFA-deficient rabbit.

Considerable evidence is available which indicates impairment of reproductive function in EFA-deficient rats of both sexes. In male rats, complete absence of sperma-

tozoa and marked degeneration of the seminiferous epithelium were observed. (4). Histological examination of pituitary glands of these rats revealed the presence of typical "signet ring" cells similar to those found in the castrate animal (5). However, Greenberg and Ershoff (6) noted no significant difference in the histological picture of the testes of rats fed a diet deficient in EFA and those fed a natural diet, but observed a reduction in the weight of male accessory glands in the deficient animals. As these changes were reversed by use of chorionic gonadotropin, the investigators concluded that the primary failure was due to insufficiency of pituitary gonadotropin(s). Female rats deprived of dietary EFA ovulated irregularly, and had prolonged gestational periods (7, 8). Histological examination of the ovaries of such fat-deficient rats revealed the presence of wheel cell or "deficiency nuclei" similar to those seen following hypophysectomy (9, 10). On the basis of this observation and quantitative analysis of pituitary cell types (10), these authors concluded that fat deficiency causes a reduction in the secretion of luteinizing hormone, and consequently results in loss of ovarian function. From the evidence presented above, it is difficult to conclude whether the hypophysis is the primary target of EFA deficiency, with changes in the seminiferous tubules occurring secondarily, or whether there is a primary hypogonadism followed by the pituitary changes.

The second object of this study was to elucidate more fully the relationship of EFA and the reproductive gland using available biochemical, histological and histochemical methods. Rabbits were chosen for this objective because they could be made EFA-deficient (1–3) and are large enough to be used subsequently for studies of semen lipids.

MATERIALS AND METHODS

Ten 3-month-old male New Zealand rabbits, weighing between 1,100 and 1,300 g, were divided into 2 groups of five each. One group was fed a commercial diet [5] and the second group was fed a fat-free pelleted diet [6] colored green, and which contained 21.1% vitamin-free casein, 4.0%

salt mixture, 58.45% sucrose, 16.45% non-nutritive bulk.[7] Each kilogram of diet contained: vitamin A, 20,000 IU; vitamin D, 26,000 IU; and (in grams) choline chloride, 6.0; nicotinic acid, 0.6; inositol, 0.3; and (in milligrams) tocopherol, 225; menadione, 2.25; thiamine·HCl, 22; pyridoxine·HCl, 22; riboflavin, 22; and Ca pantothenate, 44. Each kilogram of salt mixture contained: (in grams) Ca carbonate, 68.6; Ca citrate, 308.3; Ca biphosphate, 112.8; magnesium carbonate, 35.2; magnesium sulfate, 38.2; potassium chloride, 124.7; dibasic potassium phosphate, 218.8; sodium chloride, 77.1; ferric ammonium citrate, 15.3; cupric sulfate, 0.078; manganese sulfate, 0.20; ammonium alum, 0.092; potassium iodide, 0.04; and sodium fluoride, 0.51. This diet was known from previous experiments to induce EFA deficiency in rats. The animals were fed measured quantities of food and water ad libitum. At the end of 10 weeks when the experimental animals began to lose their fur, a biopsy was performed on the testes of animals, two from each group. The tissues were fixed in Bouin's fixative and the sections were cut and stained with hemotoxylin and eosin. A second testicular biopsy was performed on the same animals 3 weeks later. After an additional week, the animals were killed by cervical dislocation. The testes were removed immediately, cleaned and weighed. For histochemical study one-half of each testis was transferred to a Petri dish placed on dry ice; for histological study a small cross section of a portion of the remaining testis from each animal was transferred to Bouin's solution. The remaining testis tissue from each animal was weighed and transferred to individual vials containing normal saline and kept frozen until analysis was performed. The seminal vesicles were weighed and stored similarly. Prostate gland weights were also recorded but were not analyzed. All the pituitaries were dislodged, weighed and the anterior portion was then fixed in Bouin's solution for histological study. Animal body weight, food consumption and EFA deficiency

symptoms were recorded at weekly intervals. Periodically, receptive females were caged with the males to observe the libido of the latter.

Extraction, separation and estimation of total lipid and lipid fractions. The samples of testis tissue and of seminal vesicles from each animal were thawed, homogenized separately in a tissue grinder, and the lipids extracted according to the procedure of Folch et al. (11). Due to the small amount of lipids available in the seminal vesicles, quantitative determination of total lipids and lipid classes was attempted on the testes only. An aliquot of each sample was used to determine the total lipid gravimetrically, expressed as percentage of wet tissue weight. Another aliquot was used to separate neutral lipids and phospholipids on silicic acid column chromatography. The solvent system and elution techniques are described elsewhere (12).

Gas-liquid chromatography. The lipids of the testes and the seminal vesicles were converted into methyl esters by transesterification with 5% methanolic HCl. The esters were analyzed on Beckman GC-2A and Research Specialties gas chromatographs equipped with hydrogen flame detectors. An aluminum column 180 cm × 0.62 cm was packed with 20% ethylene glycol succinate and 2% phosphoric acid on Gas Chrom P, 80 — 100 mesh. The flow rate of helium was 80 ml/minute, and the column temperature was 190 to 195°. The esters were identified by equivalent chain length (13) and internal standards were used whenever feasible. If the identification was in doubt, the peak ester was isolated and hydrogenated to determine the chain length. The data are reported as area percent of total fatty acids. Amounts less than 0.1% are expressed as trace. Five samples of testes from each group were analyzed and, in the case of seminal vesicles, 5 samples from the control and 4 samples from the deficient animals were analyzed.

Histological studies. The testes and pituitary glands were fixed in Bouin's solution for 24 hours for routine tissue sectioning. The testes sections were stained by hemotoxylin and eosin, and pituitary

sections were stained by Periodic Acid Schiff (PAS) and by orange G.

Histochemical studies (glucose 6-phosphate dehydrogenase and Δ^5-3β-hydroxysteroid dehydrogenase). The fresh frozen tissues (16 µ) were cut in a cryostate at
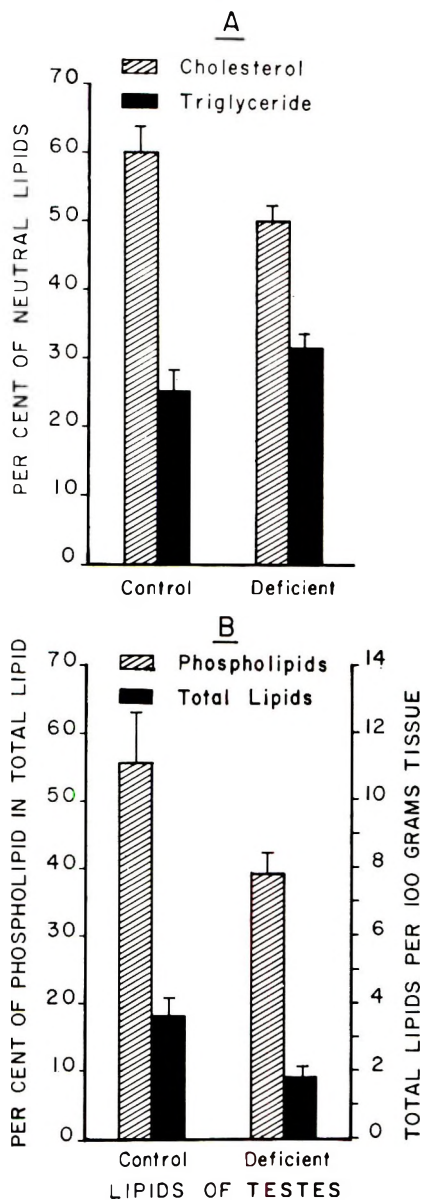
Fig. 1 Lipids of testes in the normal and EFA-deficient rabbits. (A) Percentage of cholesterol and triglyceride in neutral lipids. (B) Total lipids per 100 g of tissue and percentage phospholipids in total lipids.

−18° and placed on a cover slip to dry. The sections were incubated in a medium for 20 minutes for G-6-P dehydrogenase and one hour for 3β-ol-enzyme. Adequate controls were run in each case. The formula and procedure for preparation of medium are similar to those described by Deane et al. (14) for G-6-P dehydrogenase and Nachlas et al. (15) for 3β-ol-enzyme.

RESULTS

The average weight gain during 10 weeks for the deficient animals was 1,300 g, and 2,200 g for the controls. The food consumption of the groups was not significantly different. Feed utilization was approximately 18.0 g weight gain/100 g of diet for the controls and 10.0 g/100 g for the deficient animals. No data of water consumption were recorded. In the deficient animals the livers weighed almost twice as much as those of controls, and the surfaces were covered with small brownish spots. Hemorrhagic spots were visible in the kidneys and the spleens.

Figure 1 summarizes the analyses of total lipids and major lipid classes in the testes. Values for total lipid, percentage of phospholipids in total lipid and percentage of free cholesterol in neutral lipids, were larger for the controls. Neutral lipid and

percentage of triglycerides in neutral lipid were less for the controls. Other lipid classes, such as diglyceride, free fatty acids and cholesterol esters, were present in small amounts and were not measured.

Fatty acid content of the total lipids of testes and seminal vesicles are shown in table 1. In general, the pattern of fatty acid composition of normal rabbit testes resembles that for testes of other laboratory and domestic animals. Seminal vesicle fatty acids were perdominantly unsaturated and resembled those in other reproductive organs. The effect of deficiency upon fatty acid pattern of testis lipids is similar to that noted in rats. EFA deficiency in the rabbit caused diminished content of fatty acids related to linoleate and increased $16:1\omega7$, $18:1\omega9$ and $20:3\omega9$. These compositional changes are characteristic of EFA deficiency in several species.

Microscopic examination of the testes revealed a number of significant findings (fig. 2). In the deficient animal extensive degenerative changes were noted in the seminiferous tubules. Spermatogenesis did not progress beyond the stage of secondary spermatocytes. The intertubular spaces were wide, and the tubules appeared to be smaller than in the controls. There was no

TABLE 1

Fatty acid composition of total lipids of testes and seminal vesicles of rabbits

Fatty acids	Testes		Seminal vesicles	
	Control [1]	Fat-deficient [1]	Control [1]	Fat-deficient [2]
12:0	1.4 ± 0.2 [3]	2.3 ± 1.0	2.8 ± 0.8	2.9 ± 0.2
14:0	1.1 ± 0.2	2.8 ± 1.8	1.0 ± 0.2	1.9 ± 0.4
unknown	1.2 ± 0.2	1.9 ± 0.5	1.2 ± 0.2	2.4 ± 0.8
16:0	24.9 ± 6.0	23.1 ± 4.5	23.2 ± 3.0	24.1 ± 3.7
16:1ω7	1.7 ± 0.7	6.9 ± 1.5	2.5 ± 0.2	3.8 ± 2.0
18:0	11.1 ± 2.0	14.2 ± 4.0	15.8 ± 2.1	13.9 ± 0.8
18:1ω9	12.6 ± 2.2	18.3 ± 4.9	18.2 ± 2.0	19.3 ± 0.8
18:2ω6	10.8 ± 2.1	5.9 ± 0.5	12.6 ± 2.0	15.2 ± 0.3
18:3ω3	1.3 ± 0.4	tr	1.3 ± 0.6	2.0 ± 0.2
20:2ω9	tr	tr	absent	absent
20:3ω9	2.3 ± 0.8	19.5 ± 5.4	tr	tr
20:3ω6	5.8 ± 0.7	tr	2.9 ± 0.2	2.6 ± 0.5
20:4ω6	9.1 ± 1.9	2.7 ± 1.0	7.1 ± 2.8	6.7 ± 0.3
20:5ω3	1.8 ± 0.5	tr	3.9 ± 0.4	2.3 ± 0.2
22:4ω6	2.8 ± 0.7	tr	1.7 ± 0.4	4.0 ± 2.0
22:5ω6	14.8 ± 2.6	3.2 ± 1.2	5.6 ± 0.3	3.7 ± 1.7
22:5ω3	1.0 ± 0.3	tr	1.5 ± 0.5	1.4 ± 0.3
22:6ω3	1.3 ± 0.2	absent	3.1 ± 0.3	2.6 ± 1.1

[1] Five rabbits/group.
[2] Four rabbits/group.
[3] Averages ± SE of mean.
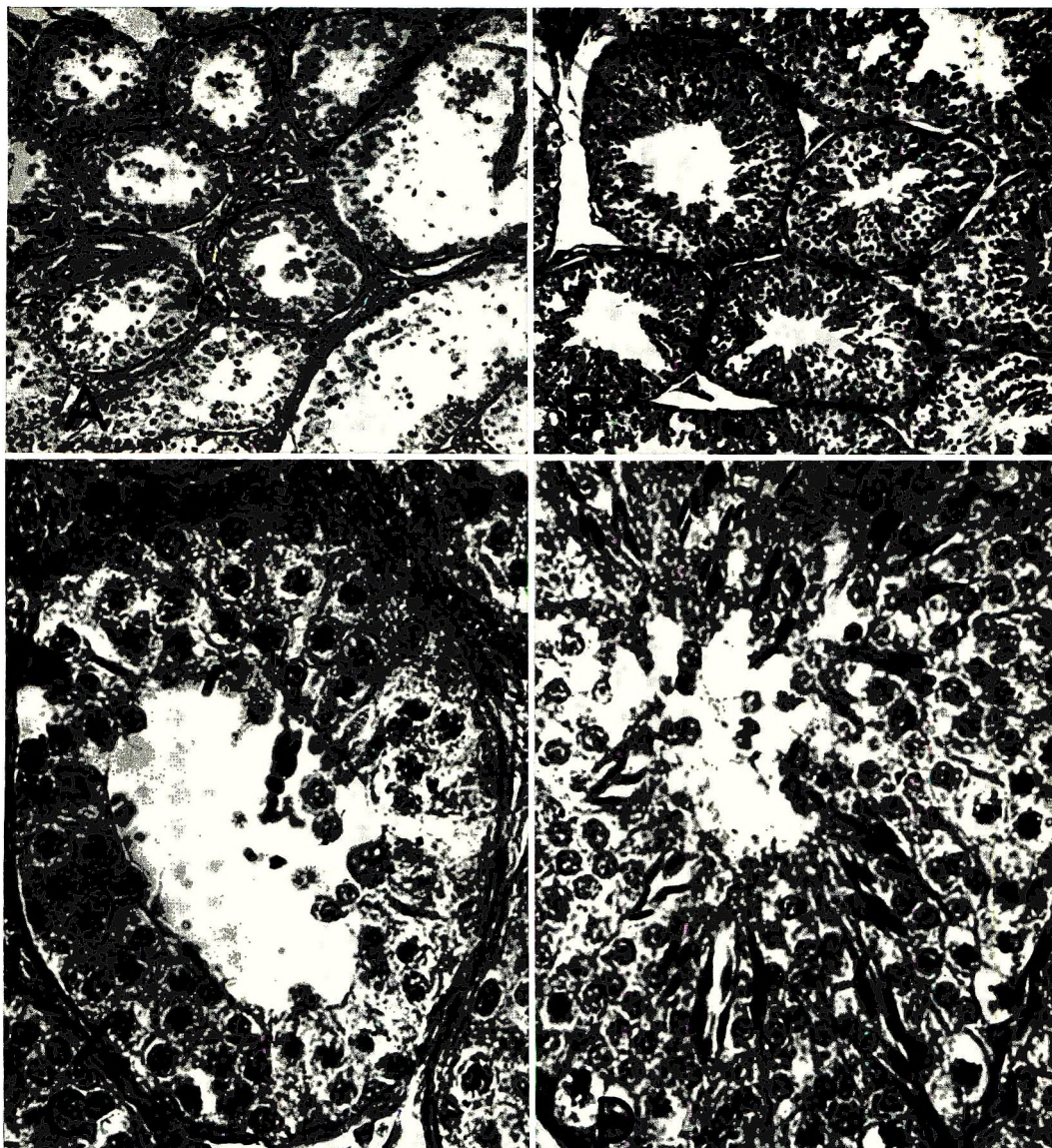
Fig. 2 Microscopic section of seminiferous tubules of a deficient and control rabbit, stained by hematoxylin and eosin. (A) Note the degenerative changes in the tubules of the deficient animal. No stage beyond secondary spermatocyte is detectable. Intertubular spaces are wide and tubules appear to be smaller. × 25. (B) Normal control. Note active spermatogenesis in all tubules. × 25. (C and D) Tubule from the same general areas shown in A and B magnified to show differences more clearly. × 400.

change in the number or size of the interstitial cells (Leydig cells). The spermatogonia and primary spermatocytes appeared normal in shape and size. A tubule (×400) from the same general area (fig. 2, C and D) shows these differences more clearly.

The histological picture of anterior pituitary stained by PAS technique (fig. 3) showed degenerative changes in the PAS-positive basophils in the deficient animals. These cells were reduced in size and partially degranulated. In addition to this,
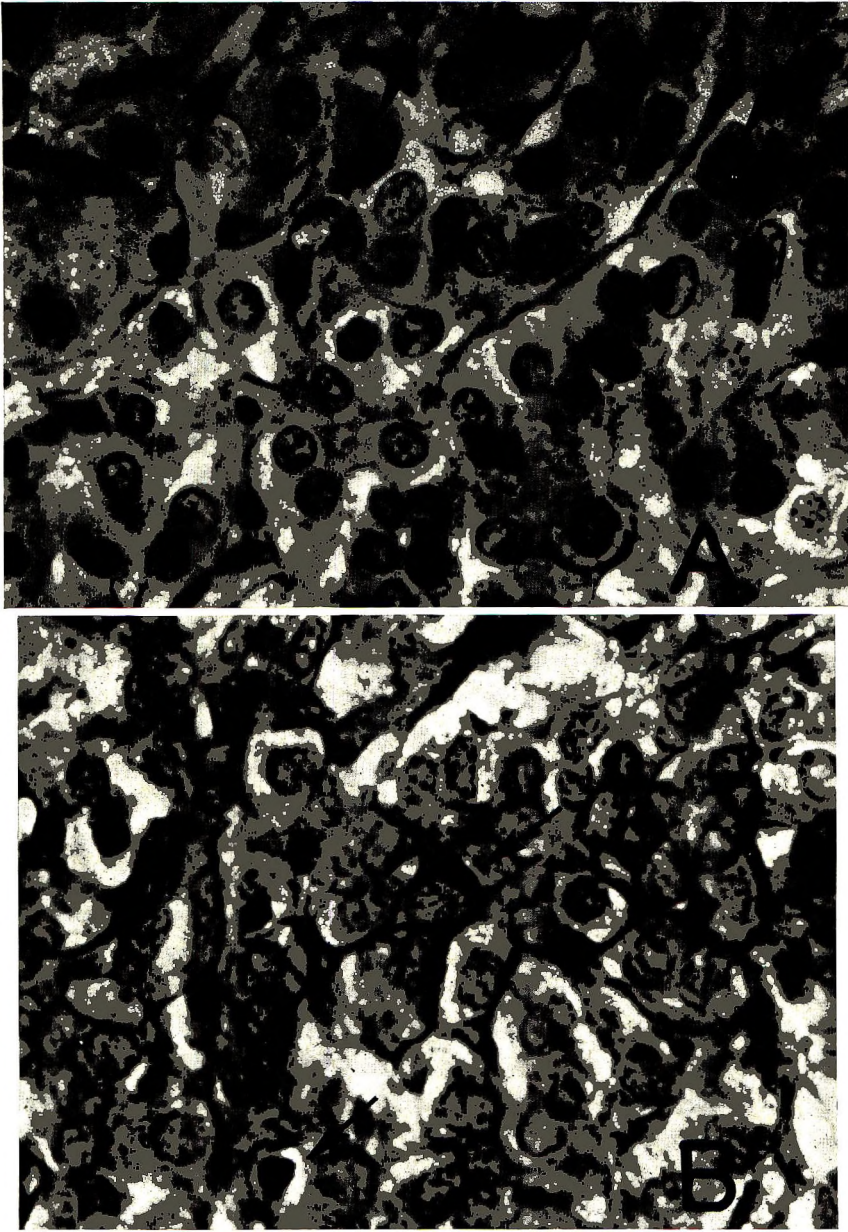
Fig. 3 (A) Pituitary of control rabbit. Note large, well-granulated basophils (arrows) and distinct cell boundaries. PAS-orange G. × 400. (B) Pituitary of rabbit fed fat-deficient diet. Basophils (arrows) are small and shrunken. Note that most of the gland is composed of large chromophobes with irregular, indistinct cell boundaries. PAS-orange G. × 400.

there was marked reduction in the number of acidophil cells and a significant increase in the chromophobes.

Histochemical studies of G-6-P dehydrogenase and 3β-ol-enzyme activity (fig. 4, A, B) showed that the enzymes necessary for synthesizing androgens were present in both groups, but whether the hormones were actually being released and in what quantities could not be ascertained.
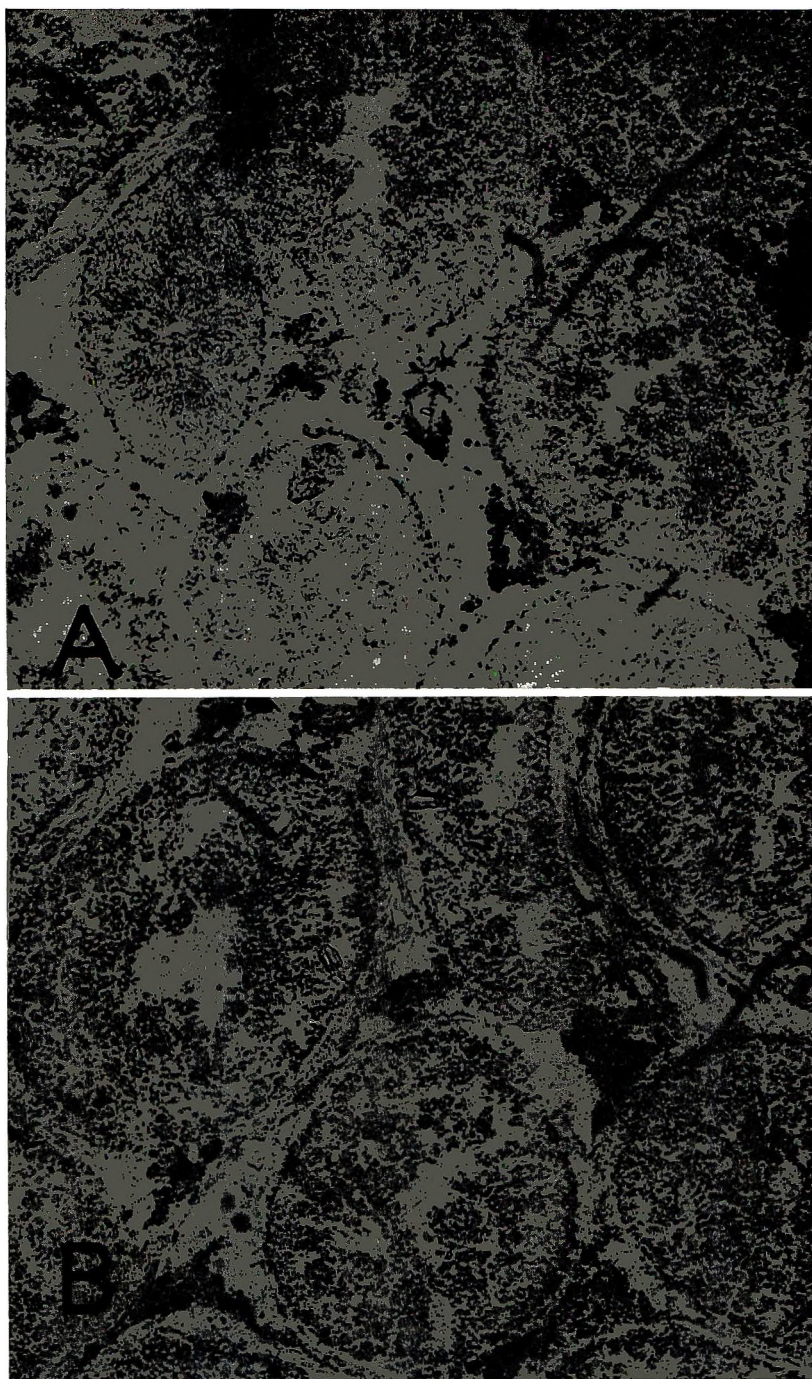
Fig. 4   (A) Activity of 3β-enzyme with 3β-OH, androst-5-en-17-one (DHA) in the testes of control rabbit. × 25. (B) Note no change in the 3β-enzyme activity in the deficient animal. × 25.

TABLE 2

Weights of organs of control and fat-deficient animals

Organ	Control	Fat-deficient	No. animals	P value
Seminal vesicles, mg	300 ± 19 [1]	248 ± 8	4	< 0.05
Prostate, mg	424 ± 22	324 ± 19	4	< 0.05
Testes, g	5.7 ± 1.1	4.3 ± 0.3	5	> 0.05
Pituitary, mg	29.0 ± 0.4	27.0 ± 1.0	4	> 0.05
Liver, g	52.0 ± 4	101 ± 8	5	< 0.05

[1] Averages ± SE of mean.

In the deficient animals the male accessory gland weights (table 2) were significantly reduced. No significant differences were noted in weights of pituitary gland and testes in either group.

DISCUSSION

This study indicates that rabbits, like rats, can develop a dietary deficiency of EFA, although a few differences in the nature of the symptoms were observed. For instance, dermatological changes were less evident in the rabbit than in the rat, although weight loss appeared earlier in the rabbit (16). Total lipids and phospholipid levels in EFA-deficient rabbits were somewhat higher than those reported by Bieri and Prival (17). The less efficient utilization of food in the deficient rabbits paralleled that observed in rats (18).

The diminished phospholipid in the testes of the deficient animals suggests that phospholipids are closely related to function. This conclusion is substantiated by observations of an increased phospholipid content in the frog ovary during production of ova (19) and in the chick brain during embryonic development (20, 21). Phospholipid increases in the growing bovine testis and decreases during testicular degeneration (12) and in the degenerating postpartum corpus luteum.[8]

As in other species studied (17, 22), the fatty acids of the rabbit testis were highly unsaturated. The effects of EFA deficiency on the fatty acids of testes included a decrease in the linoleate family of fatty acids and an increase in the oleate family. There was a marked increase in eicosatrienoic acid of testis lipid which is also characteristic of tissues of the EFA-deficient rat (23). Saturated fatty acids increased in the testis lipids of deficient animals although the longer-chain fatty acids present in

small amounts in the normal testis disappeared. The testis fatty acids of control rabbits used in this study contained eicosatrienoic ($\omega 9$) [9] acid and some long-chain fatty acids which have not been reported previously (17). The fatty acid composition of the rabbit seminal vesicle did not appear to be significantly affected by EFA deficiency.

As in the fat-deficient rat (6), the weight of the accessory reproductive organs was reduced in the deficient rabbits. This indicates a deficiency in production or release of androgens (24). Although the testes of the deficient rabbits did not change in weight significantly, they exhibited failure of spermatogenesis beyond the spermatocyte stage. Similar observations have been made in the rat (4, 25) which are compatible with failure of gonadotropic stimulation (26–28).

The moderately intense G-6-P dehydrogenase and 3β-ol-steroid dehydrogenase activity observed in the Leydig cells of the deficient animal is worthy of note because it indicates that the enzymatic mechanism for androgen production was at least partially intact. However, the absence of libido in the deficient males, and the decrease in seminal vesicle weight, were good indications that androgen release was not occurring (29).

The changes produced by fat deficiency in the pituitary are difficult to interpret without bioassays of the hormones actually present. The signet ring cells seen in the fat-deficient rat (4) and in the castrated rabbit (30, 31) were not observed in this

[8] Unpublished observations.

[9] In this notation, 20 indicates chain length, 3 indicates number of double bonds, and $\omega 9$ indicates the first double bond from the terminal methyl group is at the ninth carbon atom. In PUFA all double bonds are assumed to be *cis* and methylene interrupted. Thus $\omega 9$ denotes oleate family, $\omega 6$ is linoleate, and $\omega 3$ linolenate family.

study. The PAS-positive basophil cells were shrunken and partially degranulated. This may indicate reduced gonadotropic activity of these cells. In PAS staining it is difficult to identify the granules in the basophils producing FSH and LH; therefore, in this study all basophil granules were considered together. The degranulation of basophils indicates either reduced secretion of LH or FSH or both. Lack of LH would inhibit androgen secretion of the testes which might result in hypogonadism. Our histological results on the testes and weights of accessory glands are compatible with the changes observed in the pituitary. Therefore, in EFA deficiency in the rabbit the deficiency of androgen secretion may be due to lack of LH from the pituitary, and this possibility is now being tested experimentally in rabbits. This has been indicated by studies in EFA-deficient rats (4) in which testosterone administration repaired the testicular damage to a greater degree than did the gonadotropins. Many studies have been reported in the literature to relate reproductive failures with vitamin deficiency, undernutrition and complete starvation. None of these conditions was a contributory factor in this study; yet some of the effects, at least upon the hypophysis, are similar.

This study has demonstrated that rabbits can easily be made deficient in EFA. Beginning with half-grown animals, distinct morphological and chemical changes gave evidence that the EFA deficiency was induced in a shorter period than in rats of a similar age. Fatty acid composition of seminal vesicles showed only minor difference between groups, indicating that this tissue is more resistant to changes in dietary lipid than testes in which marked changes were caused by EFA deficiency. Histological examination of testes also showed degenerative changes, leading to the conclusion that rabbits are good subjects for the study of the role of EFA in reproduction.

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Body Weight Changes, Pancreas Size and Enzyme Activity, and Proteolytic Enzyme Activity and Protein Digestion in Intestinal Contents from Calves Fed Soybean and Milk Protein Diets [1,2]

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ABSTRACT   Milk and soybean sources of dietary protein for calves were compared on the basis of growth, pancreatic enzymes and intestinal proteolysis. Thirty-six 3- to 5-day-old calves were fed either whole milk or milk substitutes containing protein from skim milk and whey powder (all-milk), 60% supplied by a 50% crude protein soybean flour (soy-50), or 86% supplied by a 71% crude protein soybean flour (soy-71) and killed after 1, 3 or 5 to 6 weeks of feeding. Calves lost body weight with the soy-50 diet, but weight gains with the soy-71 diet equaled those with whole milk. Trypsin and chymotrypsin activities of the pancreases and intestinal contents from calves fed the soy-50 diet were less than those from calves fed the other diets. Also, more trypsin and chymotrypsin activity were destroyed, and less protein was digested during a 2-hour in vitro incubation of intestinal contents from calves fed the soy-50 diet. In vitro digestion of intestinal protein from calves fed the soy-71 diet and killed after 1 or 5 to 6 weeks of feeding equaled that in digesta from calves fed milk protein diets. Results obtained with calves fed the soy-50 diet were attributed in part to the high soybean trypsin inhibitor content of the soybean flour in this diet. The 71% crude protein soybean flour contained negligible amounts of soybean trypsin inhibitor.

Milk substitutes (replacers) containing dried milk with or without plant by-products supplemented with minerals, vitamins and antibiotics are widely used in place of cow's milk to rear young calves (1, 2). Inferior growth and feed utilization by calves fed diets containing plant nutrients (3) led to the hypothesis that certain digestive enzymes were not synthesized in sufficient quantities. Supplementation of vegetable-type milk replacers with exogenous digestive enzymes has not increased growth rate of calves (1). Williams and Knodt (4) reported the calves did not grow with rations containing raw soybeans. Physiological and biochemical changes in the pancreas and digestion in calves in response to soybean trypsin and growth inhibitors have not been studied previously. The present report compares changes in pancreas size and proteolytic enzyme activity in the pancreas and in intestinal contents of calves fed milk or soybean protein diets. Protein digestion and enzyme stability in small intestinal contents were determined in vitro.

MATERIALS AND METHODS

Holstein bull calves (3 to 5 days of age) were fed one of 4 diets and killed after 1, 3 or 5 to 6 weeks on trial (table 1). The calves were fed either whole milk, or milk substitutes containing protein from skim milk and whey powder (all-milk); 60% of the protein supplied by a 50% crude protein soybean flour (soy-50); or 86% of the protein supplied by a 71% crude protein soybean flour (soy-71).[4] The all-milk and the soy-50 (high soy) diets were from the same batches as those described previously (5). When high levels of soybean trypsin inhibitor were observed for the 50% protein soybean flour in the soy-

50 diet, it was deemed essential to introduce another soy diet (soy-71) (table 2) in which protein was supplied by a soybean flour nearly free of trypsin inhibitor. Calves were not fed this diet until after data from the other 3 diets had been collected. Calves were fed whole milk at the rate of 10% of body weight per day. The milk substitutes were reconstituted to 12 to 14% solids in water at 37° just before feeding. The amount fed (in grams air-dried diet/day) at specified ages (in days)

TABLE 1

Number of calves fed the 4 diets for each time-period indicated and included in final analyses

	Number of calves		
Diet	Feeding duration, weeks		
	1	3	5–6
Whole milk	3	4	4
All-milk replacer [1]	3	3	3
Soy-50 replacer [2]	4	3	2
Soy-71 replacer [3]	2	3	2

[1] Skim milk and whey powder.
[2] 60% of total protein supplied by a 50% crude protein soybean flour, and 40% of protein supplied by skim milk and whey powder.
[3] Promosoy, Central Soya, Decatur, Illinois; percentage composition (air-dried basis): protein, 71; fat, 0.5; fiber, 3.7; ash, 6.3; and carbohydrate, 18.1.

TABLE 2

Composition of soy-71 milk replacer diet

	kg
Soybean flour [1]	25
Fat premix [2]	33
Glucose monohydrate [3]	33.25
Vitamin-mineral premix [4]	4.0
B vitamin complex [5]	2.0
Trace mineral salt	2.0
DL-Methionine	0.5
Aurofac-10 [6]	0.25
Total	100.00
Kjeldahl protein (N × 6.25)	19.5

[1] Promosoy, Central Soya, Decatur, Illinois; percentage composition (air-dried basis): protein, 71; fat, 0.5; fiber, 3.7; ash, 6.3; and carbohydrate, 18.1.
[2] 30% fat premix (mixture of dried whey and fat), supplied by Milk Specialties, Inc., Dundee, Illinois.
[3] Cerelose, Corn Products Company, Argo, Illinois.
[4] The premix contained: (in grams) thiamine, 55; menadione, 9.9; vitamin A (30,000 IU/g) + vitamin D (2,800 IU/g) + dl-α-tocopheryl acetate (82 IU/g), 1100; K citrate, 3438; Na_2SeO_3, 0.624; $Al_2(SO_4)_3$·18H_2O, 300; H_3BO_3, 10.5; Na_2MO_4·2H_2O, 10.5; pyridoxine·HCl, 11.6; NaBr, 20.9; ascorbic acid, 57.2; inositol, 286; folic acid, 1.1; p-aminobenzoic acid, 28.6; biotin, 5.5; vitamin B_{12} (0.1% trituration of cobalamin), 31.4; (in kilograms) K_2HPO_4, 12.5; MgO, 6.24; and glucose monohydrate, 21.3 (vitamins supplied by Merck Sharp and Dohme, Rahway, N. J.).
[5] Dawes Lab, Inc., Chicago, containing (mg/454 g) riboflavin, 2,000; pantothenic acid, 4,000; niacin, 9,000; and choline chloride, 90,000.
[6] American Cyanamid Company, Princeton, New Jersey, containing 10 mg Aureomycin/454 g.

was as follows: 280, 3 to 7 days; 420, 8 to 14; 560, 15 to 21; 840, 22 to 28; 980, 29 to 35; 1120, 36 to 42. Any deviations from this schedule were dictated by calf size and incidence of diarrhea.

Calves were killed 1 to 1.5 hours after the morning feeding when maximal flow of digesta from the abomasum to the duodenum was reported to occur (6). Pancreases were removed from the calves immediately and placed on ice. Connective tissue, lymph nodes and large blood vessels were removed before weighing these glands. Pancreatic tissue slices from calves fed whole milk, all-milk and soy-50 diets were incubated for three hours at 37° in Krebs bicarbonate medium saturated with 95% O_2 and 5% CO_2. The purpose of the incubation was to study in vitro synthesis of pancreatic enzymes, but net de novo synthesis was negligible. Tissue slices plus incubation media were homogenized in 0.15 M NaCl and centrifuged at 1300 × g for 30 minutes at 5° to remove cellular debris. Data from nonincubated tissue slices were not used, since considerable enzyme activity was lost in the precipitate during centrifugation (7). Homogenization of pancreatic tissue in 0.15 M NaCl containing 0.1% Triton X-100 (7) had been adopted when calves fed the soy-71 diet were killed. Both homogenization procedures, however, gave comparable enzyme activities in the supernatant following centrifugation (7). Trypsinogen and chymotrypsinogen in the pancreases from calves fed whole milk, all-milk and soy-50 diets were activated with a crude enteropeptidase at 37° for 1 hour according to the procedure of Gorrill and Thomas (7). The activation procedure had been improved by the addition of 0.05 M $CaCl_2$ and lowering the temperature to 4° (7) when pancreases from calves fed the soy-71 diet were assayed. Since this modified procedure averaged 30% higher trypsin and chymotrypsin activities, values for pancreases from calves fed the soy-71 diet were reduced by this amount to make a valid comparison between all 4 diets. Esterase activities of trypsin and chymotrypsin were assayed spectrophotometrically (7), with tosyl arginine methyl ester (TAME) and benzoyl tryosine ethyl ester

(BTEE), dissolved in 56% methanol (v/v) as substrates, respectively.

The small intestine was tied at intervals to prevent movement of digesta before removal from the animal, and then divided into 3 sections of equal length (upper, middle, lower). Intestinal contents from calves fed whole milk, all-milk and soy-50 diets were centrifuged at $1300 \times g$ for 30 minutes at 4° to remove solid feed particles before further analysis. Contents from calves fed soy-71 were centrifuged before enzyme assays, but protein was determined on noncentrifuged samples. A portion of the contents from each section of the small intestine was stored at 4° and another portion was incubated at 37° for 2 hours. Trypsin and chymotrypsin activities were determined in both portions. The first 3 to 4 minutes of enzyme reaction rates (changes in absorbancy per minute) for intestinal contents from calves fed soy-50 were disregarded to minimize the error due to an interfering substance (7). Corrections for this nonenzymic activity of intestinal contents from calves fed soy-71 were made by running blank enzyme reactions (that is, minus TAME and BTEE).

In vitro protein digestion was calculated from the change in Kjeldahl protein (N × 6.25) during incubation of intestinal contents. Nonprotein nitrogen was removed by adding an equal volume of 11% trichloroacetic acid to from 1 to 5 ml of intestinal contents. After 30 minutes the samples were centrifuged at $1300 \times g$ for 30 minutes. The supernatant fluid was discarded; the precipitate was washed with 5% trichloroacetic acid and centrifuged again. The final precipitate was frozen to facilitate its quantitative removal from the test tubes for Kjeldahl digestion.

The 3 milk substitute diets and abomasal contents from calves fed all 4 diets were fractionated into a whey solution according to the scheme of Garlich and Nesheim (8). Abomasal and intestinal contents from calves fed the 2 soy diets were also extracted according to Alumot and Nitsan (9). The final ammonium sulfate precipitate was dissolved in 0.1 M (pH 7.6) Tris buffer (Tris(hydroxymethyl)amino methane) for trypsin inhibitor assay. The trypsin inhibitor activity of the extracts was compared with that produced by known amounts of purified soybean trypsin inhibitor (SBTI) as described previously (5). Whey extracts of the diets and abomasal contents were assayed for protein by the semimicro-Kjeldahl method (N × 6.25).

RESULTS

The soy-50 diet produced varying degrees of diarrhea in calves at all times, and the all-milk diet resulted in periodic diarrhea. Little or no scouring was evident in calves reared with whole milk or the soy-71 diet. There was no curd formation in abomasal contents from calves fed the 3 milk replacer diets and killed 1 to 1.5 hours after feeding. Abomasal contents from calves fed whole milk were almost completely coagulated. Digesta from the abomasum (table 3) and small intestine of calves fed whole milk had lower pH values than those from calves fed the other diets. Upper intestinal contents from calves fed whole milk, all-milk and soy-50 were 1.1, 0.4 and 0.7 pH units higher, respectively, than values for abomasal contents from calves fed these diets. Digesta from these 2 sections of calves fed soy-71 had the same pH (6.2). Contents of the lower intestine were always above pH 7. Age of calves had no effect on pH of digesta in the abomasum or small intestine. Calves fed the soy-50 diet lost body weight for the duration of the trial (table 3) and three died while on this regimen. The all-milk diet sustained relatively low weight gains (0.08 kg/day) compared with whole milk and soy-71.

Calves fed soy-71 had smaller pancreases than those fed the other diets ($P < 0.005$, table 3). Pancreases from calves fed soy-50 or soy-71 contained less total trypsin and chymotrypsin activity than those from calves fed whole milk ($P < 0.01$). Total activity of these enzymes per unit body weight increased with feeding duration for calves fed the milk protein diets, with an opposite trend for calves fed soy-50. Dietary treatment had no effect on pancreatic ratios of chymotrypsin-to-trypsin. The value of 0.2 approximated that noted in pancreatic juice collected from calves (5). Growth rate of calves fed for 3 or 5 to 6 weeks was not related to the total trypsin (r = − 0.20) and chy-

TABLE 3

Body weight changes, pH of abomasal contents, and size and enzyme activity of pancreases from calves fed 4 diets

Diet	Abomasal pH [1]	Body wt change	Pancreas					Ratio: Chymotrypsin trypsin
			Size	Trypsin activity		Chymotrypsin activity		
				Conc	Total	Conc	Total	
		kg/day	g/kg body wt	units[3]/mg dry wt	units/kg body wt	units[4]/mg dry wt	units/kg body wt	
Whole milk	4.68 d	0.20 a,b	0.60 a	5.69 a	747 a	1.23 a	163 a	0.22
All-milk	5.69 a,b	0.08 b	0.66 a	4.17 a,b	582 a,b	0.90 a,b	122 a,b	0.22
Soy-50	5.39 c	−0.11 c	0.57 a	3.46 b	386 b	0.72 b	79 b	0.22
Soy-71	6.17 a,c	0.33 a	0.47 b	4.93 a,b	485 b	0.95 a,b	98 b	0.19
P value	< 0.0005	< 0.0005	< 0.005	< 0.05	< 0.01	< 0.05	< 0.01	ns [5]

[1] Data represent average values from calves killed after 1, 3 or 5 to 6 weeks of feeding.
[2] Means with same superscript are not significantly different at P < 0.05 using Duncan's multiple range test (10).
[3] One unit equals hydrolysis of 1 μmole TAME/minute.
[4] One unit equals hydrolysis of 1 μmole BTEE (36% methanol v/v in reaction mixture)/minute.
[5] No difference between treatment means, P < 0.05.

motrypsin ($r = -0.19$) content of the pancreases.

Concentrations and in vitro stabilities of proteolytic enzymes in intestinal contents had large animal variations, tending to mask dietary effects. Four calves were killed during moderate or severe attacks of diarrhea. Intestinal contents from these animals contained very low trypsin and chymotrypsin activities and these data were eliminated from all analyses presented. Total trypsin and chymotrypsin activities of intestinal contents from calves fed soy-50 were lower than those from calves fed whole milk or the all-milk diet (table 4). Intestinal contents from calves fed soy-50 contained interfering substance(s) which produced an apparent esterase activity (7), the cause of which was unknown at that time. Enzyme activities were corrected for the small amount of interfering substance(s) in digesta from calves fed soy-71. Intestinal contents from calves fed whole milk and the all-milk diet, except from calves killed after 1 week of feeding, had little or none of this interference (7). For these reasons, the actual trypsin and chymotrypsin activities of intestinal contents from calves fed soy-50 would be less than the values shown in table 4. Correlation coefficients between growth rate of calves during 3 or 5 to 6 weeks of feeding and the total trypsin or chymotrypsin activities of intestinal contents were 0.25 and −0.18, respectively.

Intestinal contents resulting from the soy-71 or all-milk diets retained about 80% of the original trypsin activity during a 2-hour incubation, whereas contents arising from soy-50 retained only 64% (table 4). Only 24% of the original chymotrypsin activity of intestinal contents from calves fed soy-50 was present after incubation, whereas an average of 43 and 59% of this activity was retained in intestinal contents from calves fed milk protein and soy-71 diets, respectively. An interaction between diets and intestinal sections was found for the stability of these enzymes in incubated digesta (table 5). Contents from the upper or middle sections tended to retain more of the original trypsin and chymotrypsin activity than that from the lower section when the all-milk and soy-71 diets were the source of nutrients. In contrast,

TABLE 4

Trypsin and chymotrypsin activities, and protein concentration before and after incubation of intestinal contents from calves fed 4 diets [1]

Criteria	Diet				P value
	Whole milk	All-milk	Soy-50	Soy-71	
Trypsin [2]					
Total activity, units [3]	3732 [a]	2994 [a,b]	1764 [b]	2160 [a,b]	< 0.05
In vitro stability [4]	0.73 [a,b]	0.82 [a]	0.64 [b]	0.78 [a]	< 0.025
Chymotrypsin					
Total activity, units [5]	1038 [a]	849 [a,b]	288 [c]	612 [b,c]	< 0.0005
In vitro stability	0.40 [a]	0.45 [a]	0.24 [b]	0.59 [c]	< 0.0005
Ratio					
Chymotrypsin-to-trypsin:					
Nonincubated contents	0.34	0.45	0.26	0.33	< 0.005
Incubated contents	0.17	0.28	0.08	0.27	
Protein					
Before incubation, mg/ml	16.1	12.3	14.8	16.4	ns [6]
In vitro digestion, mg/ml	6.08 [a]	3.32 [b]	1.08 [c]	3.51 [b]	< 0.0005
Total digestion, g/2 hr	3.29 [a]	2.41 [a]	0.46 [b]	2.46 [a]	< 0.005
Enzymic efficiency of protein digestion					
Trypsin, mg digested/unit	1.42 [a]	1.29 [a]	0.48 [b]	1.75 [a]	< 0.005
Chymotrypsin, mg digested/unit	3.96 [a,c]	3.31 [a,b]	2.02 [b]	5.61 [c]	< 0.01

[1] Data represent average values from calves killed after 1, 3 or 5 to 6 weeks of feeding and digesta from entire small intestine.
[2] Means with same superscript are not significantly different at $P < 0.05$ using Duncan's multiple range test (10).
[3] One unit equals hydrolysis of 1 μmole TAME/minute.
[4] Ratio of enzyme activity of incubated-to-nonincubated intestinal contents.
[5] One unit equals hydrolysis of 1 μmole BTEE (26% methanol v/v in reaction mixture)/minute.
[6] No difference between treatment means, $P < 0.05$.

TABLE 5

Protein content and in vitro digestion, and trypsin and chymotrypsin activities before and after incubation of digesta from the upper, middle and lower sections of the small intestine from calves fed 4 diets

Interaction	Protein		In vitro enzyme stability [2]	
	Nonincubated conc	In vitro digestion [1]	Trypsin	Chymo-trypsin
	mg/ml	mg/ml		
Diet × intestinal section				
Whole milk — upper	22.7	7.80	0.68	0.36
— middle	19.6	7.75	0.82	0.42
— lower	6.1	2.69	0.70	0.41
All-milk — upper	8.6	2.64	0.83	0.54
— middle	15.5	5.65	0.89	0.53
— lower	12.7	1.69	0.74	0.28
Soy-50 — upper	11.9	0.35	0.48	0.05
— middle	20.8	1.54	0.67	0.00
— lower	11.7	1.33	0.77	0.66
Soy-71 — upper	17.6	3.66	0.91	0.65
— middle	22.6	4.42	0.62	0.65
— lower	9.0	2.43	0.81	0.48
P value	< 0.025	< 0.05	< 0.025	< 0.0005

[1] Protein digested during a 2-hour incubation of intestinal contents at 37°.
[2] Ratio of enzyme activity of incubated-to-nonincubated intestinal contents.

retention of enzyme activities was greater in the contents of the lower than in upper or middle intestine from calves fed soy-50. Little or no chymotrypsin activity was retained in incubated contents of the upper and middle intestine from calves fed soy-50. Ratios of chymotrypsin-to-trypsin activities were lower, and proportionately more chymotrypsin activity was destroyed during incubation of intestinal contents from calves fed soy-50 than in digesta from calves fed the other diets (table 4).

Protein concentration of intestinal contents was not altered appreciably by diet (table 4), but an interaction occurred between diets and intestinal sections (table 5). Contents of the lower intestine from calves fed whole milk or soy-71 contained 50% or less protein than contents from the upper and middle sections. The protein concentrations of lower intestine contents from calves fed the all-milk or soy-50 diets were equal to or greater than those in upper intestine contents.

Digestion of protein (mg/ml) during incubation of intestinal contents from calves fed whole milk was nearly 2 times greater than that from calves fed the all-milk or soy-71 diets, and 6 times greater than that from calves fed soy-50 (table 4). Total in vitro protein digestion in contents from the entire small intestine, however, ranged from 2.4 to 3.3 g/2 hours from calves fed the 2 milk protein and soy-71 diets, and only 0.46 g/2 hours from calves receiving soy-50. Differences due to diets were not altered appreciably by correcting for the final body weights of the calves. Approximately 38, 30, 25 and 8% of the protein present in intestinal contents from calves fed whole milk, all-milk, soy-71 and soy-50 diets, respectively, was digested during the 2-hour incubation. In vitro protein digestion was less in lower than that in upper and middle intestine contents when whole milk, all-milk or soy-71 were the source of nutrients (table 5). But protein digestion in lower intestine contents from calves fed soy-50 was 4 times greater than that in upper intestinal contents. Protein digestion was positively correlated with protein concentration of intestinal contents before incubation (r = 0.43, P < 0.001). But statistical adjustment by covariance analysis (10) for protein con-

centration did not alter the magnitude of dietary differences in protein digestion. Body weight changes during 3 or 5 to 6 weeks of feeding were related to total in vitro protein digestion (r = 0.47, P < 0.05).

Protein digested in vitro per unit of tryptic activity (efficiency of protein digestion) was nearly equal for intestinal contents from calves fed milk protein and soy-71 diets, but was markedly reduced (P < 0.005) in intestinal contents from calves fed soy-50 (table 4). Intestinal contents from calves receiving soy-71 had the greatest protein digested per unit of chymotrypsin activity (5.6 mg) and contents from calves fed soy-50 had the least (2.0 mg). Protein digestion per unit of the combined trypsin and chymotrypsin activities of intestinal contents from calves fed whole milk, all-milk, soy-71 and soy-50 diets was 1.10, 0.80, 1.28 and 0.41 mg, respectively. Protein digestion per unit of enzyme activity was less in digesta from the lower than that from the upper small intestine when calves were fed whole milk, all-milk and soy-71 diets, but not in digesta from calves fed soy-50.

The soy-50 diet contained an equivalent of 6.17 mg soybean trypsin inhibitor (SBTI)/g of air-dried diet (table 6). This was 40 times more trypsin inhibitor than in the all-milk or soy-71 diets. Differences of similar magnitude in trypsin inhibitor were noted in abomasal contents from calves fed these diets. The protein content of the dietary extracts which were assayed for trypsin inhibitor were nearly equal. Competition between dietary proteins in the extracts and the synthetic substrate (TAME) for the active sites of trypsin, therefore, would not account for the high trypsin inhibitor content of the soy-50 diet. Fractionation of abomasal contents from calves fed soy-50 or soy-71 by the method of Alumot and Nitsan (9) resulted in almost the same trypsin inhibitor values as those obtained by the procedure of Garlich and Nesheim (8). Abomasal contents from calves fed whole milk contained no detectable trypsin inhibitor.

Free trypsin inhibitor was present in extracts of intestinal contents from calves fed soy-50, but the amount was less in lower than in upper and middle sections

TABLE 6

Trypsin inhibitor activity in 3 milk substitute diets, and in abomasal and intestinal contents from calves fed these diets

Source of trypsin inhibitor	SBTI equivalent activity [1]		
	Soy-50	All-milk	Soy-71
Diet,[2] mg/g	6.17	0.15	0.14
Diet, mg/g protein [3]	1.56	0.046	0.052
Abomasal contents,[2] mg/ml	0.542	0.012	0.014
Small intestine contents: [4]			
upper, mg/ml	0.240 [5]	—	0
upper, mg/mg protein [6]	0.033	—	0
middle, mg/ml	0.301	—	—
middle, mg/mg protein	0.032	—	—
lower, mg/ml	0.129	—	—
lower, mg/mg protein	0.019	—	—

[1] Level of soybean trypsin inhibitor (SBTI) compared with inhibition by purified SBTI.
[2] Fractionated according to Garlich and Nesheim (8).
[3] Kjeldahl N × 6.25 in extract assayed for SBTI.
[4] Fractionated according to Alumot and Nitsan (9).
[5] Average of contents from 7 calves.
[6] Kjeldahl N × 6.25 in intestinal contents.

(table 6). A correlation coefficient of − 0.28 ($P > 0.05$) was found between free trypsin inhibitor and in vitro digestion in intestinal contents from calves fed soy-50. The procedure of Alumot and Nitsan (9) does not give the amount of trypsin inhibitor combined with trypsin in the intestinal contents. The latter may be more closely related to inhibition of proteolysis than the level of free trypsin inhibitor.

DISCUSSION

The prevalence of diarrhea in calves fed soy-50 and to a lesser extent the all-milk diet may be associated with the relatively high pH and lack of curd formation in abomasal contents from these calves, compared with those from calves fed whole milk. Shoptaw et al. (11) suggested that the diarrhea produced in calves fed a soybean flour diet was due to lack of curd formation and rapid passage of the liquid contents into the duodenum. The pH of upper intestine contents from calves fed whole milk and killed 1 to 1.5 hours postprandial (pH 5.8) was in the same range as that reported in digesta obtained from calves fitted with re-entrant duodenal cannulas (6). The greater total trypsin and chymotrypsin activities of intestinal contents from calves fed whole milk compared with those from calves fed the other diets might be explained, in part, by the lower pH of abomasal and upper intestine contents, supporting results with sheep (12).

Calves fed a diet containing 86% of the protein from a soybean concentrate high in protein and low in soybean trypsin inhibitor (soy-71) grew at rates comparable to those fed whole milk, confirming previous trials with isolated soybean proteins (13). The failure of calves to grow in this and other trials (1, 4) with diets containing 50% crude protein soybean flour may be related either to the higher levels of carbohydrate or trypsin inhibitor, or both. However, the soybean flour used in the soy-50 diet may not be representative of other soybean flours with respect to trypsin inhibitor content. The soy-50 diet contained nearly the same estimated levels of the most limiting amino acids in soybean protein, particularly methionine (14), as those in the all-milk diet (5). Lower trypsin and chymotrypsin activities in the pancreases and intestinal contents from calves fed soy-50 compared with those from calves fed the other diets confirmed the marked reduction in secretion of these enzymes in pancreatic juice collected from calves fed this same soybean diet (5). However, unknown amounts of trypsin and chymotrypsin would be combined with the SBTI in intestinal contents from calves fed soy-50.

In vitro digestion of intestinal protein was closely related to calf performance

with the various diets. However, the proportion of dietary protein intake that was digested by incubating intestinal contents ranged from only 5% for calves fed soy-50 to 20 to 30% for calves fed the other diets. These values are low compared with 75 to 85% protein digestion in digestibility trials (13). Protein digestion may occur in other parts of the digestive tract besides the small intestine. Limiting substrate was indicated since rate of protein digestion was positively related to protein concentration of intestinal contents. Also, in vitro incubation of intestinal contents may not adequately simulate conditions in vivo. The high level of SBTI in the soy-50 diet provides a logical explanation for the limited in vitro proteolysis in intestinal contents from calves fed this diet. Alumot and Nitsan (9) reported a negative correlation between proteolysis and antitrypsin level of chick intestinal contents. Reduced in vitro stability of trypsin and chymotrypsin activity and protein digested per unit of enzyme activity in contents from calves fed soy-50 compared with those from calves fed the other diets may also be associated with SBTI activity. The following evidence suggests that the SBTI in the soy-50 diet might be destroyed or inactivated as digesta traversed the small intestine of the calf: 1) greater in vitro protein digestion and less destruction of trypsin and chymotrypsin activity occurred during incubation of contents from the lower compared with that from the upper small intestine (opposite to results with calves fed the other 3 diets), and 2) contents from the lower intestine contained less free trypsin inhibitor than contents from the upper and middle sections.

One or more of the following mechanisms may be responsible for the poor growth and hyposecretion of pancreatic enzymes by calves reared with the soy-50 diet: 1) rapid passage of chyme through the upper small intestine due to constant diarrhea, 2) limited proteolysis due to the presence of SBTI activity, and 3) deficiencies of essential amino acids for protein and enzyme synthesis. Intestinal contents from calves suffering diarrhea, when killed irrespective of diet, contained very low levels of proteolytic enzymes. Under these conditions the release of secretin and pancreozymin which trigger the secretion of exocrine fluid and digestive enzymes from the pancreas (15) might be limited. This would also prevent loss of endogenous protein secretions in the feces. An adequate duodenal stimulus for the release of pancreozymin may require the release of sufficient quantities of certain stimulatory amino acid residues from the dietary protein (16). Limited proteolysis in digesta from the upper small intestine of calves fed soy-50 could therefore fail to trigger the release of pancreozymin in sufficient quantities. Finally, inhibition of intestinal protolysis would limit the supply of amino acids for synthesis of digestive enzymes as well as all other body proteins.

The specific effects of purified soybean trypsin (or growth) inhibitors on biochemical changes in calf pancreases and intestinal proteolysis have not been studied. If SBTI was of major importance in producing the results obtained in calves fed soy-50, it failed to evoke pancreatic hypetrophy and hypersecretion of digestive enzymes, which it does in rats and chicks (8, 17). Intestinal proteolysis in rats was not inhibited by SBTI (18), whereas practically no intestinal proteolysis occurred during the first 4 hours after feeding chicks raw soybeans (9). The response of swine pancreases to raw soybeans has also been reported to differ from that commonly observed in rats and chicks (19)[5] and tends to corroborate the resutls with calves. Further research is essential to elucidate the specific effects of trypsin inhibitors and other growth factors in raw soybeans on young calves.

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Choline Biosynthesis and Choline Requirement in the Rat as Affected by Coprophagy [1]

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ABSTRACT   A study was made of the influence of several dietary and environmental factors on the dietary choline requirement of young rats. Under conditions of relatively low choline requirement which involve the absence of supplementary cystine and cholesterol and moderately low levels of fat and protein in the diet, prevention of coprophagy and possibly to some extent the inclusion of penicillin in the diet decreased the rat's requirement for choline. Coprophagy prevention completely protected from the high mortality related to kidney degeneration, and liver fat levels remained normal for the first 4 weeks postweaning. Methyl-labeled methionine tracer studies showed that transmethylation to form liver choline was high in rats 2 weeks postweaning and declined to a lower level which reached a plateau at 6 to 8 weeks postweaning. Choline deficiency lowered the early transmethylation rate, but the later plateau level was the same as for normal choline-fed animals. Coprophagy prevention raised the early transmethylation rate and choline biosynthesis in the choline-deficient animals so that it equaled the normal rate. It is proposed that prevention of coprophagy protects the young rat from choline deficiency by increasing choline biosynthesis. This effect is demonstrable only in the rat during the first 4 weeks postweaning when the choline requirement is elevated and it is also observable only with dietary conditions that provide for a minimal choline requirement.

During the course of a study of the influence of soybean trypsin inhibitor upon the metabolism of methionine-2-^{14}C as reflected by the production of $^{14}CO_2$ (1) it was observed that preventing coprophagy invariably increased $^{14}CO_2$ output.[2] Since $^{14}CO_2$ formation from methionine-2-^{14}C represents a stage of metabolism which would be expected to follow the initial activation and transmethylation of the methionine, it was possible that choline biosynthesis was increased along with the increased oxidation of the carbon chain of methionine when coprophagy was prevented. This is a report of the initial studies in which this hypothesis was explored.

EXPERIMENTAL

Male albino weanling rats (Holtzman) were used in all of these studies. The basic composition of the test diets is shown in table 1. Dietary choline, provided as choline dihydrogen citrate, was fed at zero, 0.075 or 0.3% of the diet. Liver fat was measured in fresh liver by chloroform Soxhlet extraction of Na_2SO_4 dehydrated liver followed by evaporation and weighing the extract. Coprophagy was prevented by the method of Barnes et al. (2). When DL-methionine-2-^{14}C was used it was administered by intraperitoneal injection and the rats were maintained in all-glass metabolism units for the collection of CO_2. These procedures have been described previously including a tabulation of the composition of the soybean diets used in the present study (1). Methyl-^{14}C-labeled L-methionine was injected into the portal vein of ether-anesthetized rats and 15 minutes later the liver was excised and homogenized. Phospholipid was extracted and hydrolyzed and the free choline was determined quantitatively by precipitation as the Reinecke salt and then dissolved in acetone and assayed colorimetrically. Radioactivity was determined in a Packard liquid-scintillation counter.

RESULTS

Time curves of $^{14}CO_2$ production following intraperitoneal injection of DL-methionine-2-^{14}C are shown in figure 1. The methionine was given as a tracer dose of 10 μCi/100 g body weight. The rats had

TABLE 1

Composition of choline-deficient diets

	Casein diet	Soybean diet
	g	*g*
Major components		
Casein [1]	10	—
Raw soybeans [2]	—	20
Glucose monohydrate [3]	68	58
Hydrogenated vegetable oil [4]	15	15
Salts [5]	4	4
B-vitamins in sucrose	2	2
Fat-soluble vitamins in corn oil	1	1
Total	100	100

B-vitamins in 2.0 g sucrose	
	mg
Thiamine·HCl	0.40
Riboflavin	0.80
Pyridoxine·HCl	0.40
Ca pantothenate	4.00
Niacin	4.00
Inositol	20.00
Biotin	0.02
Folic acid	0.20
Vitamin B_{12}	0.003
Menadione	1.00

Fat-soluble vitamins in 1.0 g corn oil	
	mg
Vitamin A acetate	0.31
Vitamin D (calciferol)	0.0045
α-Tocopherol	5.00

[1] Diets containing either 10% or 15% casein were used, the 5% difference being corrected for by amount of glucose added; Vitamin-Test Casein, General Biochemicals, Chagrin Falls, Ohio.
[2] Raw whole ground soybeans, Clarke variety.
[3] Cerelose, Corn Products Company, Argo, Illinois.
[4] Primex, Procter and Gamble Company, Cincinnati.
[5] Hubbell, R. B., L. B. Mendel and A. J. Wakeman 1937 A new salt mixture for use in experimental diets. J. Nutr., 14: 273.

been fed a purified, 15% casein diet for 2 weeks following weaning (21 days of age). Sixteen hours prior to the tracer study food was removed and, when used, crystalline soybean trypsin inhibitor was given by stomach tube in a dose of 50 mg one hour before administration of the labeled methionine. Three rats were used for each treatment and immediately following the labeled methionine injection they were placed in all-glass metabolism units for CO_2 collection. The curves show a definite increase in CO_2 when coprophagy was prevented in the rats receiving the control casein-containing diet. It is also evident that trypsin inhibitor increased $^{14}CO_2$ output when used in conjunction with a casein diet in the same manner that has been previously described in which soybean diets were used (1). Cop-

rophagy prevention resulted in a further increase in $^{14}CO_2$ production.

A series of other dietary conditions was studied along with a replicate study of the control casein-containing diet (table 2). Coprophagy prevention increased $^{14}CO_2$ output in rats receiving a raw (unheated) soybean diet alone or supplemented with penicillin or methionine. The only condition shown in this table in which a questionable increase in $^{14}CO_2$ followed the prevention of coprophagy is with cystine supplementation. All other groups show a typical $^{14}CO_2$ increase that is associated with coprophagy prevention.

Since most experience had been obtained with unheated soybean-containing diets, a liver fat study was set up in which weanling rats were fed semipurified diets with 10% protein from raw, ground soybeans without choline added. Supplements of choline, methionine, penicillin, and cystine were added and the experimental diets were fed for 4 weeks. The effect of these diets upon liver fat and the influence of preventing coprophagy are shown in table 3. In the conventional rats choline, methionine, and penicillin were effective in maintaining lower liver fat levels when the choline-deficient diet was fed. Prevention of coprophagy resulted in low liver fat without any lipotropic supplement. Coprophagy prevention had no effect upon the accumulation of liver fat when cystine was added to the diet. This observation was particularly interesting in view of the fact that the one dietary condition in which a questionable rise in $^{14}CO_2$ from methionine-2-^{14}C resulted from coprophagy prevention was when a cystine supplement was fed.

With the encouraging results from the soybean-containing diets, a more highly purified diet was indicated and therefore a vitamin-test casein at a level of 10% in a purified diet without choline was tried. The results are shown in table 4. The high mortality of the conventional rats within 2 weeks postweaning confirms the extreme sensitivity to hemorrhagic kidneys in choline deficiency that has long been known from the work of Griffith and Wade (3). The high mortality which, of course, was due to the extremely low choline content of the casein diet, (as contrasted with the
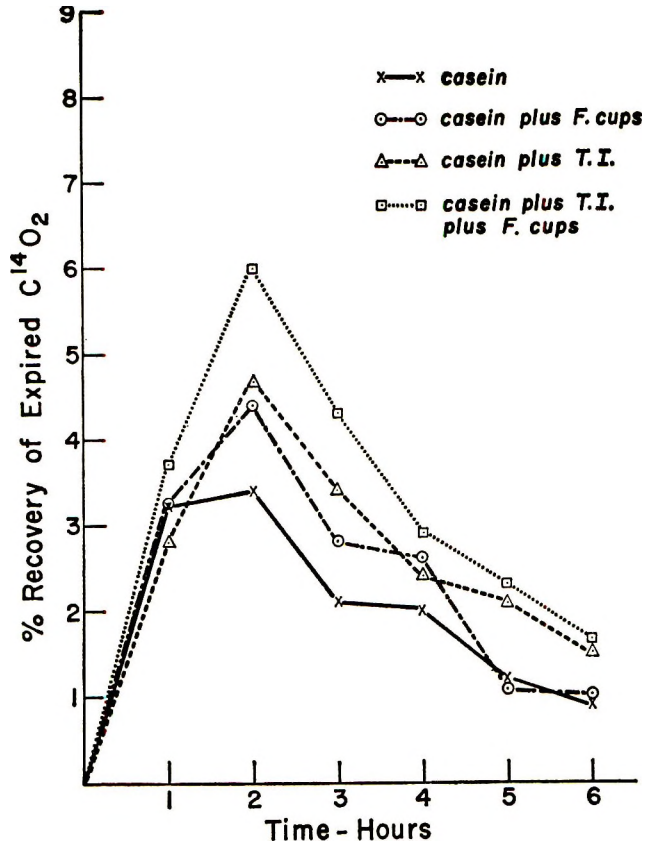
Fig. 1 Influence of preventing coprophagy with fecal cups (F. cups) upon $^{14}CO_2$ output following intraperitoneal injection of DL-methionine-2-^{14}C in rats either with or without the prior administration of soybean trypsin inhibitor (TI) by stomach tube.

TABLE 2

Effect of preventing coprophagy on ^{14}C recovery in expired CO_2 following the intraperitoneal administration of DL-methionine-2-^{14}C in rats

Diet plus supplements	^{14}C recovery, % of administered activity in 6 hours	
	Coprophagy permitted	Coprophagy prevented
	%	%
50% raw soy [1]	16.4	20.0
50% raw soy + penicillin [2]	15.7	26.4
50% raw soy + cystine [3]	16.8	18.3
50% raw soy + methionine [4]	17.9	23.8
15% casein	12.8	15.2
15% casein + trypsin inhibitor [5]	16.8	20.8

[1] Uncooked full fat soy chips.
[2] Procaine penicillin, 0.1% in diet.
[3] L-Cystine, 0.3% in diet.
[4] DL-Methionine, 0.3% in diet.
[5] Kunitz type.

TABLE 3

Effect of preventing coprophagy upon liver fat in rats fed a low protein (10% from raw soybeans), low choline diet

Diet supplement	Liver fat	
	Coprophagy permitted	Coprophagy prevented
	%	%
None	10.6 ± 0.91 [1]	5.2 ± 0.80
0.3% choline	4.0 ± 0.57	4.5 ± 0.11
0.1% DL-methionine	3.4 ± 0.76	3.3 ± 0.12
0.05% penicillin	6.5 ± 1.08	6.0 ± 0.75
0.3% cystine	11.0 ± 0.15	13.8 ± 2.80

[1] Percentage of wet weight of liver ± SE of mean.

TABLE 4

Prevention of coprophagy and mortality associated with kidney lesions of rats fed a 10% casein, choline-free diet

Dietary supplement	No. of rats dead/no. started	
	Coprophagy permitted	Coprophagy prevented
None	5/6	0/6
0.3% choline dihydrogen citrate	0/6	0/6
0.3% cystine (exp. 1)	4/6	—
0.3% cystine (exp. 2)	3/10	0/10

soybean diets used earlier) was completely abolished by the prevention of coprophagy. The results presented in this table have been confirmed many times. It is a standard procedure in this laboratory to incorporate 0.075% choline dihydrogen citrate in diets fed to conventional weanling rats for the first 2 weeks postweaning in order to study choline deficiency effects over periods longer than 2 weeks. Invariably, coprophagy prevention has protected wean-

ling rats receiving diets completely devoid of choline.

Choline biosynthesis in the liver as measured by the specific activity of liver choline following the injection of methyl-labeled methionine into the portal vein was then followed. This index of biosynthesis was compared with liver fat level for rats receiving a choline-deficient diet alone or supplemented with either choline or penicillin. The results in table 5 show that in the conventional rats, the higher level of liver fat in the unsupplemented, choline-deficient rats was associated with a lower concentration of choline and a lower specific activity of liver choline, presumably reflecting a decreased level of choline biosynthesis. In the rats with coprophagy prevented, the choline-deficient rats did not have increased liver fat and at the same time, liver choline concentration and specific activity did not decrease as it had in the conventional rats. Protection against fat increase in the liver associated with increased choline biosynthesis was also observed in conventional rats given penicillin. However, a major difference from the results obtained when coprophagy was prevented, was the continuing low liver choline concentration in the conventional rats given penicillin.

All of the results reported above were obtained within the first 4 weeks after weaning. The influence of coprophagy upon liver fat was examined during the second 4 weeks postweaning, namely, at 4, 6, and 8 weeks, and the results of this study are given in figure 2. The dietary

TABLE 5

Liver fat and the synthesis of choline in rats fed a low protein, low choline diet [1]

Diet supplement	Coprophagy permitted					Coprophagy prevented				
	Liver		Liver choline			Liver		Liver choline		
	Wet wt	Total fat		Total counts	Specific activity	Wet wt	Total fat		Total counts	Specific activity
	g	%	mg/g liver	cpm/g liver	cpm/mg choline	g	%	mg/g liver	cpm/g liver	cpm/mg choline
Choline-free	5.7	10.3	0.52	2,818	5,420	4.0	4.1	1.72	24,450	14,215
Choline-free with 0.1% penicillin	5.2	4.1	0.80	8,602	10,753	4.7	3.1	1.40	17,112	12,223
0.3% choline	4.9	3.3	1.60	17,061	10,633	4.2	3.1	1.90	18,175	9,566

[1] Methyl-labeled methionine injected into the portal vein and the liver removed for choline and fat isolation 15 minutes later. Three to 4 rats/group.
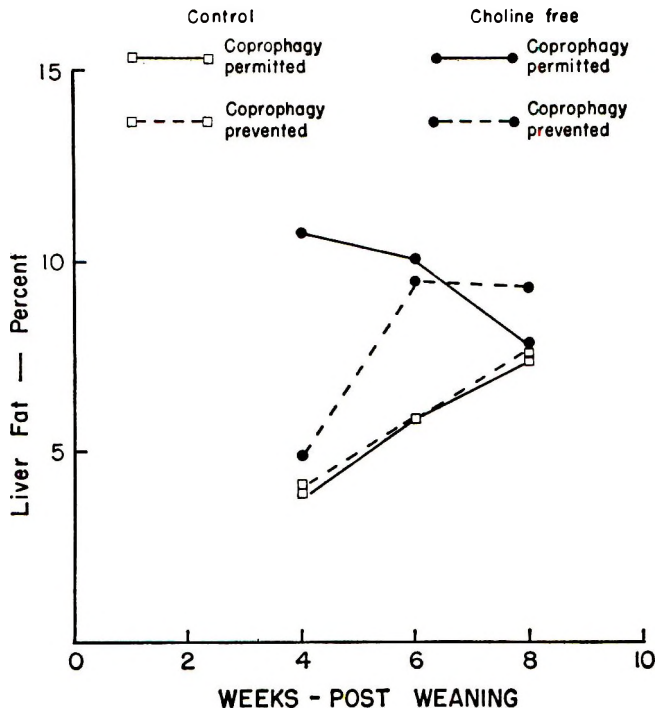
Fig. 2 Influence of preventing coprophagy upon liver fat of rats during the fourth to eighth week postweaning while receiving choline-deficient diets.

conditions used for the choline-deficient rats involved the feeding of 0.075% choline dihydrogen citrate for the first 2 weeks followed by complete removal of choline from the diet, 15% fat in the form of hydrogenated vegetable oil, 10% vitamin-test casein and the absence of any agent known to increase choline requirements, such as cystine or cholesterol. Under these conditions the liver fat was elevated, but not excessively, at 4 weeks postweaning and returned to lower levels, presumably normal, by 8 weeks. With coprophagy prevented, liver fat levels were low at 4 weeks and increased over the next 4 weeks so that at 8 weeks postweaning, coprophagy prevention no longer appeared to have any liver fat lowering effect.

The same rats that were used to obtain the liver fat values at 4, 6, and 8 weeks, were administered a tracer dose of $^{14}CH_3$-labeled methionine via the portal vein 15 minutes before killing. Liver choline specific activity was determined as described previously and correlated with liver fat values. A scatter graph showing the relationship between these 2 values at 4 weeks postweaning is illustrated in figure 3. The high correlation shown here disappeared completely at the 6- and 8-week postweaning periods. One rat was given an exceptionally high dosage of choline, 1.5% in the diet, and as might have been expected, liver choline specific activity was much lower than would be predicted from the regression line. Presumably this low value could be due to a suppression of choline synthesis or to a dilution effect due to the excess cold choline that was administered. The group receiving the high level of dietary choline was not included in the calculation of the coefficient of correlation which is shown to be − 0.90. This high choline intake resulted in abnormally low choline specific activity, probably either by a feed-back mechanism or simply by diluting the liver choline with a large amount of cold choline. Although the correlation between liver fat and liver choline biosynthesis was inversely high at 4 weeks postweaning, at 6 and 8 weeks this relationship disappeared.
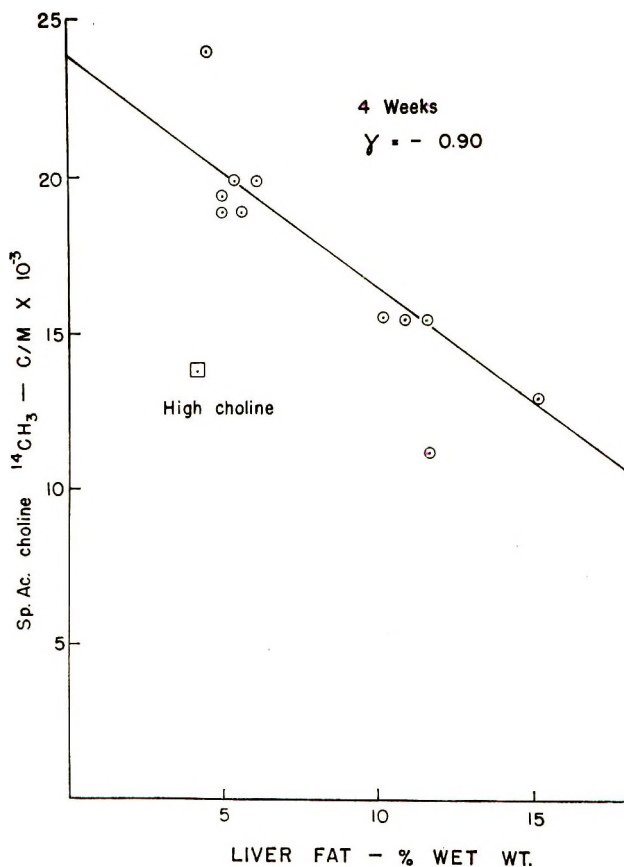
Fig. 3 Correlation of liver fat and the specific activity of liver choline following the administration of methyl-labeled methionine.

Since it was apparent that age was a critical factor in determining the effect of choline deficiency upon liver fat, the rate of liver choline biosynthesis was studied in rats of different ages. The same procedures that have been described were used and values from either 2 or 3 rats were averaged for the points shown in figure 4. There was an initial high level of choline specific activity which decreased over the subsequent 6 weeks. In the conventional rats, feeding a choline-deficient diet lowered the rate of liver choline biosynthesis below that of the control at 2 weeks postweaning, although by the sixth week the values for the control and choline-deficient animals were the same. The prevention of coprophagy did not appear to alter the choline specific activity values for the control animals; the primary effect

was to maintain choline specific activities elevated despite the dietary deficiency of choline.

Liver choline concentrations were measured and found to be lower in the conventional rats that were fed a choline-deficient diet (table 6). However, this appeared to hold only for the 2- and 4-week postweaning periods. At 6 and 8 weeks, the liver choline levels were normal. Prevention of croprophagy had some effect in maintaining higher levels of liver choline during the first 4 weeks postweaning, but the results are perhaps not as clear-cut as shown in table 5. If the total choline counts are compared for conventional and coprophagy-prevented rats during the first 4 weeks postweaning, it becomes evident that there was an increased net synthesis of choline in the coprophagy-prevented
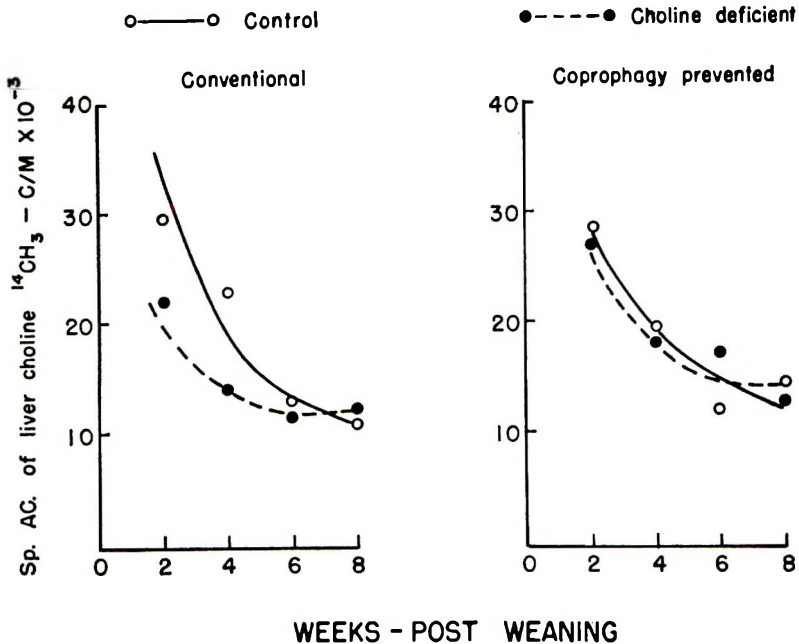
Fig. 4   Liver choline specific activity following the administration of methyl-labeled methionine in rats at different ages and the effects of preventing coprophagy.

TABLE 6

Liver fat and synthesis of choline as affected by dietary choline, prevention of coprohpagy, and age

Choline status	Weeks post-weaning	Coprophagy permitted				Coprophagy prevented			
		Liver		Choline		Liver		Choline	
		Wet wt	Total fat		Total counts	Wet wt	Total fat		Total counts
		g	*%*	*mg/g liver*	*cpm/g liver*	*g*	*%*	*mg/g liver*	*cpm/g liver*
Choline-deficient	2	4.5	13.1	1.5	32,631	4.4		1.9	53,577
	4	4.1	10.8	1.2	17,398	3.4	4.9	2.4	44,989
	6	5.1	10.1	2.7	34,995	4.7	9.5	2.6	32,704
	8	5.4	7.8	1.8	21,847	5.5	9.3	1.7	26,619
0.3% choline	2	3.5	4.8	2.4	71,103	3.2	5.4	2.8	79,736
	4	4.3	3.9	2.4	55,587	3.8	4.1	2.8	52,511
	6	4.7	5.9	2.4	29,717	4.8	5.9	2.3	28,785
	8	6.2	7.4	2.1	22,928	5.8	7.8	1.8	26,162

group. When a normal choline intake was provided, preventing coprophagy had no effect upon either choline concentration or total choline counts. This is convincing evidence that coprophagy prevention had no effect upon net choline synthesis and the specific activity comparisons in figure 4 show that there was no effect upon methyl transfer to choline under these dietary conidtions.

DISCUSSION

Under the dietary conditions used in these studies, the rat's dietary choline requirement was highest during the first 2 to 4 weeks after weaning. With the stated dietary condition the rat can synthesize sufficient choline to meet its needs when it is 8 weeks postweaning. This is in accordance with the conclusion drawn many years ago by Griffith and Wade (3). It

has been observed in this laboratory, in confirmation of reports of others, that if the dietary fat level is increased or if cystine is incorporated in the choline-free diet, fatty livers will persist to 8 weeks and beyond. The dietary requirement for choline must depend to a major degree upon the balance between the tissue needs for choline and the extent of choline biosynthesis by the tissues. In the present study it has been shown that the rate of liver choline biosynthesis as reflected by its concentration, total activity and specific activity following methyl-labeled methionine administration is high in the weanling rat and decreases to a minimal plateau level approximately 6 weeks postweaning. In view of this decreasing biosynthetic activity accompanying a decreased dietary requirement, it is concluded that the tissue need for choline must also be decreasing during the first 2 months of life.

The fact that an absorbable antibiotic, penicillin, tended to prevent fatty livers in the present study confirms the results of Baxter and Campbell (4) using aureomycin, but is contrary to the negative report of Salmon and Newberne (5). In the latter studies, antibiotics did decrease the incidence or severity of hepatic cirrhosis over a 630-day test period but did not decrease liver fat or prevent hemorrhagic kidneys in young, choline-deficient rats. These differences in the effectiveness of antibiotics are possibly explained by the severity of the choline deficiency that has been produced in the different laboratories. In the present study a relatively mild choline deficiency was used. In all probability the protective effect of penicillin would not have been detected if cystine or cholesterol had been included in the diet or if a higher fat consumption had been used or if a protein severely deficient in methionine had been substituted for casein. The liver fat results given in tables 2 and 3 show that with cystine added to the diet, coprophagy prevention had little or no effect in lowering liver fat levels. This confirms observations made in an accompanying report (6). The one measurement of protection from kidney degeneration death (table 4) shows a positive effect of coprophagy prevention in the presence of a

dietary cystine supplement. The possibility of an effect of coprophagy prevention on kidney degeneration that differs from its protection from fatty liver should be examined in more detail. Conditions that will increase the dietary requirement for choline would very likely mask a relatively weak choline-sparing agent such as penicillin. The choline-sparing effect of penicillin was accompanied by an increased liver biosynthesis of choline, although this did not appear to be as great as with coprophagy prevented. At this time no attempt will be made to explain how an antibiotic can affect liver biosynthesis. However, assuming that the effect was due to the antibacterial properties of penicillin, it appears likely that whatever the mechanism, the effect must have been elicited in the small intestine since penicillin given by mouth disappears before it completes its transit of the small intestine.

The protective effect of coprophagy prevention on choline deficiency in the very young rat is believed to have been mediated through an increased choline biosynthetic rate. The mechanism for this is as obscure as in the case of protection by penicillin, but it is proposed that the 2 effects may have similar explanations. Prevention of coprophagy is known to alter the intestinal microflora (7). This is believed to be brought about by blocking much of the microbial reinoculation of the gut that results from the ingestion of fresh feces and in a manner the end result of this might be similar to the reduction of upper intestinal tract microflora by penicillin.

Chahl and Kratzing (8) have studied the effect of environmental temperature on choline requirement in the rat. At 2° the dietary requirement for choline was found to be 25 mg and at 33° it was 100 mg/100 g food. These authors could not relate the higher requirement at elevated temperatures to decreased food intake alone and suggested that changes in utilization and degradation of choline might be involved. It is obvious that various factors other than dietary composition may influence choline requirements.

The influence of age upon choline biosynthesis which has been reported in a

preliminary note [3] is of considerable interest. A decreased rate of choline synthesis with increased body weight has been noted by Wells and Remy (9) and in their studies body weight was equated with age. In the present report this observation is confirmed. However, they also present evidence that in choline deficiency there is an increased rate of choline biosynthesis which, of course, is contrary to the present study. It will be important to determine the cause of the divergent results obtained in these 2 investigations, for any interpretation of the role of choline biosynthesis in meeting the choline requirement of the rat will be dependent upon the effect that a choline-deficient diet has on this synthetic rate.

Over the many years this laboratory has studied the various nutritional and biochemical effects of ingesting feces, the choline-sparing effect reported in this paper is the first and only nutritionally advantageous consequence of preventing coprophagy that has been consistently observed. Nature must have had some basis for the practically universal practice of coprophagy among the world's monogastric mammals.

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Effect of Dietary Supplements of Cystine upon Growth, Liver Fat and Choline Biosynthesis in the Choline-deficient Rat [1]

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ABSTRACT   Evidence to support an explanation for the antilipotropic effect of supplementary dietary cystine in choline-deficient rats was presented. The results of these studies showed that supplementary cystine aggravates a choline deficiency under dietary conditions where cystine does not improve the nutritional state of the animal. This was accomplished by using, as the sole dietary protein, isolated soy protein which has been shown not to be limiting in cystine. These experimental results are in conflict with the prevailing hypothesis that cystine acts by improving the nutritional state of the animal which causes an increase in the animal's requirement for methionine and choline. Under these same conditions, supplementary cystine caused a decrease in the synthesis of choline in the liver, thus supporting an hypothesis that cystine increases the dietary requirement for choline by limiting its biosynthesis through an inhibition of methionine transmethylation.

It has been recognized for many years that a dietary supplement of cystine given to rats receiving a choline-deficient diet causes an increase in the severity of the choline deficiency. This observation is so well-established that in the vast majority of rat studies of choline deficiency that are carried over long time-periods, supplementary cystine is included in the diet. Although increased fatty livers resulting from dietary cystine had been noted by Beeston and Channon (1), Griffith and Wade (2) were the first to describe the choline-cystine antagonism in detail, and later Griffith (3) proposed that this effect was attributed to the improvement in the state of nutrition induced by a cystine supplement which may have increased the animal's requirement for methionine and choline. Other investigators (4, 5) have failed to confirm this hypothesis. However, in the latter studies, 5% casein diets were fed to young rats, which provided a rather unusual dietary situation. Casein is known to be extremely deficient in cystine and, in fact, is rare among food proteins in this respect. At levels of casein in the diet of 8% to approximately 15%, supplements of cystine will cause young rats to grow at a more rapid rate. This, of course, is the anticipated response of the animals to dietary supplementation with the first-limiting amino acid. For some unexplained

reason, if casein is fed at a level of 5% or less in the diet, dietary supplementation with this amino acid does not bring about a growth response. It has been these conditions in which cystine addition to the diet did not increase growth rate but at the same time did increase the accumulation of liver fat in choline-deficient rats, that have led to a contradiction of the Griffith hypothesis which relates increased liver fat to nutritional state. Since evidence for an alternative explanation for the effect of cystine supplements in choline-deficient diets has not been provided, and in previous studies an unusual dietary pattern has been utilized, we have reopened an investigation of this problem.

EXPERIMENTAL

Male, weanling rats (Holtzman) were used in all experiments. They were caged individually in wire mesh-bottom cages and were fed a diet of the composition shown in table 1. The animal room was kept at a temperature of 25°. In studies with choline deficiency, this is an important variable since it has been shown that choline requirement varies with environmental temperature (6). Dietary choline

TABLE 1

Diet composition

Major components	g
Soybean protein source [1]	15.0
Glucose monohydrate [2]	63.0
Hydrogenated vegetable oil [3]	15.0
Salts [4]	4.0
B vitamins in sucrose	2.0
Fat-soluble vitamins in corn oil	1.0
Total	100.0

B-vitamins in 2.0 g sucrose	mg
Thiamine·HCl	0.40
Riboflavin	0.80
Pyridoxine·HCl	0.40
Ca pantothenate	4.00
Niacin	4.00
Inositol	20.00
Biotin	0.02
Folic acid	0.20
Vitamin B_{12}	0.03
Menadione	1.00

Fat-soluble vitamins in 1.0 g corn oil	mg
Vitamin A acetate	0.31
Vitamin D (calciferol)	0.0045
α-Tocopherol	5.00

[1] Promine, Central Soya Company, Chicago.
[2] Cerelose, Corn Products Company, Argo, Illinois.
[3] Primex, Procter and Gamble Company, Cincinnati.
[4] Hubbell, R. B., L. B. Mendel and A. J. Wakeman 1937 A new salt mixture for use in experimental diets. J. Nutr., *14:* 273.

was provided as choline dihydrogen citrate. To prevent the high mortality that results from feeding a choline-deficient diet to weanling rats, 0.075% choline dihydrogen citrate was included in the diet for certain groups of animals which are identified in the tabular material presented later. Casein was used as the source of protein in some experiments but particular impor-

tance has been attached to the use of isolated soy protein.[2] In a previous report we provided evidence that cystine was not first-limiting in this protein source (7). Food intake was measured every other day and body weights were recorded weekly. All diets were fed ad libitum. Liver fat was measured in fresh liver which was weighed, ground with anhydrous sodium sulfate, and extracted with chloroform in a Soxhlet extractor for 4 hours. The chloroform extract was filtered through fat-free paper into a dried flask and the residue dried to constant weight. This is essentially the procedure used by Longmore et al. (8). Methyl-labeled L-methionine was used for the determination of liver choline biosynthesis. The $^{14}CH_3$-labeled methionine was injected into the portal vein of an ether-anesthetized rat and 15 minutes later the liver was excised and homogenized. Phospholipid was extracted and hydrolyzed. Choline was quantitatively determined by precipitation as the Reinecke salt, which was then dissolved in acetone and assayed colorimetrically. Radioactivity was determined in a Packard liquid-scintillation counter.

RESULTS AND DISCUSSION

The first observations (table 2) were made with a choline-deficient diet containing casein at a level of 10%. Half of the animals had coprophagy prevented by the use of tail cups (9). The diets were continued for 4 weeks postweaning, when the rats were injected with methyl-labeled methionine and killed. In the conventional

[2] Promine, Central Soya Company, Chicago.

TABLE 2

Effect of cystine upon liver fat and choline in rats receiving a 10% casein diet

Diet supplement	Coprophagy permitted					Coprophagy prevented				
	Liver		Liver choline			Liver		Liver choline		
	Wt	Total fat		Total counts	Specific activity	Wt	Total fat		Total counts	Specific activity
	g	%	mg/g liver	cpm/g liver	cpm/mg choline	g	%	mg/g liver	cpm/g liver	cpm/mg choline
Choline-free	5.7	10.3	0.52	2,818	5,420	4.0	4.1	1.72	24,215	14,215
0.3% choline [1]	4.9	3.3	1.60	17,061	10,633	4.2	3.1	1.90	18,175	9,566
Choline-free 0.3% L-cystine	5.6	16.5	0.40	3,184	7,961	6.0	12.3	0.64	3,505	5,477

[1] Choline dihydrogen citrate.

animals receiving the choline-free diet, several had died with hemorrhagic kidneys and, in general, the remaining animals were in very poor condition. This probably accounts for the relatively low total counts for liver choline and the low specific activity in the first group. In the rats with coprophagy prevented, the effect of dietary cystine supplement was much more evident. Under these conditions, the rats receiving the choline-free diet did not develop fatty livers nor did any animals die with hemorrhagic kidneys. This protective effect due to preventing coprophagy has been described in another publication (10). In the rats with cystine supplement, there was a marked decrease in the specific activity of the liver choline which was accompanied by an increase in the level of liver fat. This first experiment confirmed the well-established effect of supplementary cystine in increasing liver fat. The cystine also increased the growth rate of rats. The lower specific activity of liver choline accompanying dietary supplementation with cystine suggested the possibility that a decreased biosynthesis of choline may have contributed to the increased severity of the choline deficiency. That the lower specific activity was accompanied by lower total choline and lower total choline counts rules out the possibility that the effect was one of dilution with non-labeled choline.

The next study was carried out with isolated soy protein as the dietary protein source, which was known from previous studies to be rather seriously limiting in methionine but completely lacking growth stimulation when supplemented with cystine (7). The results of this study are given in table 3. With the dietary conditions in this study, cystine supplementation resulted in an increase in liver fat but with decreases rather than increases in growth rate. There was an apparent increase in food efficiency with 0.15% cystine, but this was not evident with a supplement of 0.3% cystine. To prevent the high mortality due to choline deficiency in the first few weeks postweaning, a 0.075% choline dihydrogen citrate supplement was added to certain of the diets. Rats with this milder degree of choline deficiency were used in the evaluation of

TABLE 3

Effect of dietary cystine upon growth, food intake, liver fat and choline biosynthesis in rats fed low protein (15% isolated soybean protein) choline-deficient (0.075% choline dihydrogen citrate) diets

Diet supplement	Exp[1] no.	Wt gain	Food intake	Food efficiency	Mortality	Liver		Liver choline		
						Wt	Total fat		Total counts	Specific activity
		g	g/day	g gain/g food		g	%	mg/g liver	cpm/g liver	cpm/mg choline
None	1	32	8.9	3.6	3/6	6.1	27.3	—	—	—
	2	19	—	—	4/10	4.7	29.8	—	—	—
0.075% choline	1	61	13.3	4.6	0/6	4.2	10.5	—	—	—
	2	37	—	—	0/10	5.0	10.5	0.62	6,388	10,304
0.3% choline	1	59	12.5	4.7	0/6	5.0	4.0	—	—	—
	2	28	—	—	0/10	3.8	4.1	2.60	30,001	11,539
0.075% choline + 0.15% cystine	1	51	9.6	5.3	0/6	7.0	23.8	—	—	—
	2	25	—	—	0/10	5.7	26.1	0.68	5,113	7,519
0.075% choline + 0.3% cystine	1	40	9.6	4.2	0/6	6.1	21.0	—	—	—

[1] Two trials, 3-week experimental period. Six rats in first trial and 10 in second for mortality measurement. Six rats used for liver fat in both experiments and either 3 or 4 for choline biosynthesis in experiment 2.

choline biosynthetic rate, as indicated by specific activity of liver choline following the administration of methyl-labeled methionine. A lowered specific activity was found in those rats receiving cystine supplementation, thus confirming the preliminary observations made with the casein diets and shown in table 2. As in the previous study, total choline was low in the livers of choline-deficient rats, but the level did not appear to be related to the amount of fat in the liver. Also the total choline count was low so that the decreased specific activity in the cystine-supplemented animals could not be accounted for by a simple dilution of choline.

The 2 experiments just described confirm the observations of others (4, 5) that an antilipotropic effect of cystine can be demonstrated in the absence of any stimulation of growth rate. The fact that dietary supplementation with cystine caused a decrease in liver choline biosynthesis supports the concept that the antilipotropic activity of cystine is related to an inhibition of endogenous choline synthesis. The importance of endogenous choline biosynthesis in determining the dietary requirement for choline has been discussed in detail by Stekol (11). The dietary cystine effect that has just been described appears to provide another example of the contribution of endogenous synthesis. Presumably inhibition of biosynthesis by dietary cystine is brought about by some type of feedback which retards transmethylation. It is difficult to see how the hypothesis of Griffith can provide even a partial explanation of the present results, particularly in view of the belief that ". . . cystine aggravates the effect of choline deficiency only if the basal diet is deficient in cystine" (12). In the studies of others in which 5% casein diets were used, cystine probably was first-limiting, yet the animals did not respond to a cystine supplement because of the toxicity of excess cystine under these conditions. Urinary calculi and nephropathogenicity due to cystine excess have been known for years (13) and this phenomenon has been observed in the authors' laboratories in rats receiving diets

containing presumably optimal levels of choline.[3] In the present studies, when the soy protein diet was used cystine was not deficient and yet choline deficiency was aggravated as was evidenced by an increase in liver fat.

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Copper Metabolism in the Early Postnatal Period of the Piglet [1,2]

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ABSTRACT   A study was made to determine whether low serum copper and ceruloplasmin activity values of piglets were the result of insufficient copper or insufficient serum protein. Observations were made on naturally and artificially raised piglets. In the naturally raised piglets, serum copper and ceruloplasmin activity increased from very low values to approximately those of the adult pig in one week. Two litters of artificially raised pigs were subdivided into 3 groups, each of which was fed in one of 3 dietary sequences. Pigs fed in sequence A received a mineral water solution for the first 36 hours and thereafter received cow's milk fortified with iron and copper. Sequence B pigs received the mineral water for the first 36 hours also, and thereafter were fed cow's milk fortified with iron alone. Sequence C pigs received cow's colostrum for the first 36 hours and then cow's milk as those in sequence B. The results from the artificially raised piglets indicated: (a) that ceruloplasmin protein as measured by its activity was inducible by supplementary dietary copper; (b) that rate of synthesis of hemoglobin was increased by cow's colostrum; and (c) that one of the limiting factors with the piglet under the conditions of this experiment appeared to be source of protein in early postnatal life rather than iron and copper per se.

This investigation was prompted by observations made in studies centered around porcine neonatal nutrition conducted at North Carolina State University over the past decade. It was reported that pigs are born extremely deficient in blood serum proteins with an absence of γ-globulin and low total protein, albumin and α-globulin values (1–3). It was also observed that newborn pigs possess a low serum copper concentration (20–40 ug/100 ml serum) as compared with that of adult pigs (200–250 μg/100 ml serum). Liver copper concentration in the newborn pig is higher than in adult pigs (4). Since ceruloplasmin, a copper-containing protein, which accounts for approximately 50% of the serum copper in the pig is an α-globulin, the question arose as to whether the limiting factor in serum copper concentration was synthesis of this copper protein. Therefore, 2 experiments were conducted to determine the changes occurring during the postnatal period in copper distribution in the serum and the effect of supplemental copper on ceruloplasmin activity in the blood.

EXPERIMENTAL

The first experiment was conducted with 3 litters of naturally raised pigs at the swine barn at North Carolina State University. Immediately after birth and before the pigs were allowed to suckle, a blood sample was taken from the vena cava of each piglet. In each litter, 3 littermates were assigned to the control group, and three were injected intraperitoneally with a copper solution (1 mg copper/pig) in the form of copper-ethylenediaminetetra-acetate (EDTA). All piglets received an iron injection at one week of age, and thereafter they had free access to a commercial starter ration in automatic feeders. Additional blood samples were collected at 7, 14, 21 and 28 days of age, and total copper and ceruloplasmin activity was determined in the serum.

In the second experiment 2 litters of piglets raised artificially were used. The

pigs were taken from the sow at parturition, caught in a clean towel, and placed in individual cages equipped with an infrared heat lamp. After birth, all pigs were weighed, bled from the vena cava and injected intraperitoneally with 5 ml of porcine γ-globulin (5). Based on birth weights, 4 replications of 3 pigs each were made from each litter. The 3 pigs of each replication were allotted at random to consecutive cages made of aluminum, and each piglet was assigned at random to one of the three experimental dietary sequences listed in table 1. Criteria measured were as follows: body weight; total serum copper, by the method of Parks et al. (6); ceruloplasmin activity by the procedure of Houchin (7) as modified by Rice (8); hemoglobin by the method of Shenk et al. (9); total serum protein, estimated by refractive index, values obtained with a hand protein refractometer [5]; optical density of serum TCA precipitate, as an estimate of

true protein (1); and copper and iron concentration of the liver and spleen by direct reading in an atomic absorption spectrophotometer [6]; cytochrome oxidase activity by the method of Cooperstein and Lazarow (10); and tissue protein by the method of Lowry (11).

RESULTS AND DISCUSSION

The results obtained with the naturally raised pigs are shown in figures 1 and 2. Serum copper levels (fig. 1) of both the control group and the copper-injected group increased from an initial value of about 30 μg to approximately 200 μg/100 ml of serum which approaches a normal serum copper concentration for adult pigs. Although the serum copper level of the copper-injected group increased at a slightly faster rate than the control group,
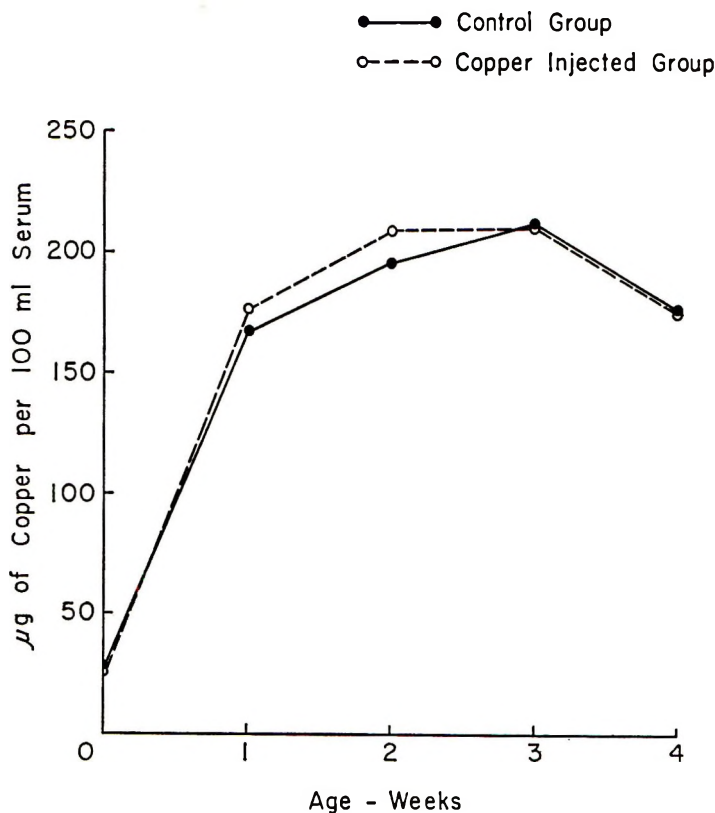
Fig. 1   Total serum copper changes during the first 4 weeks of life in naturally raised pigs.
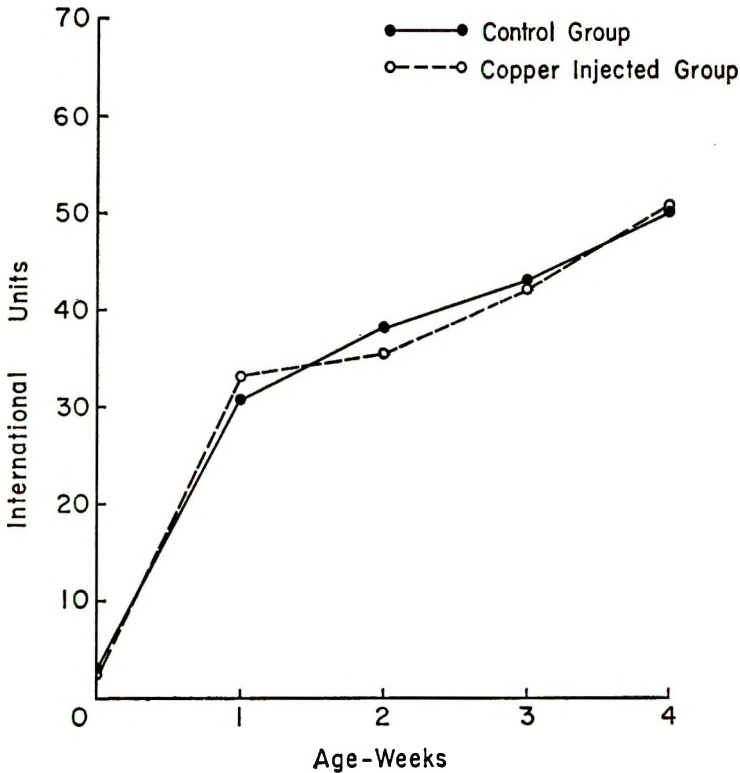
Fig. 2   Ceruloplasmin activity in naturally raised pigs.

the differences between the 2 groups is negligible. These results could be the result of either poor utilization of copper-EDTA or of the copper not being limiting in either sequence. Ceruloplasmin activity for both groups also increased from birth in a manner analogous to that of the serum copper values (fig. 2). With both criteria, moreover, the greatest increase occurred in the first week.

The experimental diets and feeding protocol for the artificially raised pigs are shown in tables 1 and 2. Essentially, the feeding regimen was divided into 2 phases. During the first phase, which extended for a period of 30 hours from the initial feeding, the experimental groups on sequences A and B were fed the mineral and vitamin solution (table 1), and the group on sequence C was fed cow's colostrum. From

TABLE 1

Experimental dietary sequences for artificially raised pigs

Time sequence	Diet A	Diet B	Diet C
0 to 30 hr	Mineral-vitamin solution [1]	Mineral-vitamin solution	Cow's colostrum
36 to 90 hr [2]	Change-over sequence to whole raw milk	Change-over sequence to whole raw milk	Change-over sequence to whole raw milk
Day 4 to 21	Whole raw milk + 20 ppm copper + 80 ppm iron	Whole raw milk + 80 ppm iron	Whole raw milk + 80 ppm iron

[1] Stock solution, part A (double concentration for 2 liters): (in grams) KCl, 10.4; KH_2PO_4, 2.4; $Na_2H(PO_4)\cdot 7H_2O$, 15.6; $MgSO_4\cdot 7H_2O$, 2.9; vitamins, 60.0; (Matrone et al., J. Nutr., 86: 155, 1965). Stock solution, part B: 0.441 g of $CaCl_2\cdot 2H_2O$ was dissolved in 1 liter of distilled water. Final solution: equal parts of stock solutions A and B were mixed immediately before feeding.
[2] Milk used in the change-over period was already mixed with the corresponding proportions of copper and/or iron specified in the last time-sequence.

TABLE 2

Feeding schedule

Feeding no.	Feeding period	Volume/ feeding	Diet A	Diet B	Diet C
	hours	*ml*			
1	zero	100			
2	6	100			
3	12	100	Mineral-vitamin	Mineral-vitamin	Cow's colostrum
4	18	100	solution	solution	
5	24	100			
6	30	100			
				Change-over sequence [1]	
			MVS-milk	MVS-milk	CC-milk
7	36	90	50%–50%	50%–50%	50%–50%
8–12 [2]	42–72	90	50%–50%	50%–50%	50%–50%
13–17	72–90	90	25%–75%	25%–75%	25%–75%
	days				
18–22	5	90			
23–27	6	100			
28–32	7	120			
33–37	8	150			
38–42	9	180	Milk + 80 ppm	Milk + 80 ppm	Milk + 80 ppm
43–47	10	210	iron + 20 ppm	iron	iron
48–88	11–18	240	copper		
89–93	19	270			
94–98	20	300			
99–103	21	360			

[1] MVS indicates mineral-vitamin solution; CC, cow's colostrum.
[2] Feeding 5 times/day, for example, 7 A.M., 11 A.M., 3 P.M., 7 P.M., *and* 11 P.M.

36 hours to 90 hours all experimental groups were changed over gradually to whole raw milk. Starting on the fifth day, 80 ppm of iron were added to the raw milk fed to all groups, and, in addition, the group on sequence A received 20 ppm of copper which was added to the milk.

The gains of the group fed the normal copper diet (sequence A) and the one fed the low copper diet (sequence B) were almost identical, whereas the group fed the cow's colostrum low copper diet (sequence C) gained significantly more than the other 2 groups (fig. 3). Since all the pigs received equal amounts of milk diet after the fourth day, the superior performance of the groups on sequence C apparently was the result of the cow's colostrum fed during the first 4 days.

There was a precipitous drop in hemoglobin values in the first 90 hours for all groups (fig. 4). Thereafter, there was a gradual rise in hemoglobin throughout the 21-day experimental period for the groups receiving the normal copper diet and those receiving the low copper diet, whereas that of the group fed the cow's colostrum increased rapidly until the seventh day and then leveled off. It might be inferred that the initial precipitous drop was associated with the fact that iron was omitted from the diet for the first 4 days; however, this phenomenon has been observed with artificially raised pigs when iron and copper were supplied in the diets from birth (12). Also noteworthy is that, presumably, feeding of cow's colostrum resulted in a rapid increase in hemoglobin level despite the raw milk diet being fed after the fourth day. This suggests that the colostral proteins either directly or indirectly influenced the synthesis of hemoglobin.

The total serum copper and ceruloplasmin activity values are shown in figure 5. The serum copper for the low copper and the cow's colostrum low copper groups increased from a value of 25 µg copper/100 ml and plateaued at 90 hours and 14 days, respectively, at a value of approximately 75 µg/100 ml serum. The normal copper group, however, followed the same trends as the other 2 groups up until the seventh day. Thereafter, it increased rapidly until by day 14 these pigs had attained the same level of serum copper as that of the adult pig. As shown in figure 5, the response
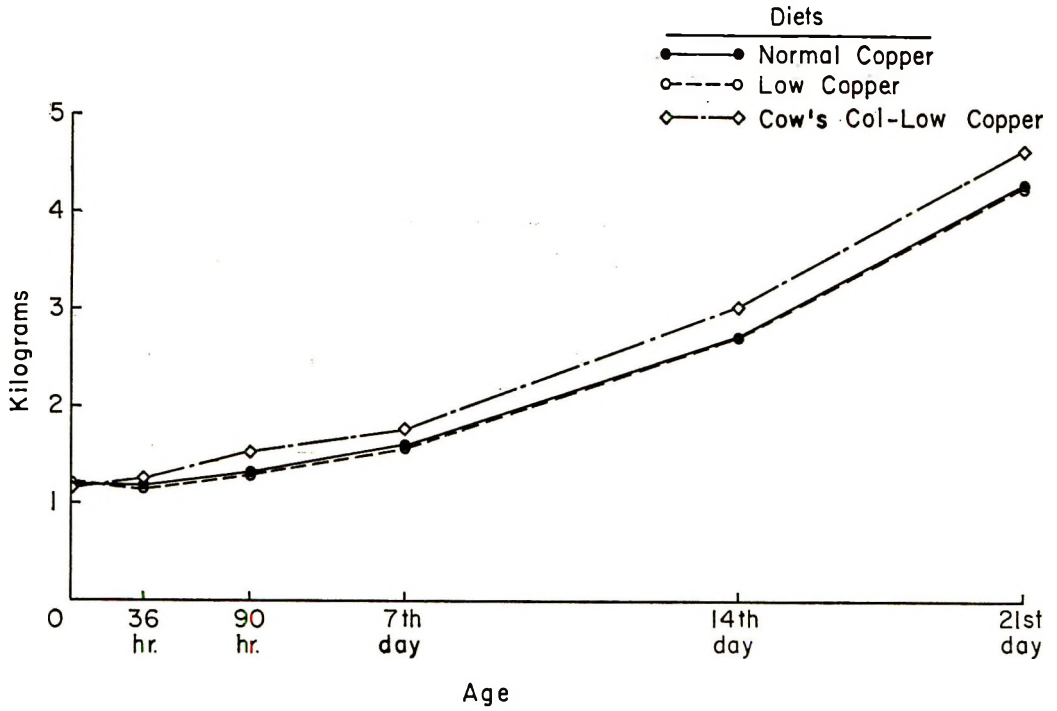
Fig. 3 Effect of diet on weight gains of artificially raised pigs.
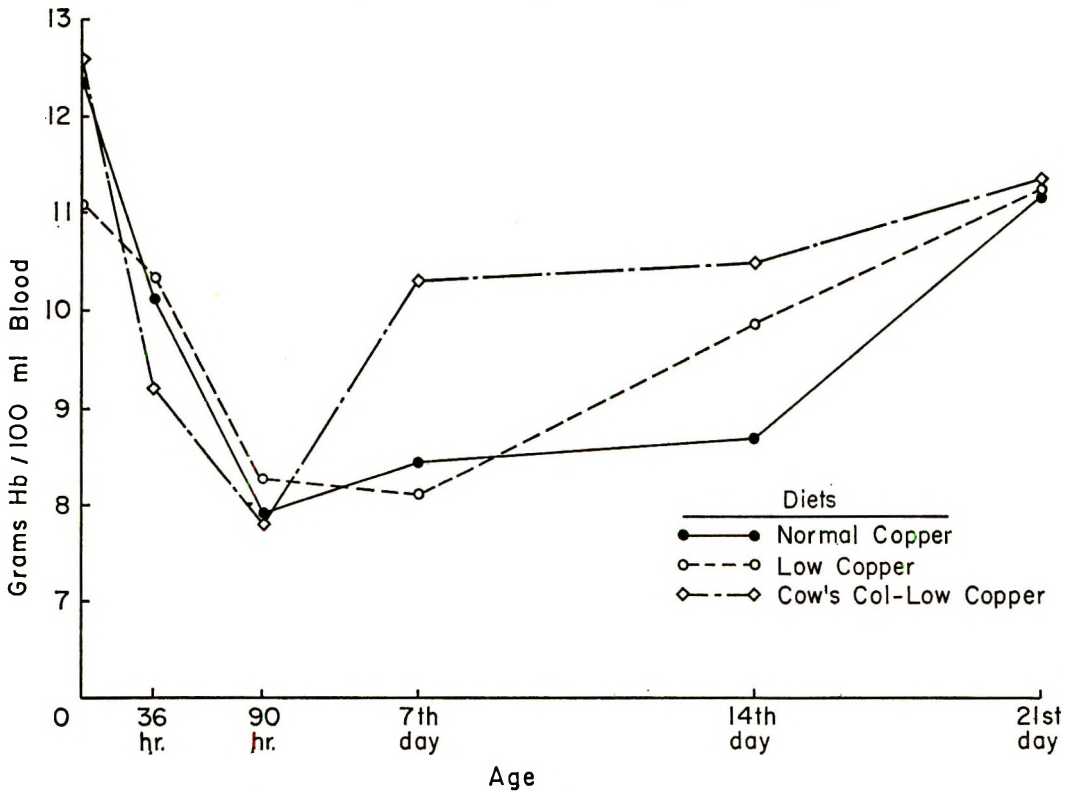
Fig. 4 Effect of diet on hemoglobin levels of artificially raised pigs.
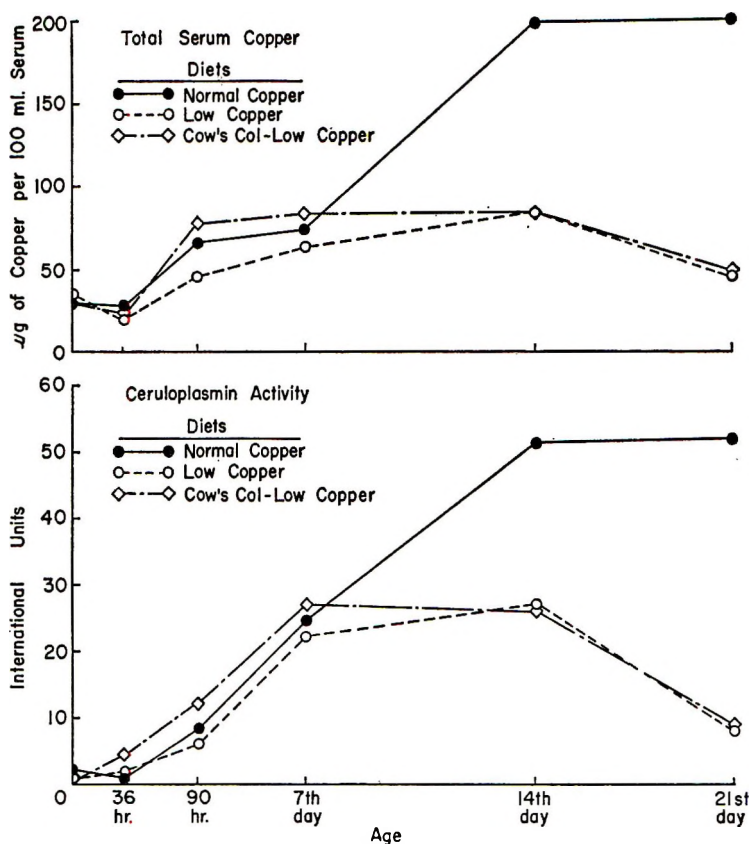
Fig. 5 Effect of normal and copper-deficient diets on total serum copper and ceruloplasmin activity of artificially raised pigs during the first 3 weeks of life.

curves for ceruloplasmin activity are similar to those of serum copper. These data suggest that copper in the diet induced the synthesis of ceruloplasmin.

Optical density of the TCA precipitate of the serum and total serum proteins are presented in figure 6. The optical density values are a relative measurement of true protein. The greatest difference obtained in this measurement was between the colostrum-fed group and those not receiving colostrum. As reported previously (1–3), the higher values obtained during the early part of the experiment reflected, in part at least, absorption of intact cow colostral proteins. Pigs in all groups, however, attained similar values at the end of 14 days.

The absorption of the colostral proteins is also reflected in the total serum protein data. The percentage of protein in the serum was increased from 3% to over 5%

in 36 hours in the group receiving colostrum and then gradually decreased to approximately 4% at the end of 14 days. The other 2 groups showed a gradual increase from 3% to 4% during the first 14 days. That the total serum proteins of the colostrum group dropped back to the level of the other 2 groups at the end of 14 days suggests that although the colostral protein was absorbed, it did not have an effect on the maturation of the protein synthesis system of the piglet.

The average heart cytochrome oxidase activity (optical density change/min/mg protein) values for the pigs on sequences A, B and C were 4.43, 6.12 and 4.99, respectively. Analysis of variance of these data, however, indicate no significant differences among groups.

Lack of copper in the diet brought about a marked reduction of liver copper
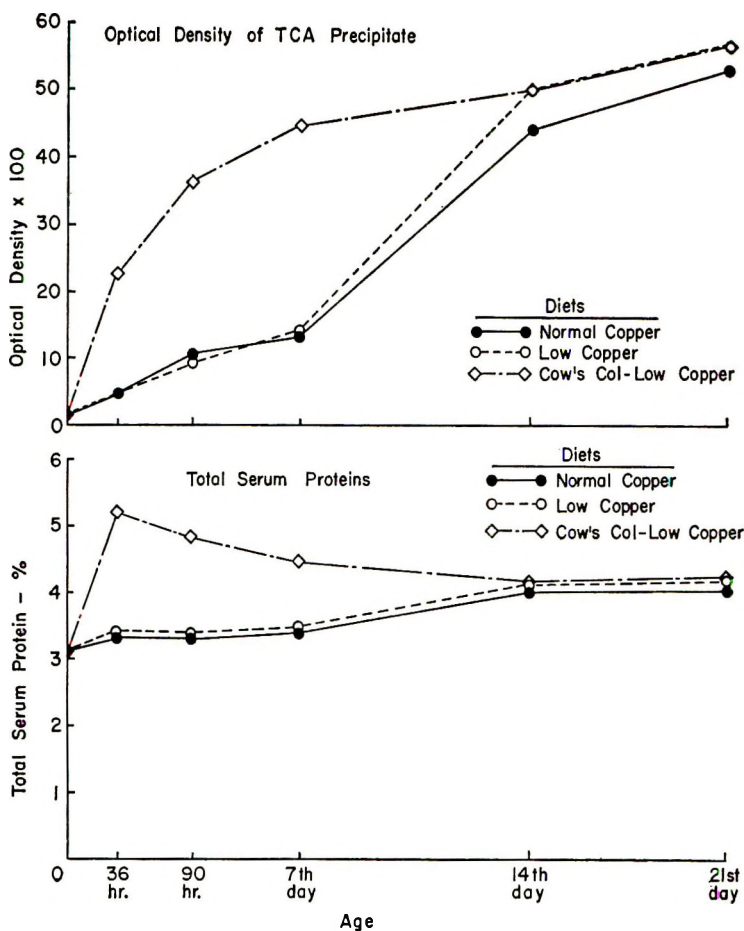
Fig. 6 Effects of diet on TCA precipitate and total serum proteins.

TABLE 3

Copper and iron of liver and spleen of 3-week old pigs artificially raised

Dietary [1] sequence	Copper				Iron			
	Liver		Spleen		Liver		Spleen	
	Conc	Total amt	Conc	Total amt	Conc	Total amt	Ccnc	Total amt
	ppm	*mg*	*ppm*	*mg*	*ppm*	*mg*	*ppm*	*mg*
A [2]	247.81	8.16	17.06	0.034	158.49	5.15	532.89	1.12
B	16.89	0.55	12.43	0.021	238.33	7.71	525.69	0.97
C	16.07 [2]	0.52 [2]	10.61	0.017	170.28	5.43	488.32	0.82

[1] Dietary sequences: A: normal copper; B: low copper; C: cow's colostrum-low copper.
[2] Values represent means of 7 observations, remaining values for B and C diets represent the means of 8 observations.

and a smaller reduction of spleen copper (table 3).

The pigs on sequences B and C without supplemental copper contained an average of 0.50 mg of liver copper at 3 weeks even though at birth the average liver copper value for piglets ranges from 1 to 2 mg (4). These results suggest that there is a copper priority for copper enzymes such as cytochrome oxidase.

The results of the liver iron analyses are shown in table 3. The iron concentration and total iron per tissue is higher for the group receiving sequence B. The group on sequence C, however, has amounts of iron similar to those of the normal copper diet. The difference between the groups on sequence A and B confirms data in the literature showing that in copper deficiency there is an accumulation of iron in the liver (13). The difference between B and C, however, elicits a different explanation. As shown in figure 4, the sequence C piglets attained a near-normal level of hemoglobin at 7 days in contrast with the other 2 groups which remained anemic. Thus, the lower level of iron in the livers of sequence C piglets probably was a result of the greater amount of hemoglobin synthesized. A significant corollary of these results is that at least during the first 3 weeks of life, under the conditions of the experiment, the limiting factor is not copper or iron but protein, and probably a particular type of protein.

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Arsenic, Germanium, Tin and Vanadium in Mice: Effects on growth, survival and tissue levels [1]

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ABSTRACT   Certain trace elements found in the human environment may produce adverse biological effects when fed in small amounts to rats and mice for their lifetime. Others may be inert or have favorable actions. Mice numbering 643 were fed a diet of rye, corn oil and dried skim milk containing small amounts of arsenic, germanium and tin and larger amounts of vanadium, in an environment designed to exclude trace contaminants. Groups of 52 to 108 or more divided as to sex were given 5 ppm of these elements as arsenite, germanate, vanadyl or stannous ions in drinking water from weaning until natural death. Chromium (1 ppm) was added to the water of those given arsenic and germanium and their controls. No element but chromium affected growth. Median life spans and longevity of the oldest 10% of males given germanium and of both sexes given arsenic were somewhat shortened. This innate toxicity was not observed in animals given tin and vanadium. All elements accumulated in one or more organs, germanium and tin in spleen with age. No element was carcinogenic. Life-term exposures of animals to trace elements may show inherent toxicities at levels tolerable to young animals.

Certain biological effects of small doses of several trace elements, given in drinking water to mice and rats from the time of weaning until death in an environment relatively free of contaminating metals, are under investigation. This report concerns one trace metal which may be introduced as a contaminant in food, tin, and three trace elements ordinarily present in most foods, arsenic, vanadium and germanium.

METHODS

The experimental conditions of the environment, and the food and water to which mice were exposed for their lifetimes have been reported in the first paper of this series (1) and have not been altered. In brief, the diet consisted of whole seed rye flour (60%), powdered skim milk (30%) and corn oil (9%), to which was added 1% iodized sodium chloride, 100 mg/kg iron as ferrous sulfate and a vitamin mixture. The drinking water came from a mountain spring and was doubly deionized; to it were added the essential trace metals, zinc (50 µg/ml), copper (5 µg/ml), manganese (10 µg/ml), cobalt (1 µg/ml) and molybdenum (1 µg/ml) as citrates, acetates or molybdate; these concentrations are similar to those present in commercial diets. Extensive precautions were taken to avoid metallic contamination (1).

Random-bred mice of the Charles River CD strain were born of pregnant females purchased from the supplier.[3] At the time of weaning 54 males and 54 females, 6/cage, were given the basic drinking water containing 5 µg/ml arsenic as sodium arsenite and 1 µg/ml chromium as chromic acetate; we have found chromium (III) to be a growth factor for rats and mice (1, 2). Another group of 71 male and 65 female weanling mice were given the water with added chromium and 5 µg/ml germanium as sodium germanate. Controls were 54 males and 54 females given the basic water with added chromium. Litters were divided among the 3 groups. The diet contained 0.46 µg/g arsenic and 0.32 µg/g germanium on a wet-weight basis.

At the time studies on tin and vanadium were begun, we were not fully aware of the essentially of chromium (III); there-

fore this element was omitted from the drinking water. Mice of the same strain numbering 54 males and 54 females were given the basic water to which were added 5 µg/ml tin as stannous chloride, and 62.5 µg/ml ascorbic acid (to inhibit oxidation to stannic tin). Controls were 34 males and 46 females. The diet contained 0.28 µg/g tin, wet weight.

A pilot study on vanadium was made, using 23 males and 29 female mice which were given 5 µg/ml vanadium as vanadyl sulfate in the drinking water. We attempted to make a diet low in vanadium, and for this purpose used defatted fish flour [4] (30%), refined wheat flour (50%), white sugar (10%) and lard (9%), with sodium chloride (1%) and vitamins added. This diet was fed to mice for 6 months; it contained 1.4 µg/g vanadium. At the end of this period, the mice were given the regular diet, which contained 3.2 µg/g vandium, on a wet basis. A similar number of controls, 25 males and 26 females,

were treated identically, without added vanadium.

Animals were weighed weekly at first and then at monthly intervals. Dead animals were autopsied, tumors and gross lesions noted and abnormal tissues sectioned for microscopic analysis. Hearts, lungs, kidneys, livers and spleens were pooled in samples of 2 to 14 from various age groups and analyzed for the elements given. Because of the small size of the samples, it was impossible to analyze all control tissues for each of the 4 elements.

Tissues analyzed for tin and vanadium were ashed at 450° in muffle furnaces and handled as reported (3, 4). In the cases of arsenic and germanium, they were ashed in a low-temperature asher [5] at approximately 105° in nascent oxygen under a partial vacuum (5, 6). Analytical methods used were: tin, the chemical method of Thompson and McClelland (3, 7); ger-

[4] Viobin Corporation, Monticello, Illinois.
[5] Tracerlab 500 A, Ashland, California.
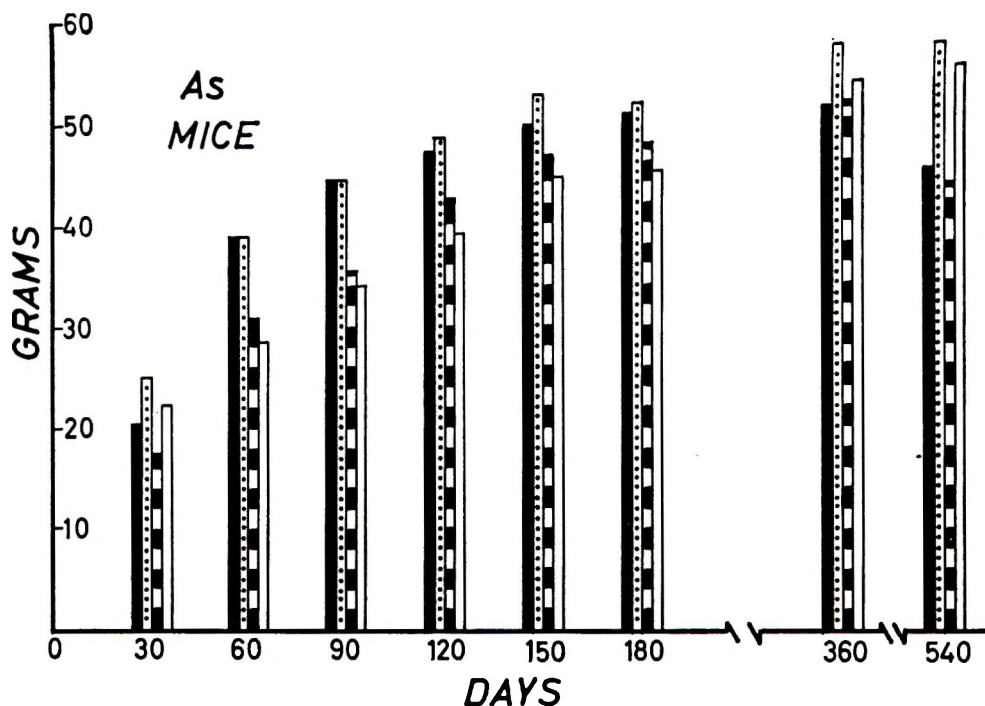
Fig. 1 Mean weights of mice given arsenic as arsenite at various ages. The solid bars represent males receiving arsenic, the dotted bars male controls. The dashed bars represent females receiving arsenic, the open bars their controls. Significant differences in weight were confined to older age males. At 360 days, male controls exceeded arsenic-fed by 4.7 g (P < 0.05) and at 540 days, by 8.9 g (P < 0.005). The difference at 540 days in the female group was 7.8 g but was not statistically significant.
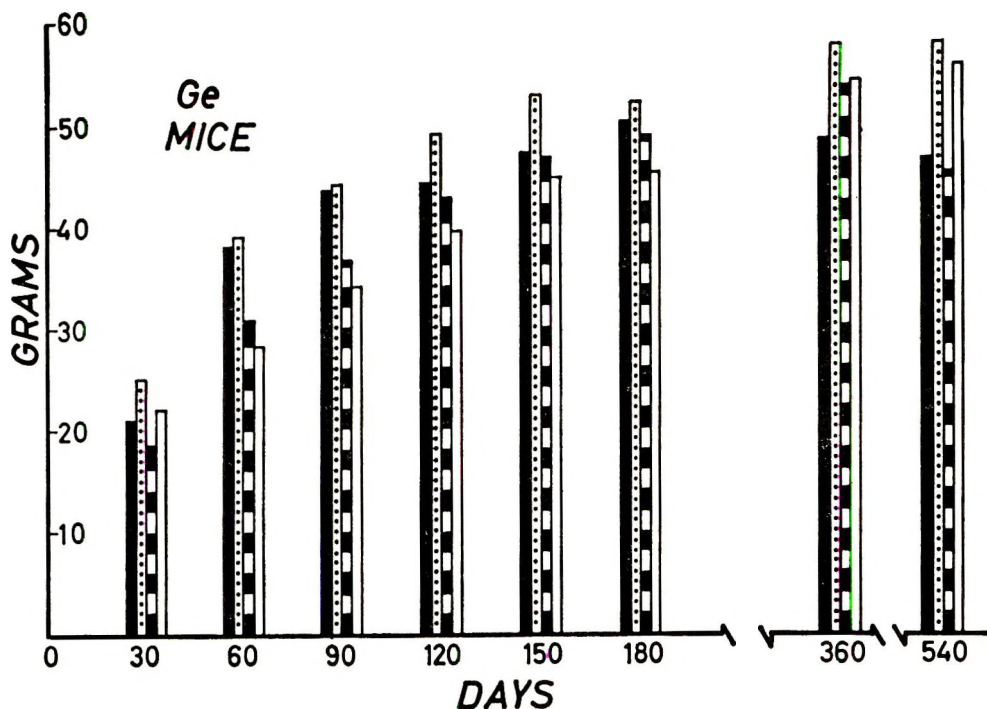
Fig. 2 Mean weights of mice given germanium as germanate at various ages. The solid bars represent males receiving germanium, the dotted bars male controls. The dashed bars represent females receiving germanium, the open bars their controls. Significant differences in weight were confined to older ages. Males at 360 days differed by 8.1 g (P < 0.005) and at 540 days by 8.7 g (P < 0.025). Females differed at 540 days by 8.9 g (P < 0.005).

manium, that of Luke and Campbell (6, 8); arsenic, that of Sandell (5, 9), modified by that of Evans and Bandemer (10); vanadium, that of Sandell (11), slightly modified (4). Sensitivities and limits of detection of these methods in our laboratory have been reported (3–6).

RESULTS

Growth rates. None of the four trace metals significantly affected growth rates of the mice, compared with their controls. There were significant differences, however, in those given chromium and those not, the former groups being heavier at all ages (P < 0.01–0.005), as reported in other series of mice (1) and rats (2). Figures 1–4 show the respective mean weights. At 18 months of age body weight decreased in females given each metal and in males given arsenic. Mice fed the fish-flour diet gained 29.3 g and those fed the same diet with added vanadium 31.0 g in 12 weeks, an insignificant difference.

Survival rates. The percentage of animals surviving at each 3-month period are shown in figures 5–8. Increased mortality was exhibited by animals fed arsenic and germanium at one or more intervals compared with controls. Tin and vanadium did not appear to affect mortality at any age. The survival curves of males receiving vanadium and tin and their controls, however, tended to be fairly straight, whereas those of males on arsenic and germanium and their controls were sigmoid-shaped. Such straight curves have been reported for male animals deficient in chromium (12, 13).

Life-span and longevity of mice. Median life spans of male mice fed arsenic and germanium were less than that of their controls by 74 and 92 days, respectively (table 1). Similarly, females fed arsenic and germanium had shortened median life spans by 76 and 35 days, respectively. No such shortening of life span occurred in mice receiving tin or
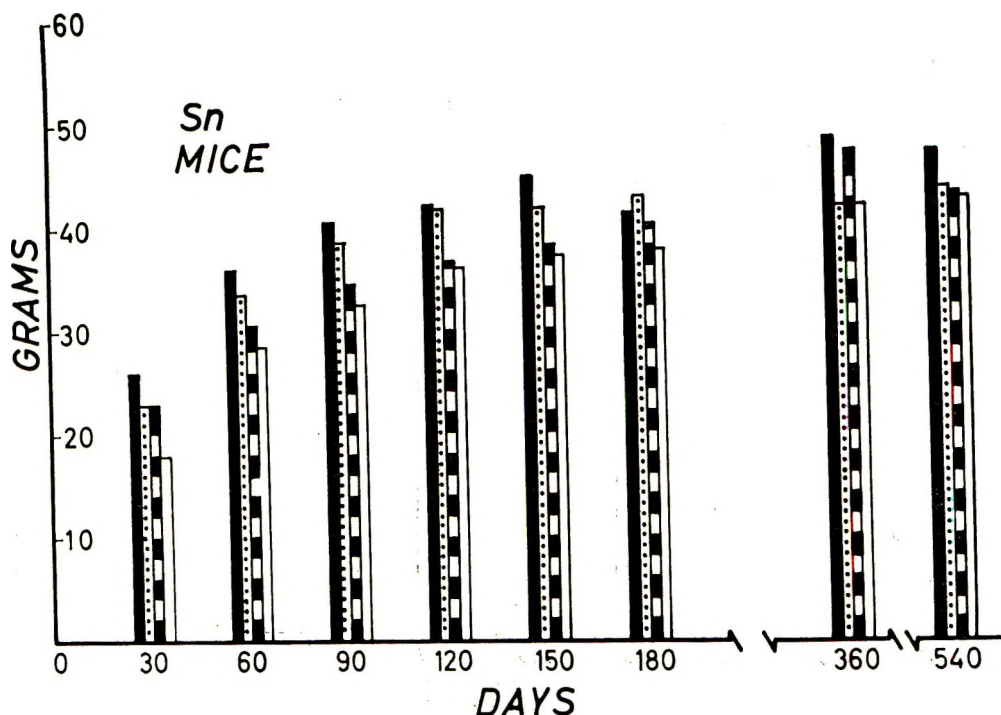
Fig. 3   Mean weights of mice receiving tin at various ages. The solid bars represent tin-fed males, the dotted bars their controls. The hatched bars represent tin-fed females, the open bars their controls. There were no significant differences between controls and tin-fed animals at any age. These mice did not receive chromium; they were significantly lighter than the controls shown in figures 1 and 2 which had 1 $\mu g/g$ in water, males differing by 2.8 to 9.02 g at various ages ($P < 0.05$–0.0005) and females differing by 2.0 to 7.7 g ($P < 0.025$–0.0005).

vanadium. Male controls had a 60-day and females a 54-day longer median life-span when given chromium than when not; similar data have been reported previously (12).

Longevity, defined as the mean age at death of the oldest 10% of animals, was significantly less in male than in female mice given arsenic and germanium, and greater in males given tin (table 2). Compared with controls, mice of both sexes fed arsenic and males fed germanium had a lessened longevity.

Accumulation of metals. In all cases, the trace metals accumulated in one or more organs of mice, in some cases in sizeable quantities (tables 3–6). Accumulation with age was demonstrated for tin and germanium (table 7); the number of samples analyzed for vanadium and arsenic were too few to estimate age-linked accumulation; although the highest values for

arsenic in spleen were noted in older mice (10 $\mu g/g$).

Incidence of spontaneous tumors. Significantly fewer spontaneous tumors, 11, were observed in mice fed arsenic, than in the controls which had 55, or those fed germanium which had 25, tin which had 22 or vanadium which had 15 ($P < 0.001$). These data are reported in detail elsewhere (14).

Other pathological changes. Examination of the microscopic sections revealed no consistent pathological changes associated with the ingestion of any of these four trace elements.

DISCUSSION

These experiments, which are confined to mice, suggest no inherent or recondite toxicity of tetravalent vanadium in the form and dose given, in terms of growth, survival, longevity, life span or patholog-
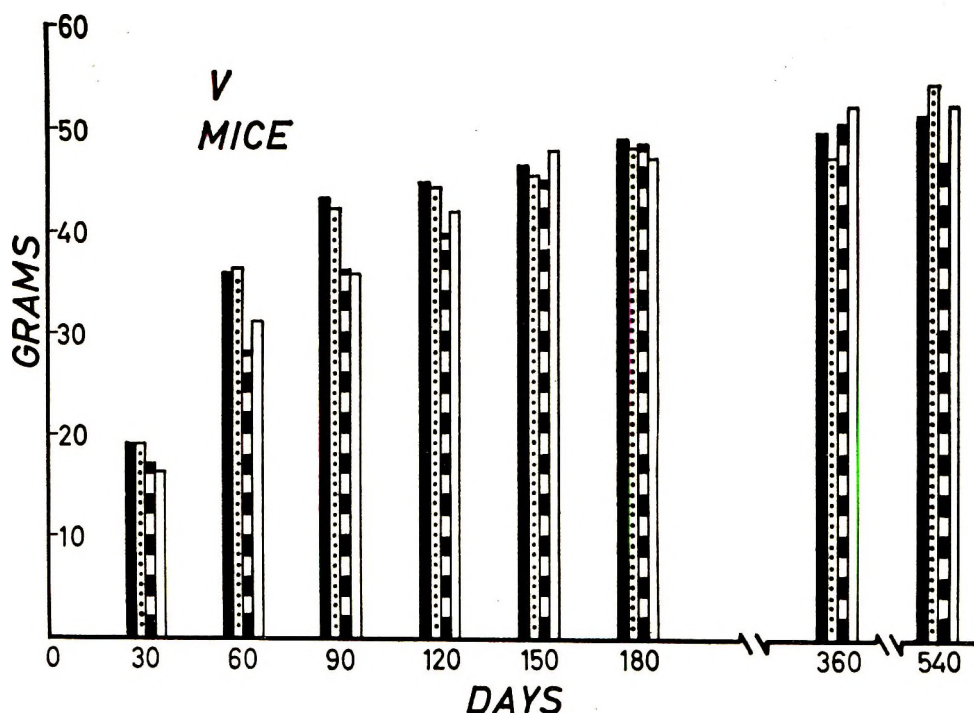
Fig. 4 Mean weights of mice receiving vanadium as vanadyl ion at various ages. Solid bars represent vanadium-fed males, dotted bars their controls; hatched bars females fed vanadium, open bars their controls. No significant differences appeared at any age. For the first 180 days these mice were fed a diet containing fish flour (see text), thereafter the regular diet. Chromium was not added to their water.
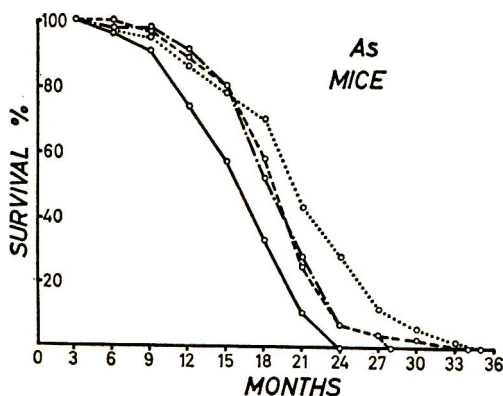

Fig. 5 Survival of mice given arsenic compared with that of controls. Solid lines show arsenic-fed males, dashed lines their controls; dot-dashed lines show arsenic-fed females, dotted lines their controls. By chi-square analysis there is a significant difference at 18 months in males ($P < 0.025$) and at 21 months in females ($P < 0.05$).
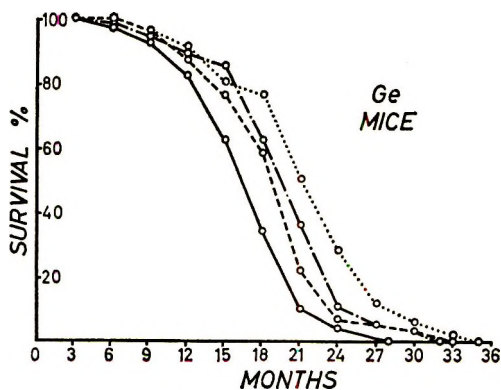
Fig. 6 Survival of mice given germanium compared with that of controls. Solid lines show germanium-fed males, dashed lines their controls; dot-dashed lines show germanium-fed females, dotted lines their controls. By chi-square analysis there is a significant difference at 18 months in males ($P < 0.005$) but not in females.
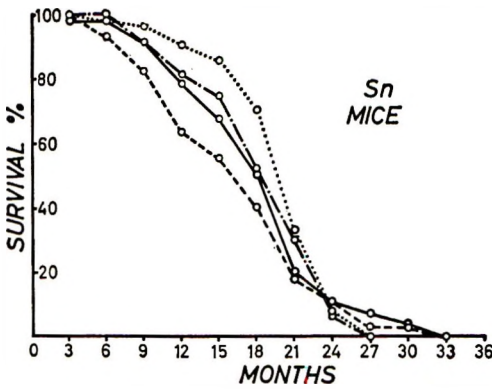
Fig. 7   Survival of mice given tin. Solid lines show tin-fed males, dashed lines their controls; dot-dashed lines show tin-fed females, dotted lines their controls. Male survival curves are relatively straight, characteristic of partial chromium deficiency. No significant difference in mortality was found at any age.
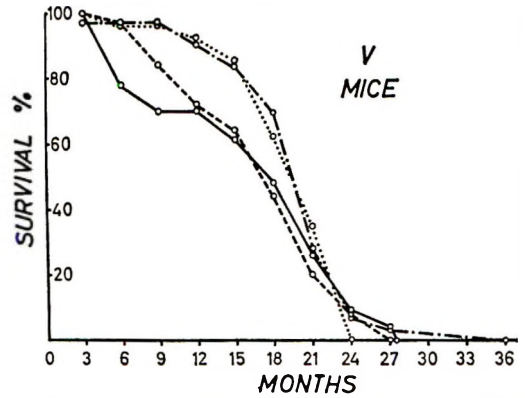
Fig. 8   Survival of mice given vanadium. Solid line represents male, dashed and dotted line female controls; dashed line represents male, dotted line female mice fed vanadium. Male survival curves are relatively straight, characteristic of partial chromium deficiency. No significant difference in mortality in each sex was found at any age.

TABLE 1

Median life spans of mice given trace elements

	Males		Females		Difference
	No. animals		No. animals		
		days		days	days
Control [1,2]	59	510	72	570	60
Vanadium [1]	23	500	29	590	90
Tin [1]	54	548	54	554	6
Control [3]	54	570	54	624	54
Arsenic [3]	54	496	54	548	52
Germanium [3]	71	478	65	589	111
Total	315		328		

[1] No chromium added to drinking water.
[2] As no significant differences appeared in the median life spans of the controls for the vanadium-fed and those for the tin-fed animals, both control groups were combined.
[3] Chromium added to drinking water, 1 μg/ml.

TABLE 2

Longevity [1] of mice given trace elements

	Male		P value [2]	Female	
	Mean age	Maximal age		Mean age	Maximal age
	days	days		days	days
Control [3]	802 ± 50.2 [4]	1007	ns [5]	735 ± 29.0	799
Vanadium	779 ± 37.6	836	ns	805 ± 93.0	1084
Tin	896 ± 35.9	990	< 0.005	761 ± 14.1	806
Control [6]	831 ± 49.6	1016	ns	910 ± 45.0 [7]	1059
Arsenic	694 ± 7.4 [8]	706	< 0.005	789 ± 22.4 [8]	849
Germanium	712 ± 29.2 [9]	835	< 0.025	829 ± 35.9	971

[1] Longevity is the mean age of the last surviving 10% of animals in each group.
[2] Significance of difference between male and female values.
[3] No chromium added.
[4] Mean ± SE of mean.
[5] Not significant.
[6] Chromium, 1 μg/ml, added.
[7] Differs from first control value, $P < 0.005$.
[8] Differs from chromium control value, $P < 0.025$.
[9] Differs from chromium control value, $P < 0.05$.

TABLE 3

Arsenic in mouse tissues, wet weight [1]

	Controls		Arsenic-fed	
	No. animals		No. animals	
		µg/g		µg/g
Kidney	2	1.30	16	1.31
Liver	29	0.02	11	0.43
Heart	11	< 0.02	13	1.41
Lung	11	0.11	11	1.35
Spleen	2	5.60	15	3.93
Tumor	1	0.93	1	0.19

[1] Tissues were pooled in lots of 2–11. Means of both sexes included. Food contained 0.46 µg/g arsenic. Eight wild mice had no detectable arsenic in kidney, 0.74 µg/g in liver and 1.1 µg/g in heart.

TABLE 4

Germanium in mouse tissues, wet weight [1]

	Controls		Germanium-fed	
	No. animals		No. animals	
		µg/g		µg/g
Kidney	11	0.12	63	3.12
Liver	3	0.49	83	2.63
Heart	2	1.62	49	1.52
Lung	2	0.35	69	3.85
Spleen	11	0.29	68	11.37
Tumor			3	2.90

[1] Means of both sexes included. Organs were pooled in lots of 2–8. Food contained 0.32 µg/g germanium. Eight wild mice had 0.36 µg/g in liver and 2.42 µg/g in spleen.

TABLE 5

Tin in mouse tissues, wet weight [1,2]

	Males		Females	
	No. animals		No. animals	
		µg/g		µg/g
Kidney	29	1.70	37	3.32
Liver	29	1.24	37	2.26
Heart	43	2.95	32	5.04
Lung	32	4.47	37	1.80
Spleen	43	1.81	37	4.47
Fat			2	0.81
Thyroid	14	2.15		
Tumor			1	5.12

[1] Organs were pooled in lots of 3–14. Food contained 0.28 µg/g tin. Mean values shown.
[2] Too few control tissues were available for tabulation; they contained less than 0.5 µg/g tin.

TABLE 6

Vanadium in mouse tissues, wet weight [1]

	Controls		Vanadium-fed	
	No. animals		No. animals	
		µg/g		µg/g
Kidney			4	5.0
Liver	6	3.38	19	2.02
Heart	18	5.42	5	25.3
Lung	24	4.80	9	9.17
Spleen	17	7.38	11	19.86
Thyroid			5	17.60
Tumor	1	1.54	1	1.90

[1] Means of both sexes included. Organs were pooled in lots of 5–12. Food contained 1.4 µg/g vanadium for first 6 months of age, thereafter 3.2 µg/g (see text).

TABLE 7

Accumulation of tin and germanium in mice with age, organs of highest concentrations, wet weight [1]

Age	Tin			Germanium	
	No. animals	Heart	Spleen	No. animals	Spleen
days		µg/g	µg/g		µg/g
< 300				4	0.42
301–400	16	3.76	0.91	10	3.23
401–500	5	4.02	2.92	23	9.88
501–600	15	3.66	3.29	27	9.17
601–700	24	5.31	2.55	8	28.68
701+	3	5.14	24.39		

[1] Mean values of concentrations in organs of all animals dying in the intervals shown are included. Organs were pooled in lots of 2–14.

ical changes, despite accumulations in heart and spleen. Because of the relatively high concentrations of vanadium in the diets (vanadium is found concentrated in all biogenic lipids studied (4)), we were unable to demonstrate physiological effects by comparing growth and survival of animals fed little or no metal with those fed normal amounts.

Although stannous tin accumulated in spleen, and, to less extent, in heart, only one effect of this element appeared. The median life span of males and females was almost identical, and longevity of males was greater than that of females. In our other series of mice, females usually lived longer than males (12). Therefore, innate toxicity of tin according to the

criteria studied was not demonstrable, despite probable accumulations.

Trivalent arsenic showed a slight tendency to accumulate in heart and lung of mice and, at the levels given, was slightly toxic in terms of median life span, longevity and survival, but not in terms of growth.

Although inorganic trivalent arsenic has been traditionally reputed to cause cancer in man, attempts to induce cancer with this element in mice and rats have consistently failed (15). The genesis and authenticity of the belief has been questioned (16). We observed fewer spontaneous tumors in mice given it at this level. The suppression of cancer incidence was apparently not associated with the decreased life span (14). Life-term experiments in rats, however, indicated no inherent toxicity of arsenic. These data will be reported.

Germanium accumulated in the spleens, kidneys, livers and lungs of mice, and at the level given, was slightly toxic in terms of life span, survival and longevity of males, but not of growth. Such subtle toxicity as appeared was confined to males. Germanium, however, has been said to show a low order of toxicity in acute experiments (16).

These experiments compared low levels of elements in the diet with higher levels in drinking water. A rough approximation of the comparable intakes can be made, assuming 7 ml of water [6] and 6 g food ingested per 100 g body weight per day (2). On this basis, the relative intakes of the controls and experimental animals per 100 g body weight per day, respectively, were: arsenic, 2.76 and 37.76 µg; germanium, 1.92 and 36.92 µg; vanadium, 8.4 to 19.2 and 43.4 to 54.2 µg; tin, 1.68 and 36.68 µg.

Because growth and survival of weanling and young mice were unaffected by any of these 4 elements, the levels given were tolerable. It is probable that longevity, late survival and life span of animals are more direct criteria of innate toxicity of elements than is the criterion of growth, especially at levels tolerated by the young.

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[6] It was almost impossible to obtain an accurate measurement of the intake of water by mice, owing to their habit of stuffing food into the drinking tubes and causing losses from the bottles. The value chosen represents the intake of mature rats weighed for a year.

Calcium Metabolism and Kinetics in Intact and Parathyroidectomized Cows Given Parathyroid Extract [1]

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ABSTRACT   Nutritional balance and ^{45}Ca kinetic studies were conducted on 3 cows during periods of experimentally altered parathyroid status. In experiments on the intact cows and on two of the cows following parathyroidectomy, the hypercalcemic response to parathyroid extract administration was consistent, and the degree of the response was comparable to that of the dog (as defined by the U.S. Pharmacopoeia) provided the dose was expressed as a function of body weight, $kg^{0.75}$. Cessation of parathyroid extract administration was followed by hypocalcemia. The kinetic data indicated that the hypercalcemic response to parathyroid extract was associated with an increase in bone resorption, the subsequent decline in plasma calcium concentration with a decrease in calcium absorption from the gut. Fecal calcium was increased during the period of parathyroid extract administration and during the immediate postinjection period. Increased fecal calcium during the hypercalcemic period induced by parathyroid extract administration appeared to be due to both an increase of endogenous fecal calcium and decreased absorption, whereas in the hypocalcemic period the increased fecal calcium was due solely to decreased calcium absorption.

The mechanisms of calcium homeostasis in the cow are challenged at parturition when negative calcium balance, hypocalcemia and a clinical syndrome, parturient paresis, may develop (1, 2). In most mammals, the parathyroid gland plays an important role in calcium homeostasis. Yet certain experimental evidence has tended to minimize the importance of the parathyroid glands in calcium homeostasis of the adult cow. Plasma calcium levels within the normal range have been reported in lactating thyroparathyroidectomized (3) and nonlactating parathyroidectomized cows (4). Elevations of only 1 to 2 mg/100 ml in plasma calcium concentration following parathyroid extract administration to non-parturient cows (5, 6) appear small when compared with plasma calcium levels over 20 mg/100 ml achieved by injection of the extract into dogs (7). In addition, parathyroid extract has failed to increase the plasma calcium concentration of parturient cows (6) and has lacked a beneficial effect in cows afflicted with parturient paresis, a hypocalcemic syndrome (5).

However, changes in plasma calcium concentration influence the plasma level of parathyroid hormone in cows (8). This suggests, in contrast with the above observations, that the parathyroid gland has the potential ability to play a role in calcium homeostasis in the cow. This role will be further determined by the underlying mechanisms and degree of the calcemic response to parathyroid hormone. The present paper reports responses in calcium metabolism of intact and parathyroidectomized cows given parathyroid extract as studied by nutritional balance and tracer kinetic methods.

METHODS

Experiments were conducted on 3 six-year-old nonpregnant, nonlactating cows in various parathyroid states (table 1). They were kept in metabolism stalls and fed 6.36 kg/day of a ground mixture of timothy hay (4 parts), corn and cob meal (8 parts), alfalfa and bromgrass hay (2 parts), and NaCl (0.14 parts). Before a balance trial, 2 weeks were allowed for equilibration

TABLE 1

Types of experiments

Cow no.	Breed	Body wt	Condition	Duration	Beginning date	PTE [1]	[45]Ca injection date
		kg		*days*		*USP units/day*	
J–2	Jersey	427	Intact	8	1/7/64		
			Intact+PTE [1]	7	1/15/64	3000	
			Post-PTE	2	1/22/64		
J–3	Jersey	369	Intact	8	9/15/64		
			Intact+PTE	7	9/23/64	3000–5000	
			Post-PTE	5	9/30/64		
		398	PTX [2]	16	8/4/65		8/9/65
			PTX+PTE	10	8/20/65	5000 [3]	8/23/65
			Post-PTE	5	8/30/65		9/1/65
G–4	Guernsey	427	Intact	7	5/7/64		
			Intact+PTE	7	5/14/64	3000	
			Post-PTE	5	5/21/64		
		522	PTX [2]	11	2/6/65		2/8/65
			PTX+PTE	8	2/17/65	5000 [3]	2/18/65
			Post-PTE	5	2/25/65		

[1] PTE, parathyroid injection, USP.
[2] PTX, parathyroidectomized; surgery was performed on cow J–3 on 7/30/65, and on cow G–4 on 8/25/64. Although complete parathyroidectomy was not confirmed in cow G–4, an estimated 92% of its parathyroid tissue was removed (4).
[3] Divided into 3 equal doses at 8-hour intervals.

with the ration. The content of the Ca and P in the diet met the recommendation of Morrison (9), and the Ca-to-P ratio was approximately 1.0. Distilled water was provided ad libitum or tap water consumption was measured so as to calculate calcium intake in the drinking water. Urine was collected by means of an indwelling self-retaining catheter (Bardex-Foley with 75-ml inflatable fluted ovoid balloon, size 26 French) into a 24-liter polyethylene carboy. Feces were collected in a pan lined with 6-mil polyethylene sheeting.

Urine and fecal collections were made daily at 8 AM. The feces were mixed with a power stirrer, weighed, and a 250-g aliquot was saved in an airtight plastic container for analysis. The urine volume was measured, and a 100-ml aliquot was frozen for analysis.

Heparinized blood samples were collected through indwelling jugular catheters of Teflon tubing (1.2 mm I.D.) before feeding at 8 AM and 4 PM and again at midnight. The plasma was separated by centrifugation within 1 hour and frozen.

Parathyroid extract [2] was injected either subcutaneously or intramuscularly once daily at 8 AM or 3 times a day at 8 AM, 4 PM, and midnight (table 1). Parathyroidectomy was accomplished with ap-

proximately 25% ablation of the thyroid gland (4).

Duplicate samples of the fecal aliquot were weighed into tared dishes and dried in an oven at 105° for 48 hours to determine dry matter content. The resultant dry feces were ground and stored. Duplicates samples (approx. 1 g) of the dry feces were redried to a constant weight in tared crucibles, and ashed in a muffle furnace overnight at 550° to 600°. The ash was dissolved in HCl (1 + 4), evaporated to dryness to dehydrate the silica, redissolved, and brought to a constant volume before filtering (10).

Calcium analysis of samples from the intact cows was performed by chelometric titration using Cal-red as the indicator (11). In the experiments with the parathyroidectomized cows, calcium analysis was by atomic absorption spectrophotometry.[3]

Plasma radioactivity was counted in 0.5-ml samples dried in a 2.5-cm diameter planchet. Standards were prepared by diluting an aliquot of the injected isotope solution with non-radioactive plasma. [45]Ca in urine and fecal ash solution was

determined by precipitating the calcium as the oxalate before counting (12). Aliquots of the injected isotope solution were similarly prepared for counting standards. Constancy of mass was attained in the precipitates of standards and samples by precipitating exactly 1.3 mg of calcium. If insufficient calcium was present in the sample, carrier calcium was added. All samples were counted with a thin-window, gas-flow Geiger detector in a low background counting system (background < 2 count/min). The activity of the samples was corrected for background and radioactive decay since the day of injection.

Tracer kinetics. $^{45}CaCl_2$ in aqueous solution with a specific activity of 1 to 4 Ci/g of Ca [4] was diluted so as to contain 1 mCi/10 ml. An injection of 1.0 mCi was made via a catheter in the mammary vein at 10 AM on each injection date (table 1). In addition to regular 8-hour samples, jugular samples were obtained at 2.5, 5, 10, 15, and 30 minutes and 1, 1.5, 2, 3, 6, 9, 12, 18, 24, and 36 hours after each ^{45}Ca injection to determine the radioactivity disappearance curve in the plasma. The results were plotted as the percentage of dose per millimole of plasma calcium.

[4] Obtained from Oak Ridge National Laboratory, Oak Ridge, Tennessee.
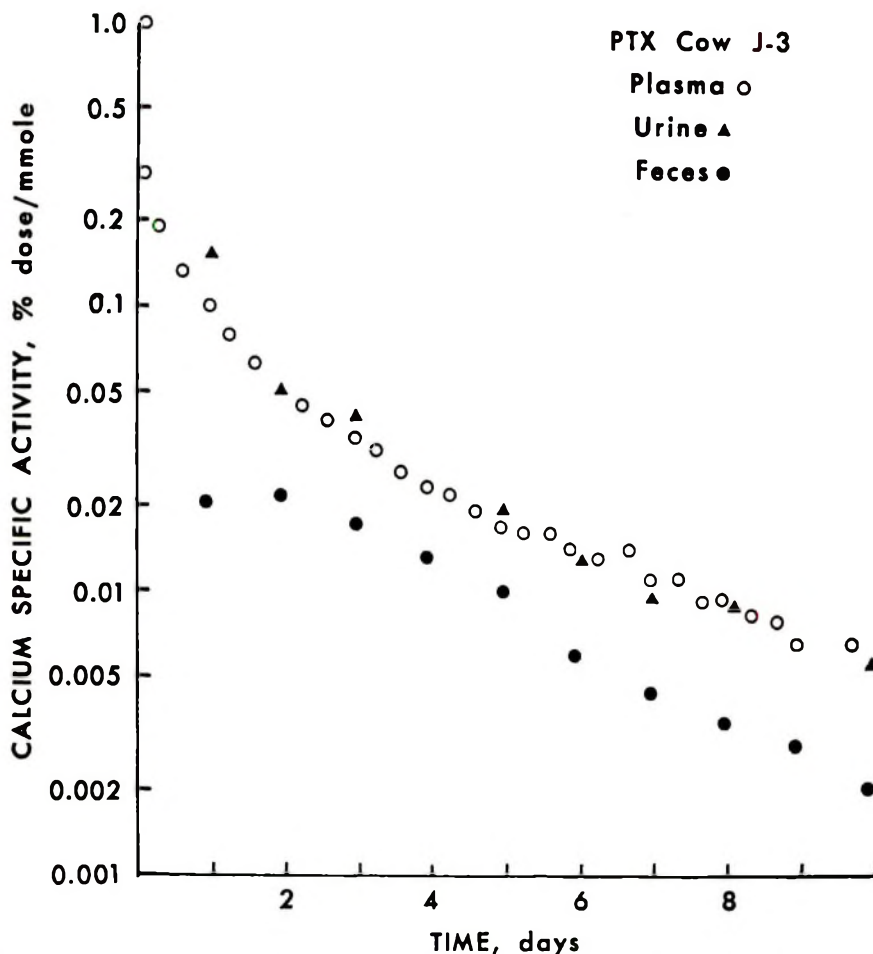
Fig. 1 Decline of radioactivity in the plasma and excreta of a parathyroidectomized cow J-3 following an intravenous ^{45}Ca injection on 8/9/65. Note that the slowest component of the plasma calcium radioactivity disappearance curve, observed in these experiments, did not become apparent until 4 days postinjection.

The data were analyzed using the second of the simplified methods and scheme 1 of calcium metabolism as proposed by Aubert et al. (13). In effect, we extrapolated the last segment (96–168 hours postinjection) of the curve, obtained by a semi-logarithmic plot of plasma calcium specific activity (SA$_P$) against time (fig. 1), back to the time of ^{45}Ca injection, that is, zero time. The slope of this portion of the curve and the Y intercept value were determined by

least squares. The fractional turnover rate $(-k)$ was calculated from the slope of this line and the pool size was calculated by dividing the injected dose by the SA$_P$ at time zero as determined from the Y intercept. The product of the fractional turnover rate times the pool size was taken to represent total inflow of calcium into the plasma sampled pool. Total outflow of calcium from the pool was assumed equal to total inflow (13). The total flows are
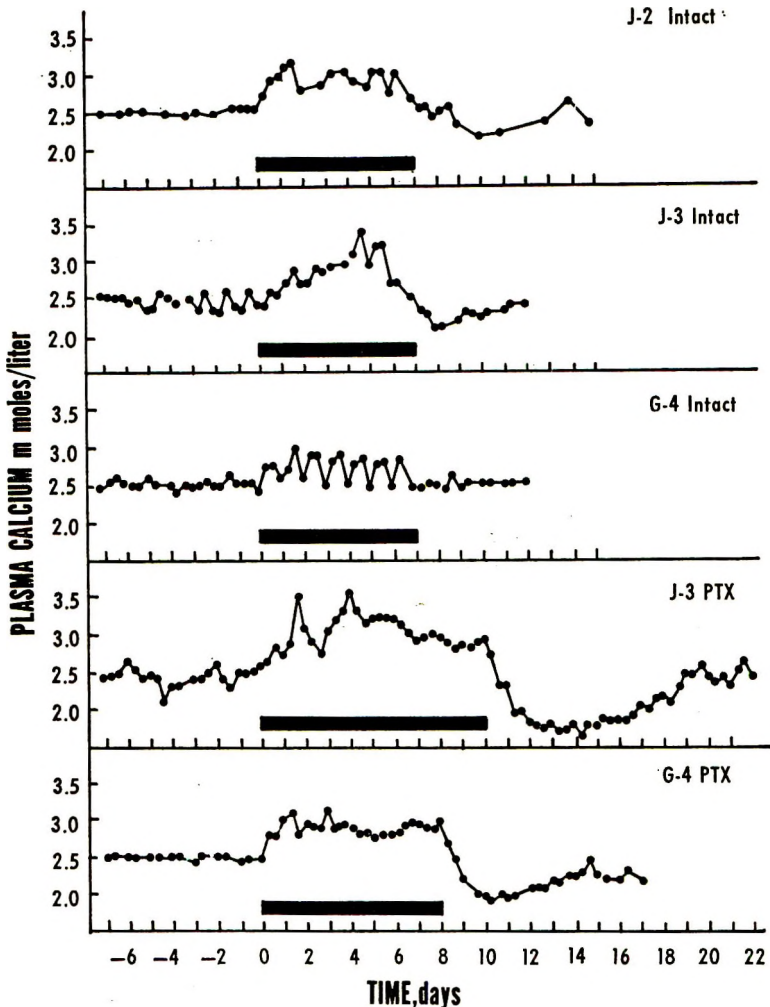
Fig. 2 Plasma calcium responses to parathyroid extract administration in intact and parathyroidectomized cows. The period of parathyroid extract administration is indicated by the solid black bar above the abscissa. Parathyroid extract was injected either subcutaneously or intramuscularly once daily in the intact cows. The dose rate for intact cows, J-2 and G-4, was 3000 USP units/day, and the dosage for cow J-3 was increased from 3000 to 5000 USP units/day beginning with the third dose. The parathyroidectomized (PTX) cows received 500 USP units/day divided into 3 equal doses at 8-hour intervals.

considered to represent the various parameters of calcium metabolism as follows:

total inflow = Ca absorption +
bone resorption
total outflow = endogenous fecal Ca +
urinary Ca + bone accretion

Since all values except flows to or from bone are determined experimentally, bone resorption and accretion can be calculated by difference.

Endogenous fecal calcium and calcium absorption were determined according to the following equations:

endogenous fecal Ca= (SA_f/SA_P) ×
fecal Ca
Ca absorption = dietary Ca intake +
endogenous fecal Ca − fecal Ca

where SA_f represents the specific activity of the fecal calcium. The mean of 4 plasma samples, taken at 8-hour intervals during the 24-hour period in which the feces were collected, was used to represent SA_P.

RESULTS

Plasma calcium. The administration of parathyroid extract consistently produced an elevation of plasma calcium in both intact and parathyroidectomized cows (fig. 2). The increases ranged from 0.48 to 1.0 mmoles/liter. The peak concentration of plasma calcium was reached 24 to 40 hours after the first injection of parathyroid extract. The single daily injections in the intact cows led to a fluctuation of plasma calcium concentration in relation to the time of injection, being the highest 12 to 18 hours post-injection then declining before the next dose. Injections at 8-hour intervals in the parathyroidectomized cows tended to eliminate this cyclic daily fluctuation so that hypercalcemia was main-
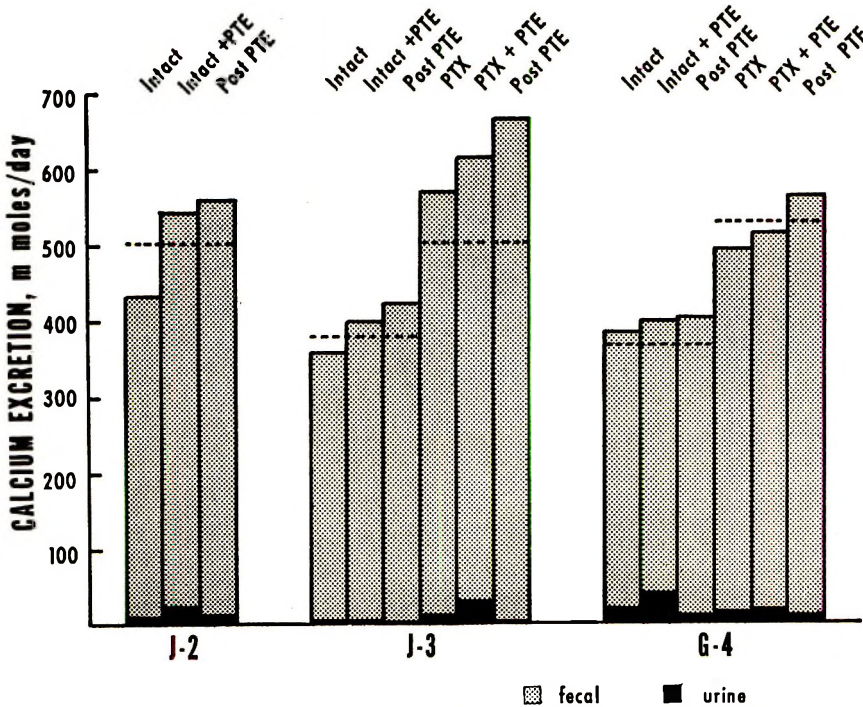
Fig. 3 Calcium excretion in intact and parathyroidectomized cows given parathyroid extract. The dashed line indicates the dietary calcium intake. The values for the period before and after parathyroid extract (PTE) administration are the mean of at least 7 daily collections for the former and at least 5 days for the latter, with one exception. The study on intact cow J-2 was continued for only 2 days after the cessation of PTE treatment. The values during PTE administration to the intact cows are the average of a 7-day period. Values during PTE administration to the parathyroidectomized (PTX) cows are the average of 10 days for cow J-3 and 8 days for cow G-4.

TABLE 2

Calcium kinetics in parathyroidectomized cows given parathyroid extract

Cow no.	J-3									G-4					
Condition	PTX[1]			PTX + PTE[2]			Post-PTE			PTX[1]		PTX + PTE[2]		Post-PTE	
Date (month/day) initial	8/10	8/13	8/16	8/20	8/24	8/27	8/30	9/5	9/8	2/9	2/12	2/17	2/22	2/25	3/3
final	8/13	8/16	8/20	8/23	8/27	8/30	9/5	9/8	9/21	2/12	2/15	2/22	2/25	3/3	3/6
Mean plasma Ca, mmoles/liter	2.56	2.44	2.44	2.90	3.20	2.90	1.96	2.08	2.44	2.51	2.48	2.87	2.90	2.20	2.28
Dietary Ca intake, mmoles/day	503	503	503	505	504	504	504	504	503	532	532	532	532	532	532
Fecal Ca, mmoles/day	538	599	540	456	575	791	667	624	537	443	505	465	543	559	482
Pool size, mmoles	—	1913	—	—	—	1696	—	749	—	—	2629	—	2908	—	—
Fractional turnover rate, days^{-1}	—	0.216	—	—	—	0.252	—	0.300	—	—	0.162	—	0.167	—	—
Total inflow, mmoles/day	—	413	—	—	—	427	—	225	—	—	427	—	485	—	—
from gut (absorption)	198	146	142	220	183	51	20	48	120	223	168	222	163	97	169
from bone (resorption)	—	267	—	—	—	376	—	177	—	—	259	—	322	—	—
Total outflow, mmoles/day	—	413	—	—	—	427	—	225	—	—	427	—	485	—	—
to gut (endogenous)	233	242	179	172	254	338	183	168	153	134	141	155	174	124	119
to urine	13	10	6	12	28	31	12	4	4	17	12	16	16	11	9
to bone (accretion)	—	161	—	—	—	58	—	52	—	—	274	—	295	—	—

[1] PTX, parathyroidectomized; surgery was performed on cow J-3 on 7/30/65, and on cow G-4 on 8/25/64. Although complete parathyroidectomy was not confirmed in cow G-4, an estimated 92% of her parathyroid tissue was removed (4).

[2] PTE, parathyroid injection, USP, 5000 units/day intramuscularly or subcutaneously, divided into 3 equal doses at 8-hour intervals. Treatment periods were 8/20 to 8/30 for cow J-3 and 2/17 to 2/25 for cow G-4.

tained throughout the period of parathyroid extract administration. Following cessation of extract administration a decrease in plasma calcium to below control levels was observed (fig. 2). This was most pronounced in the parathyroidectomized cows where the plasma calcium fell to as low as 1.75 mmoles/liter and required over a week to return to the control level.

Urinary calcium. The daily urinary excretion of calcium ranged from 5 to 20 mmoles per day in both intact and parathyroidectomized cows (fig. 3). Parathyroid extract administration led to an increase of urinary calcium in all but one trial. The maximal increase was only 2.5-fold.

Fecal calcium. Fecal calcium excretion increased during parathyroid extract administration in all but one trial (G-4 intact) and was elevated in the period following extract administration in all instances (fig. 3). The increases which occurred during parathyroid extract treatment expressed as a percentage of control values, that is, the period preceding extract administration, ranged from 4 to 23%. The increase above control levels during the post-parathyroid extract period ranged from 15 to 30%.

Calcium kinetics. After 5 to 7 days of parathyroid extract administration to the parathyroidectomized cows, bone resorption and endogenous fecal calcium increased (table 2). In cow J-3 bone accretion and calcium absorption clearly decreased during the last 3 days of parathyroid extract administration, and calcium absorption decreased further following the cessation of extract administration. In cow G-4, changes in calcium absorption during parathyroid extract administration were less clear-cut; however, a slight decline began during the latter period of extract administration and became more pronounced after treatment ceased (table 2). In both cows, calcium absorption remained depressed for approximately one week after the period of extract treatment and then returned toward pretreatment levels. Data on bone calcium flows during the post-parathyroid extract period, available from cow J-3 only, show a decrease of both bone resorption and accretion (table 2).

DISCUSSION

Calcium excretion. Although urinary calcium was slightly increased by parathyroid extract administration, the bulk of the increase in calcium excretion during and following parathyroid extract administration, which was observed in both intact and parathyroidectomized cows, was via the feces(fig. 3). The kinetic data from the parathyroidectomized cows (table 2) suggest that the increased fecal calcium during parathyroid extract administration was the result of both decreased absorption and increased endogenous secretion, whereas the elevated fecal calcium output following parathyroid extract treatment appeared to be solely the result of diminished calcium absorption.

The decline in calcium absorption may not have been due to a direct effect of parathyroid extract upon the gut since it did not become pronounced until after several days of extract administration. Also, calcium absorption declined still further and persisted for several days after extract administration was discontinued (table 2).

Plasma calcium homeostasis. Results of previous investigations have tended to minimize the role of the parathyroid glands in calcium homeostasis of cows (see introduction). In our experiments, the consistent elevation of plasma calcium following parathyroid extract administration (fig. 2) demonstrates that parathyroid extract is effective in the cow. Furthermore, the kinetic data suggest that increased bone resorption was involved in the elevation of plasma calcium (table 2). This is consistent with a direct effect of parathyroid hormone on bone as demonstrated by a variety of in vitro and in vivo techniques using other species of animals (14). These observations, together with the recent demonstration that the parathyroid glands of the cow respond to changes in plasma calcium concentration (8), lead us to conclude that the parathyroid glands are capable of performing an important role in the calcium homeostasis of the cow.

Failure to produce hypercalcemia of the degree reported in other species (7) appears to be related to the dosage used rather than a refractory response in the cow. The degree of hypercalcemia induced by parathyroid extract administration in

our cows was similar to that observed in the dog, provided the dose rates are expressed as a function of body weight, $kg^{0.75}$. The Pharmacopoeia defines 100 units of parathyroid extract as raising the plasma calcium by 0.25 mmole/liter in a 10-kg dog (16), a dose rate of 17.8 units/$kg^{0.75}$. Using cow J-2 for an example, the dose rate per day was 3000 units/427 kg, or 31.9 units/$kg^{0.75}$, and its plasma calcium increased 0.43 mmole/liter (table 1 and fig. 2). On this basis, the dose for the cow was 1.8 times that of the dog, and the increase in plasma calcium concentration observed in the cow was 1.7 times that defined in the dog.

An association between hypocalcemia and diminished calcium absorption in cows has been previously suggested by experiments involving food deprivation (16) and hyoscine-induced intestinal hypomotility (17). In our experiments, hypocalcemia associated with decreased calcium absorption was observed in the parathyroidectomized cows after cessation of parathyroid extract administration, and the subsequent return of calcium absorption toward pretreatment values was paralleled by a rise in plasma calcium concentration (fig. 2 and table 2). The observation of increased fecal calcium in the intact cows during the period immediately following cessation of extract administration (fig. 3) suggests that diminished calcium absorption may have contributed to the hypocalcemia also observed in the intact cows (fig. 2). These results further support the contention that under certain conditions calcium absorption may be important in the maintenance of a normal plasma calcium concentration in cows (17).

ACKNOWLEDGMENTS

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Percutaneous Absorption of Vitamin B_{12} in the Rat and Guinea Pig

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ABSTRACT   The percutaneous absorption of vitamin B_{12} by the rat and guinea pig was investigated. Growth experiments in weanling rats indicated that the vitamin was efficiently absorbed in an active form. Studies with ^{57}Co-labeled cyanocobalamin demonstrated that absorption was more efficient from an ethanol solution than from water or dimethyl sulfoxide and was independent of the quantity of vitamin applied to a given area. The skin was found to act as a reservoir absorbing large amounts of cyanocobalamin and releasing it slowly to other tissues. Absorption was similar in the 2 species studied.

Vitamin B_{12} is very difficultly absorbed from the gastrointestinal tract despite the fact that of all the essential nutrients it alone has a known specific mechanism to facilitate its absorption. There are reports that the vitamin is absorbed sublingually (1) and by nasal inhalation (2), but none indicate whether it is absorbed into or through the skin.

Because of the reports that dimethyl sulfoxide (DMSO) enhances the percutaneous absorption of many organic molecules, experiments were conducted in the laboratory rat to determine whether DMSO would bring about the absorption of vitamin B_{12}. Somewhat unexpectedly, it was observed that the vitamin was well absorbed through the skin from either a DMSO, an ethanolic or aqueous solution. The present report describes the results obtained in demonstrating this phenomenon, some of the factors which influence it, and its occurrence in the guinea pig as well as in the rat.

EXPERIMENTAL

Growth experiments. The first experiments were performed to determine whether cyanocobalamin applied to the skin in DMSO would stimulate growth of weanling rats consuming a vitamin B_{12}-deficient diet. Male rats of the Holtzman strain were separated into groups of eight, housed individually and allowed free access to food and water. The percentage composition of the diet was: soybean meal, 60; glucose, 23.9; salt mixture (3), 4; partially hy-

drogenated fat,[2] 10; cod liver oil, 2; thyroid powder,[3] 0.1; and a vitamin addendum [4] lacking only vitamin B_{12}. With such a diet it had been established previously that 0.1 µg cyanocobalamin/rat/day usually produced a maximal growth response (4). On the assumption that only a small fraction of the topically applied vitamin would be absorbed, 5 µg of cyanocobalamin in 0.03 ml of DMSO or water were applied daily in experiment 1 (table 1) to a 6-cm² shaved area on the backs of the animals. They were weighed at 5-day intervals and finally at 28 days when they were killed.

In experiments 2 and 3 (table 2) only 0.1 µg of cyanocobalamin in 0.03 ml of solution was applied to the rat, the experiments being identical in all other respects.

Tracer experiments with radioactive vitamin B_{12}. To quantitate the effect more accurately and to extend the initial observations, experiments were performed using vitamin B_{12}-^{57}Co. In experiment 4 (table 3) absorption from water, 90% DMSO and ethanol were measured using 2 concentrations, 0.025 µg and 2.5 µg cyanocobalamin in 0.03 ml of solution and 2 contact periods, 2 and 5 days. The solutions, each containing several tenths of a microcurie of radioactivity per dose, were

TABLE 1

Effect of 5 μg cyanocobalamin applied to skin on growth of weanling rats (exp. 1)

Diet	Topical application	Avg 28-day wt gain [1]	P value
Basal		156 ± 14.1 [2]	—
Basal + 0.1% thyroid powder		134 ± 23.8	—
Basal + 0.1% thyroid powder + 10 μg cyanocobalamin/kg diet		182 ± 36.2	0.01
Basal + 0.1% thyroid powder	5 μg cyanocobalamin in 0.03 ml DMSO [3]	188 ± 23.2	0.01
Basal + 0.1% thyroid powder	5 μg cyanocobalamin in 0.03 ml H_2O	185 ± 17.0	0.01
Basal + 0.1% thyroid powder	0.03 ml DMSO [3]	113 ± 24.5	—
Basal + 0.1% thyroid powder + 10 μg cyanocobalamin/kg diet	0.03 ml DMSO	185 ± 25.4	0.01

[1] Eight rats/group.
[2] SD.
[3] DMSO indicates dimethyl sulfoxide.

applied to a 6-cm² shaved area on the backs of male Holtzman rats weighing about 200 g and the applied areas were covered by gauze bandages held in place by adhesive tape circling the bodies of the animals. The rats were housed individually in metabolism cages to permit the collection of urine and feces, and were allowed unrestricted access to laboratory ration and water. After the designated time they were killed, the skin was excised from the area of application and the bandage and skin section were washed with portions of detergent solution.[5] Combined skin and bandage washings were diluted to 100 ml with water, as were urine samples, and the fecal samples were suspended in 100 ml water for assay. All radiometric measurements were made by gamma-ray scintillation counting. Urine, feces, washings and whole carcasses were measured in a Tobor large-volume counter, and the washed skin samples were measured in the Autogamma instrument for small volume.

In rat experiment 5 absorption of cyanocobalamin from ethanolic solution was compared at 4 concentrations (0.025, 0.25, 2.5 and 25 μg/0.03 ml) at 3 contact times; 2 hours, 2 days and 5 days. Experiment 6 was designed to investigate the role of the skin as a depot or reservoir of vitamin B_{12} after topical application. Four groups of 5 rats each were treated as described previously with 0.03 ml of ethanolic solution containing 2.5 μg of radioactive cyanocobalamin. The animals were killed at 5, 10,

19 and 40 days and appropriate measurements made. Urine and feces of all surviving animals were collected daily.

The purpose of the seventh and final experiment was to show that the observed absorptive process is not peculiar to the rat. Ten female guinea pigs of approximately 300 g in weight were subjected to experimental procedures similar in all details to those of experiments 6 with the following exceptions. One animal died on the sixth day and was examined at the time of death, 4 were killed on day 13 and the remaining five were killed on day 24.

RESULTS

The average 28-day weight gains in the growth experiments (exps. 1–3) are shown in tables 1 and 2 together with standard deviation. Also reported are probability values of differences, based on comparisons with basal diet + 0.1% thyroid powder, calculated by the method of analysis of variance. Growth responses obtained (table 1) with daily topical application of 5 μg of cyanocobalamin were significant (P = 0.01) and equivalent to ad libitum feeding of 10 μg/kg diet. Even the low dose of 0.1 μg/day when applied topically to the skin caused a maximal growth response. The DMSO and aqueous solutions were equally active.

Group average absorption results as percentage of dose in experiment 4 are re-

TABLE 2

Effect of 0.1 μg cyanocobalamin applied to skin on growth of weanling rats

Diet	Application to skin	Avg 28-day wt gain [1]		P value	
		Exp. 2	Exp. 3	Exp. 2	Exp. 3
Basal + 0.1% thyroid powder		139 ± 19.4 [2]	116 ± 22.3	—	—
Basal + 0.1% thyroid powder	0.1 μg cyanocobalamin in 0.03 ml DMSO [3] topical	157 ± 22.8	151 ± 12.2	—	0.01
Basal + 0.1% thyroid powder	0.1 μg cyanocobalamin in 0.03 ml H$_2$O, topical	160 ± 9.9	142 ± 22.7	—	0.05
Basal + 0.1% thyroid powder	0.1 μg cyanocobalamin in aqueous solution, subcutaneous	165 ± 15.5	162 ± 21.2	0.05	0.01
Basal + 0.1% thyroid powder + 10 μg cyanocobalamin/kg diet		152 ± 31.8	164 ± 12.8	—	0.01

[1] Eight rats/group.
[2] SD.
[3] DMSO indicates dimethyl sulfoxide.

TABLE 3

Effect of solvent on percutaneous absorption of cyanocobalamin-[57]Co in the rat (exp. 4)

	Application			Percutaneous absorption of cyanocobalamin-[57]Co [1]				
Vitamin B$_{12}$	Vehicle	Days	Skin	Carcass	Urine	Feces	Total absorption
μg			avg % of dose	avg % of dose	avg % of dose	avg % of dose	avg % of dose
0.025	H$_2$O	2	2.23 ± 0.56 [2]	3.02 ± 5.42	0.06 ± 0.04	0.57 ± 1.21	5.87 ± 6.16
		5	2.27 ± 0.88	0.19 ± 0.07	0.06 ± 0.05	0.13 ± 0.03	2.65 ± 0.94
0.021	EtOH	2	35.3 ± 12.3	0.56 ± 0.28	0.04 ± 0.03	0.10 ± 0.04	36.0 ± 12.53
		5	21.6 ± 10.7	0.80 ± 0.38	0.08 ± 0.06	0.20 ± 0.08	22.7 ± 10.78
0.021	90% DMSO	2	3.52 ± 0.60	0.10 ± 0.07	0.03 ± 0.02	0.09 ± 0.02	3.74 ± 0.63
		5	2.39 ± 0.41	0.37 ± 0.26	0.06 ± 0.05	0.18 ± 0.06	3.0 ± 0.58
2.5	H$_2$O	2	7.0 ± 3.3	5.95 ± 3.63	0.04 ± 0.03	1.38 ± 1.46	14.4 ± 6.64
		5	8.53 ± 4.0	0.40 ± 0.29	0.05 ± 0.05	0.21 ± 0.11	9.2 ± 4.30
2.1	EtOH	2	20.7 ± 7.7	0.51 ± 0.58	0.03 ± 0.02	0.13 ± 0.17	21.4 ± 8.16
		5	29.3 ± 7.6	2.69 ± 1.87	0.16 ± 0.10	2.39 ± 2.60	34.6 ± 11.09
2.2	90% DMSO	2	5.57 ± 6.07	4.69 ± 5.77	0.08 ± 0.11	1.75 ± 2.49	12.1 ± 8.52
		5	2.64 ± 0.79	1.07 ± 1.85	0.08 ± 0.09	0.88 ± 1.72	4.7 ± 3.52

[1] Six rats/group.
[2] SD.

corded in table 3. These data show absorption from all 3 vehicles but that absorption from ethanol is definitely superior to that from water or DMSO which were again essentially equal. The apparent lower total absorption observed at 5 days as compared with 2 days is probably the result of variations between animals (note high incidence of large standard deviations). Such variability was not encountered frequently in later experiments. This may also be due to variable retention of vitamin by the bandage.

The data obtained in the performance of experiment 5 are compiled in table 4 and show that the dose-response (total absorption) curves are approximately of a log-log type, characteristic of an adsorption process. This is true of all 3 time-intervals studied; and the slopes of the log-log curves are about equal. Initial absorption was rapid, and remained essentially constant between 2 hours and 2 days, but increased approximately 2.5-fold between 2 and 5 days. Individual absorption was variable but under the conditions of the experiment does not appear to be a function of the quantity of the vitamin applied.

Table 5 is a compilation of group average values obtained in experiment 6 expressed as percentage of dose. These figures show that the skin acts as a storage depot for vitamin B_{12}, gradually releasing the vitamin for use in other body tissues. This is shown by 1) the gradual decrease in skin radioactivity with time; 2) the concomitant increase in carcass activity; and 3) the continuous elimination of radioactivity in urine plus feces. Thus increases in elimination of radioactivity in excreta and increases in carcass concentrations occur at the expense of a parallel decrease in skin radioactivity. Excretion during the first 5 days is negligible, but increases thereafter reached a maximum in about 20 days and were still high 40 days after topical application. Animals varied considerably in the permeability and retentivity of their skin to radioactive vitamin B_{12}. In many cases high skin retentivity was accompanied by low urinary output.

The results of experiment 7 are summarized in table 6. Despite great variability between animals, the carcass and excreta activities of the guinea pig increased

TABLE 4

Effect of concentration and time on percutaneous absorption by the rat of cyanocobalamin-^{57}Co applied in ethanolic solution (exp. 5)[1]

Conc	Duration	Percutaneous absorption				Total absorption	
		Skin	Urine	Feces	Carcass		
μg	hours	avg % of dose	avg % of dose	avg % of dose	avg % of dose	% of dose	μg
0.025	2	14.8 ± 6.3[2]	—	—	1.15 ± 1.53	15.9 ± 5.78	0.0040 ± 0.0015
0.25	2	8.3 ± 2.3	—	—	0.10 ± 0.04	8.4 ± 2.28	0.021 ± 0.006
2.5	2	8.2 ± 1.6	—	—	0.22 ± 0.04	8.4 ± 1.54	0.21 ± 0.04
25	2	9.1 ± 2.2	—	—	1.20 ± 1.20	10.3 ± 1.87	2.6 ± 0.47
	days						
0.025	2	19.4 ± 10.6	0.14 ± 0.07	0.77 ± 0.51	2.47 ± 1.17	22.8 ± 11.28	0.0057 ± 0.0028
0.25	2	3.5 ± 1.7	0.12 ± 0.07	0.16 ± 0.11	0.43 ± 0.20	4.2 ± 1.78	0.01 ± 0.004
2.5	2	7.7 ± 2.5	0.22 ± 0.24	0.58 ± 0.54	1.43 ± 0.91	9.9 ± 3.50	0.25 ± 0.09
25	2	5.0 ± 2.0	0.19 ± 0.11	1.04 ± 0.30	2.88 ± 1.99	9.1 ± 3.85	2.3 ± 0.97
0.025	5	27.5 ± 5.3	0.31 ± 0.53	1.83 ± 2.90	2.57 ± 3.57	32.2 ± 12.06	0.0080 ± 0.0030
0.25	5	9.9 ± 5.3	1.49 ± 1.56	4.62 ± 4.64	4.51 ± 1.48	20.4 ± 6.61	0.056 ± 0.018
2.5	5	13.0 ± 5.1	0.82 ± 0.72	2.84 ± 2.34	5.1 ± 4.66	21.7 ± 10.46	0.54 ± 0.26
25	5	18.5 ± 7.8	0.31 ± 0.17	4.49 ± 3.45	3.46 ± 1.37	26.8 ± 10.31	6.7 ± 2.58

[1] Four rats/group.
[2] SD.

TABLE 5

Percutaneous absorption of cyanocobalamin-^{57}Co (2.5 μg) showing depot effect (exp. 6) [1]

Duration of experiment	Urine and feces, days									Skin	Carcass	Wash + bandage	Total
	1–5	5–10	10–14	14–19	19–24	24–28	28–34	34–40	Total				
	avg % of dose									avg % of dose			
days													
5	0.09 ±0.03 [2]	—	—	—	—	—	—	—	0.09 ±0.03	26.0 ± 4.9	0.62 ±0.25	65.8 ± 8.1	92.5 ± 5.9
10	0.08 ±0.04	0.54 ±0.80	—	—	—	—	—	—	0.62 ±0.81	19.1 ±11.8	1.01 ±1.04	75.9 ±13.2	96.6 ± 5.8
19	0.17 ±0.10	0.33 ±0.13	0.44 ±0.39	0.41 ±0.33	—	—	—	—	1.35 ±0.76	3.66 ± 1.60	1.19 ±0.67	87.8 ± 4.8	94.1 ± 4.7
40	0.06 ±0.05	0.28 ±0.37	1.28 ±1.78	1.50 ±1.86	0.96 ±0.94	0.77 ±0.59	0.77 ±0.67	0.65 ±0.56	6.25 ± 6.15	3.26 ± 4.11	3.19 ±1.60	79.4 ±10.28	92.1 ± 5.2

[1] Five rats per group.
[2] SD.

at the expense of a diminishing skin radio-activity, just as observed with the rat. This behavior indicates that the skin of the guinea pig also serves as a depot source for vitamin B$_{12}$. Values for skin plus bandage washings are recorded in tables 5 and 6 as an indication of the radioactivity accountability achieved in these experiments. Although somewhat variable, an average of 96% of the activity is accounted for.

Preliminary experiments with radioactive hydroxocobalamin applied to the rat indicated considerably higher percutaneous absorption of cyanocobalamin.

DISCUSSION

Cyanocobalamin appears to be readily absorbed through the skin. This is evident from the growth study as well as from the tracer measurements.

Furthermore the stimulation of growth of rats receiving a vitamin B$_{12}$-deficient diet shows that the topical vitamin is absorbed in a form active for growth production. It was unexpected that the percentage absorbed remained fairly constant over the wide range of concentrations used.

In the growth studies we were concerned that although the most inaccessible area of the back was used as the site of application, some of the vitamin might be transferred to the paws of the rat and thence to its mouth. Since in experiments 2 and 3 the quantity applied was that previously shown to be the minimum required to produce maximal growth when given parenterally (4) it did not appear likely that oral ingestion was an important factor.

In view of an earlier report (5) that 50% of a subcutaneous dose of vitamin B$_{12}$ was excreted in the urine and 6% in the feces, the reversed ratio of these parameters in the present experiment may require a word of explanation. In the former experiment in which the vitamin was administered parenterally, the vitamin probably appeared immediately in the blood stream in high concentration and consequently was excreted to a large extent by the kidney. However, during slow percutaneous absorption, blood levels probably never become excessively high, but over a period of time the vitamin is excreted in the bile and appears in the feces.

TABLE 6

Percuaneous absorption of cyanocobalamin-^{57}Co (2.5 µg) from ethanol solution by the guinea pig (exp. 7)

Guinea pig no.	Percutaneous absorption of cyanacobalamin-^{57}Co					
	Skin	Carcass	Urine + feces	Total retention [1]	Wash + bandage	Total
	% of dose	% of dose	% of dose	% of dose	% of dose	% of dose
(10) [2]	(7.1)	(0.17)	(0.07)	(7.4)	(87.4)	(94.8)
1 [3]	13.2	0.40	0.15	13.8	83.6	97.4
2	6.7	0.51	0.18	7.4	91.6	99.0
3	4.61	2.47	2.07	9.1	90.1	99.2
4	17.9	0.73	0.25	18.9	92.6	111.5
Avg ± SD	10.6 ± 6.1	1.03 ± 0.97	0.66 ± 0.94	12.3 ± 5.17	89.5 ± 4.05	101.8 ± 6.53
5 [4]	5.62	4.84	9.7	20.2	77.5	97.7
6	1.29	2.25	3.41	6.95	86.5	93.5
7	2.43	0.57	0.18	3.18	96.0	99.2
8	1.20	0.99	0.34	2.53	101.8	104.3
9	2.32	0.73	0.26	3.31	98.7	102.0
Avg ± SD	2.57 ± 1.80	1.88 ± 1.78	2.78 ± 4.10	7.2 ± 7.45	92.1 ± 9.97	99.3 ± 4.14

[1] Sum of skin, carcass and urine + feces values.
[2] Died on sixth day, not included in average.
[3] Nos. 1–4 killed on day 13.
[4] Nos. 5–9 killed on day 24.

Hyman (1) has reported that absorption of the vitamin does not occur sublingually in pernicious anemia patients but Monto and associates (2) observed absorption by such patients by the nasal route. It would be unexpected indeed if intrinsic factor were found to play a part in percutaneous absorption.

The observation that large quantities of the vitamin pass rapidly into the skin from whence it is slowly released to other tissues presents possibilities for a long-lasting pharmaceutical preparation if subsequent clinical experiments show that these results are translatable to man. The literature on the effect of massive parenteral doses of vitamin B$_{12}$ on certain peripheral disorders such as trigeminal neuralgia is voluminous and controversial. Clear-cut cases of vitamin B$_{12}$ deficiency, i.e., those responding to physiological doses of the vitamin, have been recorded in which the symptoms were not those of classic vitamin B$_{12}$ deficiency (6, 7).[6,7,8] Hence it is possible that any or all effects of vitamin B$_{12}$ may be due to correction of nutritional deficiencies rather than to pharmacological effects. The large amounts of the vitamin required may be due to poor utilization or to inefficient transport to the site where needed. For this reason it is possible that topical application in the treatment of a disease such as trigeminal neuralgia may be more effective than parenteral administration.

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Specific Hunger for Thiamine in the Rat: Selection of low concentrations of thiamine in solution [1,2]

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ABSTRACT   A study was made to determine whether rats given a thiamine-free diet can differentiate between thiamine-containing and thiamine-free solutions and obtain sufficient thiamine in this manner to maintain growth. Rats fed a thiamine-free diet and given a choice between a thiamine-containing (0.4 μg/ml) and a thiamine-free solution were able to obtain sufficient thiamine to support a growth rate close to that of their littermates fed control diets. When the rats were given a choice between a solution at pH 3.5 containing thiamine and an identical thiamine-free solution, a clear preference was shown for the thiamine solution. When the choice was between thiamine at pH 3.5 and distilled water, there was no preference, but the rats increased their fluid intake to the point where they obtained sufficient thiamine for normal growth. The absence of a preference may be indicative of an avoidance of the acid solution. When the choice was between thiamine and oxythiamine, used as a taste control, there was no preference nor was there an increase in fluid intake, and the rats failed to grow. This latter case in particular is taken as evidence for a hypothesis that the thiamine-deficient rat can detect thiamine via a specific receptor present in the oral cavity.

Since Harris and co-workers (1) first discovered that vitamin B-deficient rats would select vitamin B-adequate foods from their environment, the existence of a specific hunger for thiamine has been demonstrated many times (2–6). Little difficulty has been encountered in demonstrating a specific hunger for thiamine contained in solid diets, but investigators have not been able to demonstrate a specific hunger for thiamine in solution (6); however, Luria (7) reported that thiamine-deficient rats will press a lever for a thiamine-containing solution more times than nondeficient rats.

The obvious difference between the situation where thiamine is in the diet and the situation where thiamine is in solution is the separation in time and space between thiamine and food in the latter case; and different responses from deficient animals in these 2 situations would imply something fundamental in the mechanism by means of which a choice is made. A conditioned response (in the classic Pavlovian manner) should be developed in the same way whether the thiamine is chosen from a diet or from solution, if such a response is in fact learned. Moreover, the development of a specific hunger response to a nutrient in solution has been demonstrated by Richter (8) in the case of sodium preference by adrenalectomized rats, and by Richter and Eckert (9) in the case of calcium preference by parathyroidectomized rats.

The present study was undertaken to reinvestigate the possibility of a specific hunger for thiamine in solution, and to further elucidate the mechanism which could lead to the development of such a response.

MATERIALS AND METHODS

Solutions containing thiamine or oxythiamine [3] were adjusted to pH 3.5 by the addition of hydrochloric acid to provide minimal decomposition during experimental trials. Concentrated stock solutions of thiamine and oxythiamine were stored in the cold and used to prepare the working solutions. Working solutions of thiamine and oxythiamine were prepared fresh daily

TABLE 1

Diets

	Control	Thiamine-deficient
	%	%
Casein	22.0	22.0
Sucrose	16.4	17.0
Dextrose	30.0	30.0
Dextrin	14.8	14.8
Salt mix H [1]	5.0	5.0
Vitamin mix [2]	1.0	1.0
Choline	0.2	0.2
Corn oil	10.0	10.0
Thiamine mix [3]	0.6	—

[1] Salt mix B (Harper, A. E. 1959 J. Nutr., 68: 405) plus $Na_2SeO_3 \cdot 5H_2O$, 1.5×10^{-3}% of salt mix.
[2] Vitamin mix supplied the following/100 g dry diet: vitamin A, 400 IU; vitamin D, 40 IU; dl-α-tocopheryl acetate, 25 IU; and (in milligrams) menadione, 0.05; riboflavin, 0.5; niacin, 2.5; pyridoxine, 0.5; Ca pantothenate, 2.0; folic acid, 0.05; biotin, 0.02; and vitamin B_{12}, 0.003.
[3] 200 μg thiamine/g sucrose.

at concentrations of 0.4 μg/ml. The experimental diets were prepared according to table 1.

Male rats [4] weighing 80 to 90 g were divided into 7 experimental groups (see table 2 for experimental design). They were housed in individual steel cages with wire-mesh bottoms, in a temperature- and humidity-controlled room with a constant light-dark cycle of 12 hours. The rats were fed ad libitum. The diet was provided in porcelain feeding cups and changed daily. Body weights were recorded every other day of the test period.

A thiamine-deficient control group was provided by the 7 animals fed the deficient diet that had access to water only. The remaining animals were offered the choices indicated in table 2. Fluid intakes were recorded daily. The solutions were in graduated cylinders fitted with rubber stoppers

and drinking spouts. The position of the cylinders was varied from left to right on consecutive days to eliminate side preferences as controlling factors.

The rats offered the choice between the thiamine solution and either the pH 3.5 solution or distilled water were the test groups. Those with the choice between thiamine and oxythiamine were included as a taste control. The taste of thiamine and oxythiamine appeared identical to a number of individuals in the experimenters' laboratory. However, it was impossible to know a priori whether the taste would appear identical to normal or thiamine-deficient rats. Although oxythiamine is an antimetabolite of thiamine, it acts strictly by competitive inhibition, does not have access to the nervous system, and has an inhibition ratio greater than 100:1 (10). Therefore the animals in this study would have to drink in excess of 100 ml of the oxythiamine solution for every milliliter of thiamine solution for any significant antimetabolite effect to occur.

RESULTS

Rats fed a thiamine-deficient diet showed a significant preference for a solution containing 0.4 μg thiamine/ml over a pH 3.5 solution (group 2A in fig. 1 and table 3). None of the other groups showed a statistically significant preference for the thiamine solution. The choice of thiamine by group 2A was reflected in a growth rate comparable to that of rats fed the control diet, particularly after the choice was established (fig. 2). Although the weight gain of group 2A can be correlated with
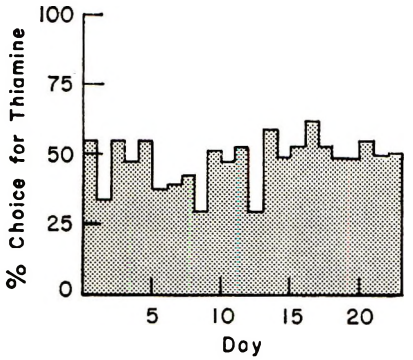
[4] Holtzman, Madison, Wisconsin.

TABLE 2

Experimental design of studies to measure preference for solutions of thiamine

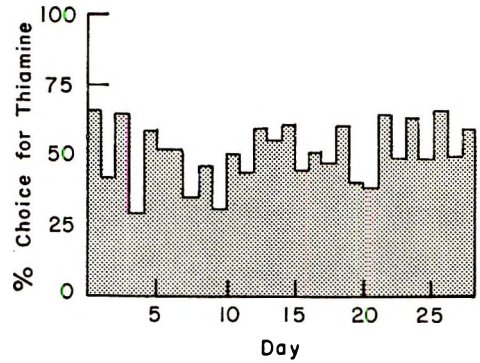| Group	Animal nos.	Diet	Choice [1]
A	1–7	thiamine-deficient	H_2O
1	8–13	control	thiamine [2] vs. oxythiamine
1A	14–19	thiamine-deficient	thiamine vs. oxythiamine
2	20–25	control	thiamine vs. pH 3.5 [3]
2A	26–31	thiamine-deficient	thiamine vs. pH 3.5
3	32–37	control	thiamine vs. H_2O
3A	38–43	thiamine-deficient	thiamine vs. H_2O

[1] Both solutions were presented simultaneously and the position of the bottles altered from left to right on consecutive days.
[2] Thiamine and oxythiamine solutions (0.4 μg/ml) were prepared in solutions of distilled water adjusted to pH 3.5 with hydrochloric acid.
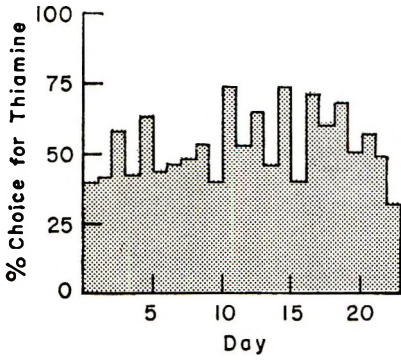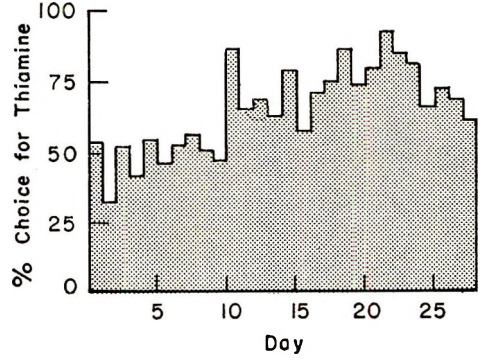[3] pH 3.5 solutions were prepared by addition of hydrochloric acid to distilled water.

Fig. 1   Mean daily group preference for thiamine-containing solution (milliliters thiamine-containing solution consumed/milliliters total fluid intake).

TABLE 3

Mean group preference for thiamine-containing solution over entire experimental period

Group [1]	Choice for thiamine	Chi square [2]
	%	
1	47.2 ± 5.5 [3]	6.5
2	51.7 ± 3.6	6.3
3	42.0 ± 8.5	10.3 [4]
1A	49.8 ± 4.4	4.7
2A	63.7 ± 5.0	50.0 [5]
3A	46.5 ± 5.8	7.2

[1] See table 2 for group description.
[2] Assuming expected mean equals 50%.
[3] Mean ± SD.
[4] Just significant, 0.95 < P > 0.90.
[5] Very highly significant, P > 0.999.

the choice for thiamine, another explanation is required for the growth response of group 3A (rats fed the deficient diet but given a choice between thiamine and distilled water), since these animals did not show a thiamine choice. The animals in group 1A (deficient diet, choice between thiamine and oxythiamine) neither made a choice for thiamine nor maintained body weight.

Figure 3 shows the growth response of typical animals from group 2A (animal 30) and group 3A (animal 43), since responses of individual animals may sometimes be of greater significance than the overall group response. Although animal
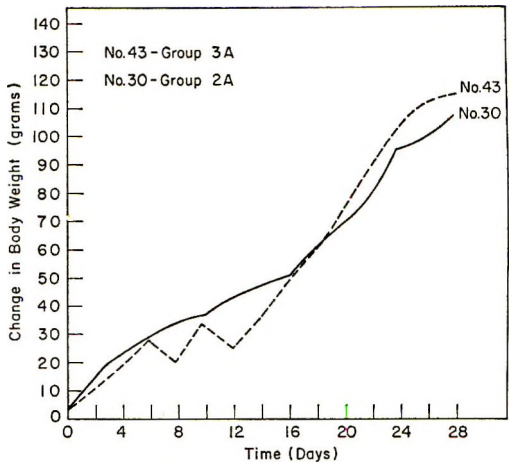
Fig. 3   Daily change in body weight for individual rats fed thiamine-deficient diets.

43 did not show a preference for the thiamine solution (fig. 4), it ingested sufficient thiamine to account for the weight gain shown in figure 3 by increasing its total daily fluid intake, thereby randomly receiving even more thiamine than animal 30 (fig. 4). The concentration of thiamine in solution (0.4 µg/ml) was such that 20 to 25 ml of the thiamine solution would have had to have been consumed to approximate the minimal daily requirement. Over the course of the experiment, animal 43 had a total fluid intake of 861 ml (194 µg thiamine), and animal 30 had a total fluid intake of 549 ml (165 µg thiamine).

DISCUSSION

The results indicate that the preference of thiamine-deficient rats for thiamine-containing solutions depends upon their ability to distinguish thiamine in their environment. When the choice was simple, that is, between thiamine or no-thiamine in otherwise identical solutions, the preference was highly significant. When the choice was between thiamine at pH 3.5 and distilled water, there was no preference, but the rats increased their overall fluid consumption to the point where they obtained sufficient thiamine to maintain a steady growth rate. It took a longer period of time for this latter group of rats (group 3A) to achieve a steady rate of growth than for the group 2A which showed thiamine preference. Since the control group
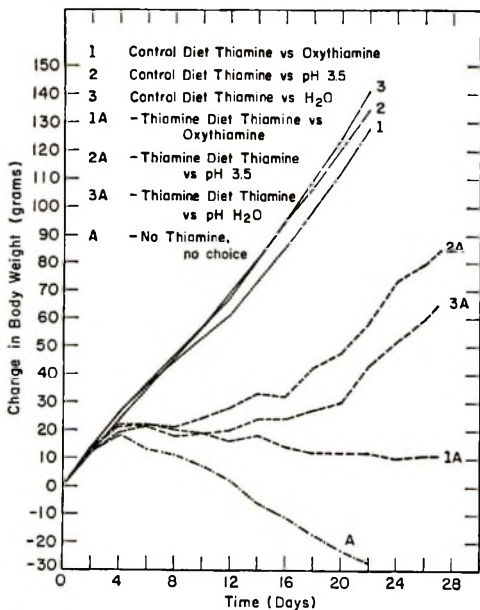

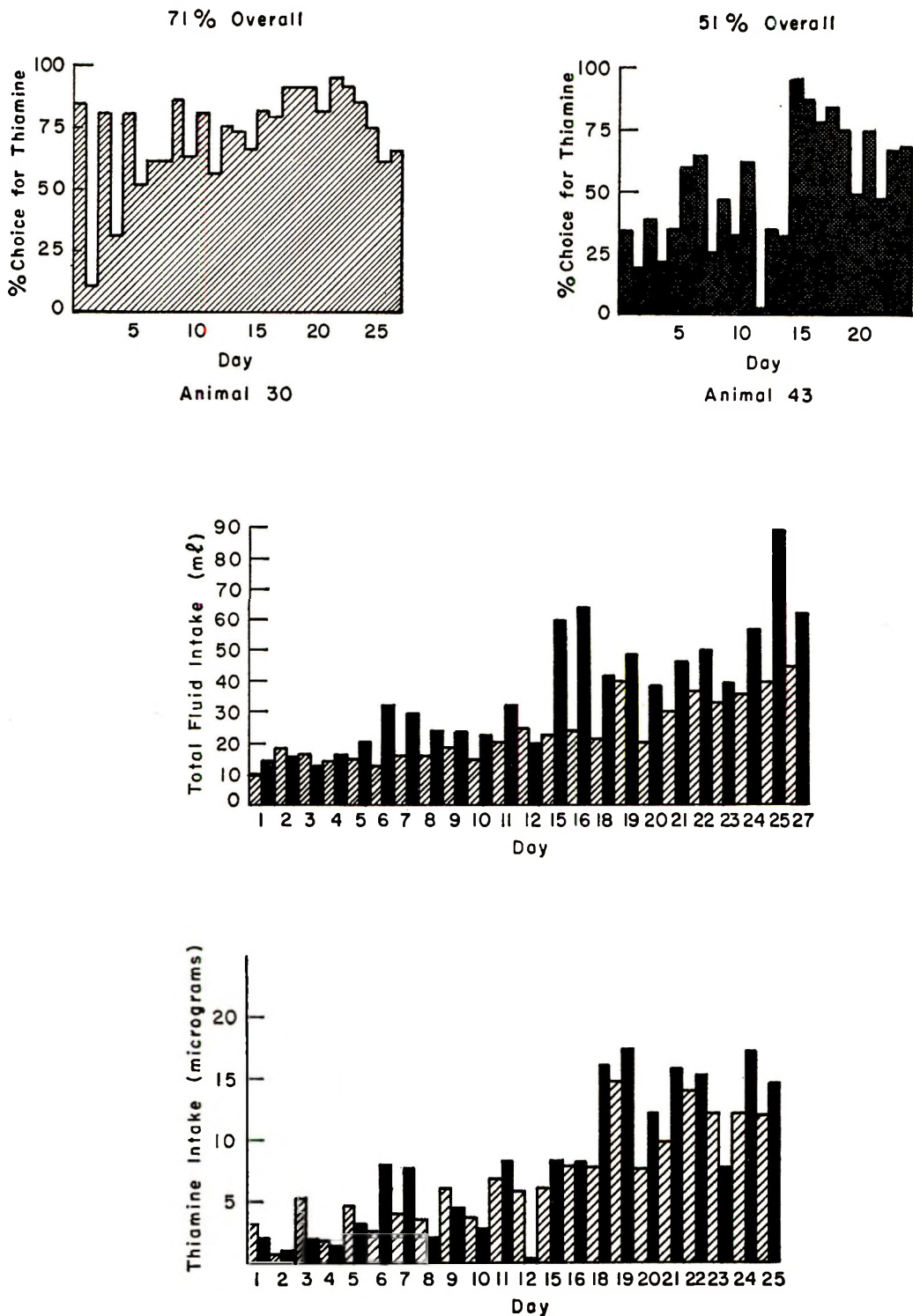Fig. 2   Daily change in body weight.

Fig. 4 Comparison of rat 30 (group 2A, deficient diet) and rat 43 (group 3A, deficient diet). Percentage of choice for thiamine (milliliters thiamine-containing solution consumed/milliliters total fluid intake), total fluid intake, total thiamine intake (daily).

(group 3) for the thiamine in pH 3.5 solution versus distilled water showed a slight rejection of the acidic medium, it may be that this effect had an overriding influence on the ability of the deficient group to make a choice.

The group given the choice between thiamine and oxythiamine never achieved a normal rate of growth and, in fact, continued to lose weight over the course of the experiment. The failure of this group to maintain thiamine intake through increased fluid intake could possibly be related to an aversion to acidic solutions. There did not, however, appear to be any marked difference in total fluid intake between the control group (group 1) given the choice between thiamine and oxythiamine and any of the other control groups (groups 2 and 3). If we accept the hypothesis that these 2 compounds have similar tastes to both normal and thiamine-deficient rats, then this result would indicate an important role for the sense of taste in the ability of the thiamine-deficient rat to distinguish between thiamine- and non-thiamine-containing solutions.

Rozin et al. (6) failed to detect a preference for a thiamine solution versus water in 21-day thiamine-deficient rats. In our experiments a preference was detectable when both thiamine and water solutions were at pH 3.5, and the animals were presented the choice before the time when they were in the deficient state. This method of presentation allowed the rats continual access to the thiamine-containing solution, and it is significant that the preference was not demonstrated until sufficient time had elapsed for the deficiency state to develop (fig. 1). It is possible that 21-day deficient rats, which in our experiments lost approximately 30% of their initial body weight, consume so little water in the face of an unacceptable diet that the opportunity to make the preference is lost; although the same investigators (6) reported that deficient rats would select a thiamine-containing solid diet (in this case the food would serve as its own reinforcement) and the choice might be much simpler than one in which the reinforcement to eating is contained in an otherwise nutrient-free medium (that is, the drinking water).

The observation that animals in various nutrient-deficient states are able to select the missing nutrient from their environment raises the question of the mechanism by means of which the response can be made. Numerous hypotheses have been proposed to explain this effect, including that of association of a feeling of "well-being" by the animal with the missing nutrient by Harris et al. (1) and Scott and Verney (4). Smith et al. (11) proposed that adrenalectomized rats learned to select a sodium diet through a need-reduction mechanism, and Richter (12) proposed a lowered threshold for sodium in the oral cavity which resulted in an enhanced taste sensitivity for sodium ions. Of particular relevance is the observation (7) that thiamine-deficient rats lever-press for thiamine-containing solutions more often than nondeficient control rats. This indicates an immediate satisfaction of a physiological need by the thiamine solution for the deficient animals and an awareness of the presence of thiamine. Reinforcement of the thiamine-deficient animals, either to make a choice or to increase their fluid intake, must occur a short interval after the solution is sampled in order for the response to be learned.

A possible explanation of the experimental results presented in this paper and of the observations by Rozin (13) and Rodgers and Rozin (14) that previously deficient and thiamine-repleted rats continue to show a thiamine preference, is that the onset of thiamine deficiency either activates a specific receptor for thiamine in the oral cavity or causes such a receptor to appear. This would account for the ability of animals to make a selection and learn a response using the sense of taste as a detector. The continued thiamine preference in repleted animals might be explained if the receptor, once activated due to thiamine deficiency, becomes slowly inactivated over a period of time during which an adequate supply of thiamine is available to the animal.

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Effects of Diet and Other Factors on Methionine Adenosyltransferase Levels in Rat Liver [1]

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ABSTRACT   The effects of some nutritional and hormonal factors on the level of the activity of methionine adenosyltransferase in rat liver were studied with the following results: 1) Thyroidectomy and cortisone treatment increased the activity of this enzyme whereas adrenalectomy and administration of bovine growth hormone or triiodothyronine lowered its activity in the liver. 2) The activity also changed with dietary protein content. Intact animals fed a high protein diet had high activity but the adrenalectomized (or hypophysectomized) animals had low activities whatever the protein content of their diet. 3) Excess methionine added to a 27% casein diet caused little change in the activity. However, rats fed a protein-free diet supplemented with methionine or its 2-hydroxy analogue (but not glycine) retained a higher level of activity than those on the protein-free diet. 4) Starvation for 2 days brought about a decrease in the activity which returned to the normal level in the subsequent 2 days.

Recent progress in the investigation of biochemical reactions concerned with methylation continues to emphasize their importance (1, 2). S-Adenosylmethionine occupies a central position as methyl donor in these biological reactions (2). It may also play a role in the regulation of the metabolism of single carbon units (3) and other intermediates (4). Methionine-adenosyltransferase (ATP:L-methionine S-adenosyltransferase, EC 2.5.1.6., henceforth referred to as the transferase) is the enzyme responsible for the catalysis of the synthesis of S-adenosylmethionine from ATP and methionine (5). In this communication we shall present the results of our survey of some of the nutritional and hormonal factors involved in the regulation of the hepatic levels of activity of this transferase, the liver being the animal (rat) tissue with a high concentration of the enzyme and of S-adenosylmethionine (6, 7).

EXPERIMENTAL

Animals.   Female rats of the Long-Evans strain weighing 145 to 200 g at the time of killing were used. They were maintained with a commercial ration [3] and tap water, but all the experimental animals were given diet N (table 1) ad libitum for 7 days before killing, except where otherwise indicated. The hypophysectomized, adrenalectomized and thyroidectomized rats used were obtained from commercial sources. [4] The adrenalectomized and hypophysectomized animals were provided with 1% of NaCl and 5% of glucose in drinking water, respectively.

Materials.   The animals were given cortisone acetate, [5] triiodothyronine, [6] or growth hormone. [7] Other groups of rats fed diet F (protein-free) were given supplements of either methionine, glycine or calcium 2-hydroxyl-4-methylthio-*n*-butyrate. [8]

Enzyme assay.   The assay procedure was adapted from the method of Cantoni and Durell (8) as modified by Mudd (9). The rats were decapitated without previous fasting except in the starvation experiments. Their livers were homogenized in a Waring blender with 2.5 volumes of 10 mM acetic acid containing 5 mM 2-mercaptoethanol and 0.2 mM EDTA. The homogenate was centrifuged for 30 minutes with 0.65 ml of a reaction mix-

TABLE 1

Composition of experimental diets [1,2]

Diet [3]	N	H	L	F
Casein	27	64	8	—
Starch	59	—	78	—
Sucrose	—	22	—	—
Dextrin, white	—	—	—	84
Yeast, brewer's	—	2	—	—
Oil, corn	10	8	10	9
Agar	—	—	—	3
Salt mixture, USP XIV [4]	4	—	4	4
Salt mixture, no. 2, USP XIII [5]	—	4	—	—

[1] The source of all diets was Nutritional Biochemicals Corporation, Cleveland.

[2] All diets were supplemented with vitamins made by adding 1 kg of the following mixture to 45.5 kg of diet: (in grams) vitamin A conc (200,000 units/g), 0.25; α-tocopherol, 5.0; ascorbic acid, 45; inositol, 5.0; choline chloride, 75; menadione, 2.25; p-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine·HCl, 1.0; thiamine·HCl, 1.0; Ca pantothenate, 3.0; (in milligrams) biotin, 20; folic acid, 90; and vitamin B_{12}, 1.35. Glucose was added to this mixture to make 1 kg.

[3] Diet M was the same as diet F but supplemented with 1.5% of DL-methionine; diet G as F with 0.75% of glycine; diet HO as diet F with 1.7% of 2-hydroxy-4-methylthio-n-butyrate.

[4] Contained: (in per cent) calcium carbonate, 6.86; calcium citrate, 30.8; calcium biphosphate, 11.3; magnesium carbonate, 3.52; magnesium sulfate, 3.83; potassium chloride, 12.5; dibasic potassium phosphate, 21.9; sodium chloride, 7.71; and 1.62 of a mixture containing: (in per cent) cupric sulfate, 0.48; ferric ammonium citrate, 94.3; manganese sulfate, 1.24; ammonium alum, 0.57; potassium iodide, 0.25; and sodium fluoride, 3.13.

[5] Contained: (in per cent) calcium biphosphate, 13.6; calcium lactate, 32.7; ferric citrate, 2.97; magnesium sulfate, 13.7; dibasic potassium phosphate, 24.0; sodium biphosphate, 8.72; and sodium chloride, 4.35.

ture, pH 7.6, of the following composition: 20 mM ATP, 20 mM L-methionine, 8 mM reduced glutathione, 300 mM $MgCl_2$, 200 mM KCl, and 130 mM Tris-HCl buffer, pH 7.6. The reaction was terminated by the addition of 0.25 ml of 6% $HClO_4$. After centrifugation, a 0.5 ml-aliquot of the supernatant was shaken with 21.5 ml of Tris-HCl buffer (15 mM, pH 8.4) and 3 ml of a 50% suspension of Dowex-1x10 (chloride form, 200–400 mesh) in a graduated test tube. The centrifuged supernatant was then read at 260 mμ in a Beckman-DU spectrophotometer. A blank without substrate was run for each sample. A molar extinction coefficient of 15,000 was used to calculate the concentrations of S-adenosylmethionine. One unit of activity is defined as the amount of enzyme catalyzing the formation of 1 μmole of S-adenosylmethionine in 30 minutes at 37° in accord with other investigators (8, 9). Protein concentrations were determined by the

modified biuret method (10) using crystalling bovine serum albumin as standard.

The livers from each group of 4 rats were pooled and the enzyme activity of each pooled sample was assayed in duplicate. Average values from duplicate experiments are reported. The individual values, when calculated as a percentage of the control values, differ from the averages within ± 5%. Intact rats fed diet N were used as controls in each experiment. The actual values for the controls (68 rats) are as follows: 68 ± 1.5 units/g of protein, or 11.2 ± 0.29 units/g of wet liver, or 42 ± 1.16 units/100 g of body weight at the time of killing. Data calculated on the initial body weight when starting the experiments are included only in table 2 and one figure, since under the experimental conditions they do not alter the picture significantly. The effect of growth hormone on the body weight of the experimental animals as well as other details of the experimental procedures are described in the legends if they do not appear in Results.

RESULTS

Effect of diet and starvation

Protein content. After feeding groups of 4 rats each with diets containing zero to 64% of casein for 7 days, differences in both the specific and total activities were evident. With a high protein diet the enzyme activity was high, whereas with a low protein diet the enzyme activity was low when compared with that of rats fed a balanced diet. The animals fed a protein-free diet had the lowest activity (fig. 1).

Effects of methionine and related compounds. The high transferase activity observed in the preceding experiments with rats fed diet H could have resulted from the presence of an excess of its substrate, methionine. Therefore we tested diet N supplemented with 1.22% of DL-methionine, an amount equivalent to that supplied by diet H. No significant change in the enzyme activity was observed; but rats that were fed diet M (a protein-free diet containing an amount of methionine equivalent to that in 45% of casein diet) for 7 days had a higher level of the transferase than those fed diet F. Its specific

TABLE 2

Effects of methionine and related compounds on the activity of the methionine adenosyltransferase of rat liver [1]

Diet [2]	Enzyme activity			
	Units/g protein	Units/g liver wet wt	Units/100 g body wt at killing	Units/100 g initial body wt
	% of initial activity			
F	62	54	50	38
M	108	88	83	64
HO	90	82	75	55
G	71	60	57	41

[1] Enzymatic activities in this table and the figures are compared under different conditions and when the activities are calculated on different bases. The absolute values for normal controls are given in the last paragraph under "Experimental."
[2] Fed for 7 days. The rats fed these diets all ate approximately the same amount of food/week. For a description of the diets see table 1.
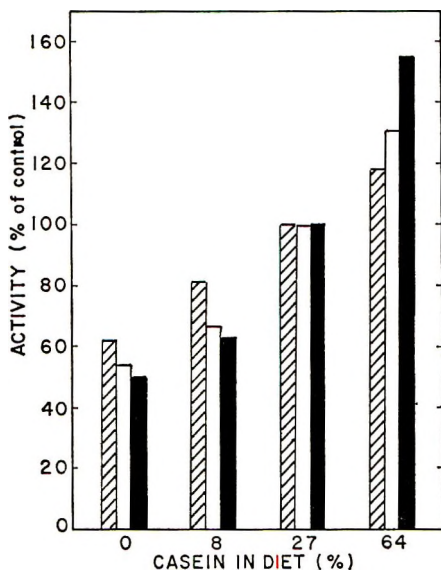

Fig. 1 Effect of dietary protein on the activity of methionine-adenosyltransferase in rat liver. Rats were fed for 7 days with diets F, L, N and H containing, respectively, 0, 8, 27, and 64% of casein before the enzyme activities of the livers were assayed. Diagonal lines per g protein; □ per g wet liver; ■ per 100 g body weight.

activity in the livers of rats receiving diet M was slightly above the normal value; nevertheless the total activity was lower than that for the controls fed diet N. A glycine supplement was almost completely ineffective, whereas the hydroxy analogue of methionine was partially effective in maintaining the transferase level (table 2).

Effect of starvation. When rats were fasted for various times up to 4 days the results shown in figure 2 were obtained.

In the first 2 days of fasting, both the specific and total activities decreased. Subsequently there was an increase toward the normal levels. On the fifth day the activity per gram of liver was 142% of the control value. In this experiment a net increase in the activity from a lower level was observed.

Effect of hormones

Effect of cortisone and adrenalectomy. Adrenalectomy decreased whereas the administration of cortisone increased the
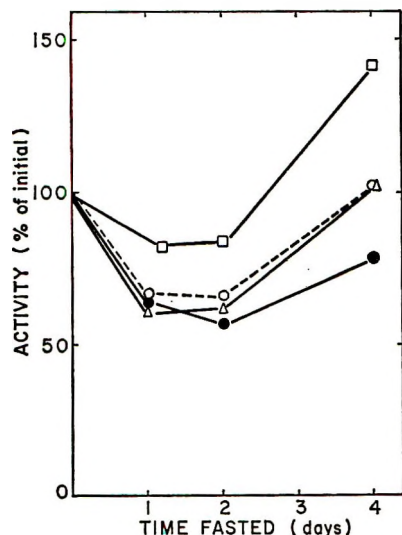

Fig. 2 Effect of starvation on the activity of methionine-adenosyltransferase in rat liver. □———□ per g wet liver; ○− − − −○ per 100 g of body weight at time of killing; ●———● per 100 g of original body weight; △———△ per g protein.

transferase activity in rat liver. Moreover, the response of the adrenalectomized animals to cortisone was found to be greater than that of the intact ones (fig. 3).

Effects of hypophysectomy and of growth hormone. The removal of the pituitary gland brought about little change in the activity of the transferase. A slight increase was observed 3 weeks after hypophysectomy. Assayed 2 weeks later, when the body weight was almost stable, the total activity per unit of body weight was a little below the normal level. However, the effect of growth hormone was quite apparent; the administration of 4 units of growth hormone daily for 4 days to the hypophysectomized rats decreased the transferase activity to about 70% of that of the untreated group (fig. 4). At the same time the average body weight of the rats increased from 145 to 163 g, a gain of 12%.

Effect of thyroidectomy and triiodothyronine. The effects of thyroidectomy and administration of 3,3′,5-triiodo-L-thyronine (T_3) are shown in figure 5. The removal of the gland caused an increase of the transferase activity which quickly fell to a level below normal following a single injection of T_3. The intact rats responded to T_3 in a similar way. After the administration of T_3 to the adrenalectomized rats the enzymatic

Fig. 4 Effects of hypophysectomy and growth hormone administration on the activity of methionine-adenosyltransferase in rat liver. The experimental rats were hypophysectomized 3 (H3) or 5 (H5) weeks before killing. Growth hormone dissolved in 0.066 M potassium phosphate buffer (pH 8.0, 8 units/ml) was administered intraperitoneally at the rate of 4 units /rat/day for 4 days. Controls were given the vehicle only. Average body weight of rats before growth hormone treatment was 145 g. Average body weight increase for the treated and untreated groups was 12% and 0%, respectively. Diagonal lines per mg protein; □ per g wet liver; ■ per 100 g body weight.

activity in the liver reached quite low values.

Combined effect of hormones and diet

In an attempt to gain some insight into the mechanism or mechanisms responsible for the increase of rat liver transferase activity caused by cortisone and by the high protein diet, the possible interdependence of these 2 factors was tested. 1) The effect of feeding adrenalectomized or hypophysectomized rats with diets N and H was compared. As shown in figure 6, the response to the high protein diet was almost completely abolished by the removal of either the adrenal or pituitary gland. 2) The effects of cortisone administration to the rats fed diets with different protein content were compared with the results shown in figure 7. The response occurred in the rats fed a protein-free diet as well as in those that received a normal diet. An additive effect was observed when rats were given both a high protein diet and cortisone.

DISCUSSION

In the discussion which follows we shall consider mainly the effects of diet and other forms of treatment on the relative specific activity of the methionine-adenosyl
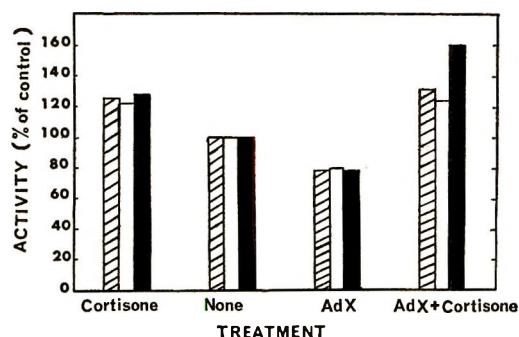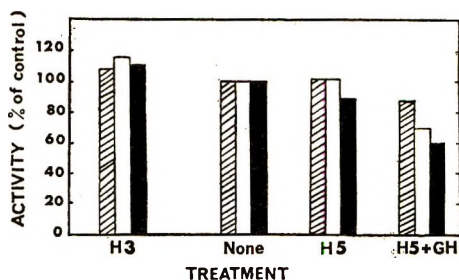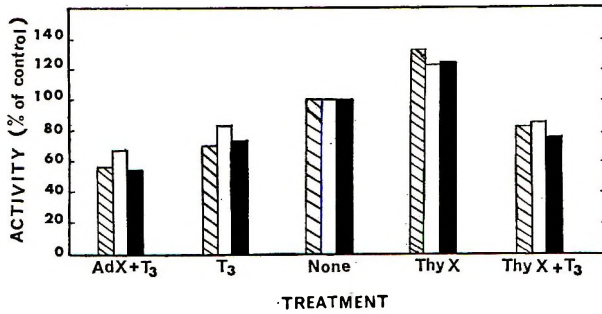
Fig. 3 Effects of adrenalectomy (AdX) and cortisone administration on the activity of methionine-adenosyltransferase in rat liver. Bilateral AdX was performed 18 days before the rats were killed. Animals were maintained with saline fed in the drinking water. Cortisone acetate, 100 mg/kg daily for 2 days, was given intraperitoneally in a suspension in 0.9% of NaCl. The controls were given the vehicle at the same time. Diagonal lines per mg protein; □ per g wet liver; ■ per 100 g body weight.

Fig. 5 Effects of thyroidectomy (ThyX) and the administration of T_3 on the activity of methionine-adenosyltransferase in rat liver. ThyX and AdX were performed 5 and 20 days, respectively, before killing. A single dose of T_3, 1 mg/kg, was administered intraperitoneally in a suspension in 0.9% of NaCl, 0.2 mg/ml. The rats were killed 24 hours later. Controls were given the vehicle only. Diagonal lines per mg protein; □ per g wet liver; ■ per 100 g body weight.
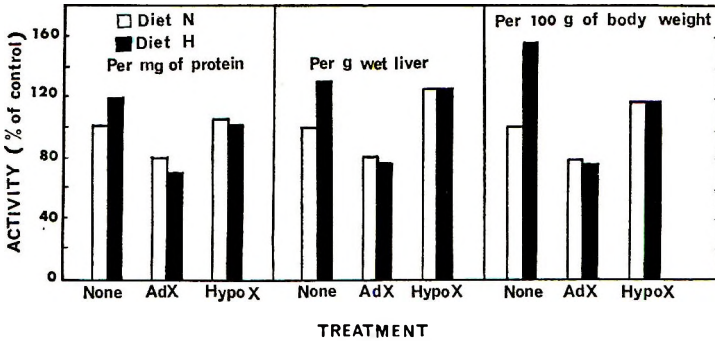

Fig. 6 Hormonal dependence of the response of methionine-adenosyltransferase activity to dietary protein. Two weeks after adrenalectomy (AdX) or hypophysectomy (HypoX), the rats were fed diet N or H for 7 days before killing.
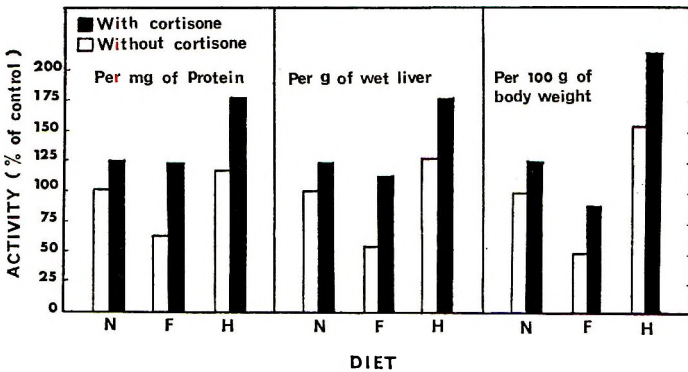

Fig. 7 Influence of dietary protein content on the effect of cortisone on the activity of methionine-adenosyltransferase in rat liver. Rats were fed diet N, F, or H for 7 days before killing. For cortisone treatment, see figure 3.

transferase in rat liver, that is, the relative differences in the activity of the enzyme per gram of total liver protein. However, it may be noted at this point that greater differences between controls and experimental animals are often observed when the data are expressed in other ways, for example, when the data are compared on the basis of units per 100 g of rat at time of killing. Manifestly, this situation prevails because the loss or gain of enzyme in different cases may be either the same, less than or more than the loss of total liver protein.

The influence of the level of dietary protein on the liver transferase is noteworthy. In going from a protein-free diet (F) to a high protein diet (64% of casein, H) the specific activity of the enzyme increases by a factor of two. This difference is apparently not entirely a result of the increase of available methionine with the high protein diet because when the normal diet (27% of casein) was supplemented with an equivalent amount of free methionine there was no difference in the transferase activity. This result is similar to results of other studies made with arginase (11) and threonine dehydrase (12). When a protein-free diet rather than a normal protein diet was supplemented with methionine the results were different. Enzyme levels were increased, perhaps because of a substrate stabilization mechanism. At least it has been shown that the transferase is stabilized by S-adenosylmethionine (9) and that S-adenosylmethionine levels decrease with starvation and increase after administration of methionine (13). The result of feeding the hydroxy analogue of methionine (2-hydroxy-4-methylthio-n-butyric acid) is in agreement with this interpretation. Enzyme levels are increased almost as much as with methionine. It has been shown that the analogue is converted to methionine (14). As might be anticipated supplementation of the protein-free diet with glycine proved to be ineffective.

The behavior of the transferase when rats were fasted is of considerable interest. Levels of the enzyme fall after one day but subsequently rise again to the initial level of specific activity. Evidently the enzyme is more easily depleted than is the total protein of the liver. It behaves like a component of the labile protein of the organ (15). The subsequent rise in the level of the enzyme may be interpreted as resulting from the stimulation of the pituitary and adrenal glands (16, 17).

The most noteworthy change produced by a hormone is that caused by cortisone, the administration of which causes a rise in the level of the transferase. Adrenalectomy causes a decrease. Thus the effect of cortisone is anabolic with respect to the transferase and other proteins in liver whereas its effect is catabolic in peripheral tissues like muscle (18). The adrenal cortex also has a great effect on the response of the transferase to the protein content of the diet; the adrenalectomized animal is unresponsive to a high protein diet. The enzyme level stays low. In the hypophysectomized animal the behavior of the enzyme level with a similar change in diet is less noteworthy.

When normal animals are treated with cortisone there is a great increase in enzyme level regardless of whether the diet is high or low in protein, but with a high protein diet together with cortisone the highest levels of transferase observed in these studies were attained. Evidently the amino acids necessary to promote enzyme synthesis may be of endogenous origin, as in the cortisone-treated animal fed a protein-free diet.[9]

The effects of adrenalectomy and cortisone are the opposite to the effect of castration and androgen on the transferase. Castration results in a rise in the enzyme level which is reduced again when the missing hormone is administered (19). Thus the protein anabolic hormone causes a decrease, whereas the catabolic hormone causes an increase in the transferase activity. The effect on carcass protein is the opposite of the effect on liver protein.

The effect of the dose of T_3 used in these studies was a catabolic one with respect to the transferase. Thyroidectomized animals showed quite high levels which were reduced by the administration of T_3.

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The Supplementary Value of Algae Protein in Human Diets [1]

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ABSTRACT   Nutritive value of algae protein for maintaining nitrogen balance in human adults fed 6 g N/day from algae alone or algae combined with other intact proteins was determined during 10 experimental periods of 5 days each. The protein sources studied were: algae (*Chlorella pyrenoidosa*), fish flour, soybean flour, dried whole egg, rice and gelatin. All diets supported positive nitrogen balance (mean values ranging from +0.30 to +0.77 g N/day), except the diets containing algae alone or algae and gelatin (mean values, −0.06 and −0.38 g N/day, respectively). While the intact proteins of fish, soybean and egg fed singly resulted in nitrogen retentions of similar magnitude, only fish protein appeared to be improved slightly by supplementing with 2 or 4 g nitrogen from algae. Nitrogen retention in response to the algae-rice combination was comparable to that observed when the high quality proteins, fish, soybean and egg were fed. Apparent nitrogen digestibility for the 6 g algae nitrogen diet was improved from 66% to 71 to 75% when part of the algae was replaced by other proteins. The latter values compared favorably with digestibilities of the single intact proteins.

A growing world concern is the acute demand for food to meet the needs of an ever-increasing human population. The realization that nutrition is an important factor influencing the health and productivity of man and the awareness of potential limitations in the agricultural capacity of the cultivatable land areas of the world, stress the need to explore possibilities of utilizing unconventional materials as sources of food. Unicellular green algae have been suggested as such a potential food, principally because of their high protein content and favorable amino acid composition (1–4).

While the strong bitter taste and intensive green color of unicellular green algae make acceptability difficult, some workers have demonstrated that dried algae powder was well accepted when added to conventional foods in small quantities (5, 6). Powell et al. (7) reported that young men were able to consume diets containing 100 g or less of algae, although certain symptoms such as abdominal distention were observed even at the 10- and 20-g levels of feeding. Dam et al. (8) reported similar symptoms in a group of subjects fed unextracted green algae mixed in conventional foods. These subjects were subsequently unable to consume ethanol-extracted algae concentrated in a single dietary item. However, with a different group of subjects, Dam et al. (8) reported that although a diet providing 6 g nitrogen daily principally from ethanol-extracted algae was inadequate for support of nitrogen balance in adult humans, nitrogen equilibrium or positive balance was attainable when algae nitrogen was increased to 7 or 10 g daily. Even at the 10-g nitrogen level, no adverse symptoms associated with algae feeding were observed.

The purpose of the present investigation was to determine the supplementary value of algae protein in human diets, that is, the ability of algae protein to replace high quality proteins from fish, egg and soybean and the ability of algae protein to improve low quality proteins from rice and gelatin.

EXPERIMENTAL

The study consisted of a 2-day depletion period, a 3-day adjustment period

and 10 experimental periods of 5 days each. An outline of the experimental plan is presented in table 1. The diets fed during the depletion, adjustment and experimental periods provided 2.0, 6.0 and 6.0 g N/subject/day, respectively, from the various protein sources and approximately 0.66 g N from other natural foods. The composition of the diet used during the 10 experimental periods is shown in table 1. The protein sources used were algae (*Chlorella pyrenoidosa*), fish flour, soybean flour, dried whole egg, rice and gelatin. Caloric intake was adjusted to maintain body weight approximately constant; resulting intakes were 45 kcal/kg for men and 40 kcal/kg for women. Fat provided approximately 20% of the total caloric intake of each subject. A nonprotein, low calorie carbonated beverage [3]

[3] Furnished gratis by Shasta Beverages, San Francisco.

TABLE 1

Experimental plan

| Period | No. of days | Primary dietary protein | | Total N intake [1] |
		Source	Amount	
			g N/day	*g N/day*
Depletion	2	rice [2]	2.0	2.66
Adjustment	3	rice [2]	2.0⎫	
		+	⎬	6.66
		egg flour	4.0⎭	
Experimental diets [3]				
1	5	algae [4]	6.0	6.66
2	5	fish flour [5]	6.0	6.66
3	5	soybean flour [6]	6.0	6.66
4	5	dried whole egg [7]	6.0	6.66
5	5	algae [4]	2.0⎫	
		+	⎬	6.66
		fish flour [5]	4.0⎭	
6	5	algae [4]	4.0⎫	
		+	⎬	6.66
		fish flour [5]	2.0⎭	
7	5	algae [4]	2.0⎫	
		+	⎬	6.66
		soybean flour [6]	4.0⎭	
8	5	algae [4]	2.0⎫	
		+	⎬	6.66
		dried whole egg [7]	4.0⎭	
9	5	algae [4]	2.0⎫	
		+	⎬	6.66
		rice [2]	4.0⎭	
10	5	algae [4]	2.0⎫	
		+	⎬	6.66
		gelatin [8]	4.0⎭	

[1] The basal diet provided 0.66 g N/day in addition to that provided by the primary dietary protein. It consisted of the following: beef bouillon (7.0 g dry powder), tomato juice (80.0 g), pineapple (100 g), cabbage (50 g), wheat starch muffins (varied), butter (varied), jelly (varied), coffee or tea (varied), Diet Cola (Royal Crown Cola Company, Columbus, Georgia), (varied). Vitamin supplements provided the following amounts per day: vitamin A, 5000 USP units; vitamin D, 500 USP units; (in milligrams) thiamine, 2.0; riboflavin, 2.5; ascorbic acid, 50.0; pyridoxine, 1.0; nicotinamide, 20.0; pantothenic acid, 1.0; and vitamin B_{12}, 1 μg.
A mineral supplement provided daily: (in grams) $CaCO_3$, 1.635; KH_2PO_4, 1.588; $MgCO_3 \cdot Mg(OH)_2 \cdot 3H_2O$, 0.972; $FeC_6H_5O_7 \cdot 6H_2O$, 0.1152; $CuSO_4 \cdot 5H_2O$, 0.0095; KI, 0.0002; $MnCl_2 \cdot 4H_2O$, 0.0088; and $ZnCl_2$, 0.0023.
[2] Belle Patna extra long grain rice, 6.8% protein, was furnished gratis by Comet Rice Mills, Houston.
[3] Experimental diets were assigned at random to subjects during periods 1 through 10.
[4] *Chlorella pyrenoidosa* 71105, 65.4% protein (N × 6.25), was furnished gratis by General Dynamics Corporation, Electric Boat Division, Groton, Connecticut.
[5] Isopropanol-extracted fish flour (North Atlantic red hake), 82.9% protein, was furnished gratis by United States Department of Interior, Fish and Wildlife Service, Bureau of Commercial Fisheries Technological Laboratory, College Park, Maryland.
[6] Lecithinated low fat soybean flour, 50.2% protein, T. Pavo Company, Inc., Minneapolis.
[7] Dried whole egg, 47.1% protein, was furnished gratis by David City Creamery, David City, Nebraska.
[8] Jiffy-Jell, plain gelatin, General Foods Corporation, White Plains, New York.

TABLE 2

Subject information

Subject	Sex	Age	Height	Weight		Caloric intake
				Initial	Final	
		years	cm	kg	kg	kcal/day
A	M	23	179.1	68.4	69.2	3068
B	F	20	167.6	84.5	82.3	2825
C	F	20	175.3	65.9	73.2	2773
D	M	32	172.7	76.8	78.5	3431
E	F	20	168.9	66.0	65.9	2641
H	M	18	172.7	55.0	53.8	2519

TABLE 3

Effect of protein source on nitrogen balance and apparent nitrogen digestibility

Diet	Mean nitrogen balance/day for subject							Apparent digestibility
	A	B	C	D	E	H	Mean	Mean
	g/day	g/day	g/day	g/day	g/day	g/day	g/day	%
Adjustment	−0.31	1.36	2.34	0.75	0.74	−0.55	0.72	74
Experimental:								
Algae	−0.18	−0.58	1.00	0.44	0.40	−1.44	−0.06	66
Fish flour	−0.03	1.19	1.45	0.13	0.80	0.02	0.60	77
Soybean flour	0.07	1.16	1.06	0.49	1.18	0.09	0.67	80
Whole egg	0.04	1.00	0.66	1.23	1.00	−0.24	0.62	82
Algae (4 g) + fish (2 g)	0.52	0.21	1.14	1.97	0.90	−0.14	0.77	71
Algae (2 g) + fish (4 g)	0.36	0.30	1.21	0.83	2.49	−0.53	0.77	75
Algae-soybean	−0.39	0.52	1.06	−0.95	1.25	0.32	0.30	72
Algae-egg	−0.04	1.22	0.13	0.34	0.34	1.24	0.54	75
Algae-rice	1.06	−0.31	1.75	0.20	0.75	— [1]	0.68	74
Algae-gelatin	−1.00	0.07	−0.03	−0.37	0.31	−0.83	−0.38	75

[1] Subject H was ill during this period.

was allowed ad libitum. Vitamin and mineral supplements as defined in table 1 were given to the subjects throughout the study. Three men and 3 women, all of American nationality, described in table 2, served as subjects for the study. All subjects were considered to be in good health on the basis of a physical examination by a physician. They engaged in their usual activities during the study.

The criterion used in evaluating protein adequacy was nitrogen retention. Nitrogen content of urine, feces and individual food items was determined by the Kjeldahl method (9).

RESULTS

Individual and mean nitrogen balance data of the experimental subjects fed various single proteins or protein combinations are shown in table 3. Even though a diet providing 6 g of total nitrogen is generally considered marginal and might be expected to support nitrogen balance only in the case of high quality proteins, mean nitrogen balances were positive in response to all dietary treatments with the exception of algae alone and the algae-gelatin combination. Nitrogen balances achieved with the latter diets were significantly lower ($P < 0.05$) than with the algae-soybean diet, whereas nitrogen balance with the 3 diets were inferior to those observed with the other intact proteins and protein combinations. Nitrogen retention achieved in response to the algae-rice diet was comparable to that observed when high quality proteins from fish, soybean or egg were fed.

As noted in earlier studies in this laboratory (8), high fecal nitrogen values were observed when the diet provided 6 g algae nitrogen. Calculation of apparent nitrogen digestibilities (table 3) of the

various experimental diets indicated that the diet containing 6 g algae nitrogen had the lowest digestibility, 66%, whereas lower levels of algae (2 or 4 g nitrogen) fed in combination with other proteins resulted in digestibility coefficients ranging from 71 to 75%. The latter values compare favorably with digestibilities of the single intact proteins of fish, soybean and egg.

DISCUSSION

The demonstration in the present experiment that algae can replace one-third of the protein of egg and up to two-thirds of the protein of fish without impairment in nitrogen retention of adult human subjects, suggests its possible usefulness in extending high quality food proteins. That the protein quality of rice was definitely improved by partial replacement with algae protein was indicated by a comparison of the results of the present study with those reported by Chen et al.[4] in the same laboratory. Nitrogen balance achieved with a diet providing 6 g nitrogen (two-thirds rice, one-third algae) was +0.68 g/day in contrast with −0.01 g/day when rice alone provided an equivalent amount of nitrogen. Algae is considered an excellent source of both lysine and threonine (10) and the beneficial effect of these amino acids in improving the protein quality of rice has been demonstrated repeatedly (11–14). Since cereal proteins in general are deficient in lysine, algae protein may be useful in supplementing other cereal proteins. However, the failure of algae to improve soybean protein cannot be explained since soybean, like rice, is suspected to be low in threonine and lysine on the basis of amino acid composition values (15). The observation that algae combined with gelatin was not more effective than algae alone was not unexpected in view of the conspicuous amino acid deficiencies in gelatin.

Poor digestibility is one of the most undesirable characteristics of algae protein. Fecal nitrogen excretions observed in all human feeding studies have been high, ranging from approximately 25 to 40% of the total nitrogen intake (7, 8). In the present study, the highest digestibility coefficient (82%) was obtained when the subjects were fed 6 g nitrogen

from egg alone and the lowest (66%) in response to feeding 6 g nitrogen from algae alone. The relatively higher digestibility coefficients, 71 to 75%, observed when algae was fed combined with other proteins suggests that algae is utilized more efficiently when fed in small quantities or when fed in combination with proteins of higher digestibility than when fed alone. Since introduction of algae to human diets may be accomplished more successfully, at least for the present, as a supplement to other food proteins, the poor digestibility of algae protein may not be a significant problem in human feeding.

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Quantitative Measurement of Alcohol Dehydrogenase Activity within the Liver Lobule of Rats after Prolonged Ethanol Ingestion [1]

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ABSTRACT   Using quantitative microchemical methods for analysis in conjunction with microdissection of lyophilized sections of liver, alcohol dehydrogenase activity, total protein and total lipid content were measured in periportal and central areas of lobules. The livers were from female rats consuming 37% of their calories as either ethanol or a glucose solution along with pair-fed amounts of both fluid and a stock ration for 3 to 4 months. The 2 groups of rats grew at the same rate and when killed, their body and liver weights were almost identical. In the glucose-fed group of rats, the total lipid content in the periportal and central areas of the liver lobule were identical and the activity of alcohol dehydrogenase in the centrolobular area was significantly greater than that in the periportal area. As a result of chronic ethanol ingestion, there was a slight but significant increase in centrolobular total lipid content and a significant decrease in alcohol dehydrogenase activity in both areas of the lobule so that the enzyme remained significantly more active in the central area than in the periportal area.

Among alcoholic subjects, it appears that the infiltration of hepatic parenchymal cells with fat may be the pathologic precursor of Laennec's cirrhosis (1, 2). An extensive literature (3–6) indicates that fatty metamorphosis of the liver induced by alcohol may be secondary to both the toxic effects of alcohol and the metabolic effects of the products of ethanol oxidation.

Studies from this laboratory using quantitative microchemical methods have shown recently that in livers of man and the rat, the activity of alcohol dehydrogenase in the periportal area of the lobule is three-fifths of that in the centrolobular area.[3] For this reason, and because alcohol dehydrogenase is probably the rate-limiting enzyme in the oxidation of ethanol by the liver (7–9), it was concluded that following acute ethanol intoxication in the normal rat and man, the centrolobular accumulation of fat may be related more to the metabolic effects of the products of ethanol metabolism than to any toxic effect, the latter effect being more likely to appear in periportal areas of the lobule.

But such a conclusion may not apply to the chronic ingestion of ethanol. The activity of alcohol dehydrogenase in liver homogenates has been reported (10–12) to be altered after the chronic ingestion of large amounts of ethanol and it is not known whether such treatment significantly changes the periportal-central distribution of alcohol dehydrogenase activity within the lobule.

In the present study, quantitative microchemical methods were used to measure the intralobular distribution of alcohol dehydrogenase activity and total lipid content in the livers of carefully pair-fed rats following the chronic ingestion of large amounts of ethanol or isocaloric amounts of glucose along with a diet believed to be adequate in lipotropic agents (13, 14).

EXPERIMENTAL METHOD

Animals and diets.   Twelve female rats of the Sprague-Dawley strain [4] weighing 97 to 130 g were paired into 2 groups according to body weight and maintained in

separate wire-bottom cages at an ambient temperature of 22 to 28°. The experimental group of 6 rats was fed ground stock ration [5] with ethanol as the only source of drinking fluid ad libitum. The concentration of ethanol in the fluid available to this group was increased gradually from 10% (v/v) to 25% over a period of 6 weeks after which the animals consumed an average of 37% of their calories as ethanol for a period of 3 to 4 months. During this 3- to 4-month period the average daily intake of ground stock ration remained close to 8.3 g in both the experimental and pair-fed control groups. According to data supplied by the manufacturer,[5] the stock ration contains 25% protein, 50% carbohydrate and 5% fat with 340 kcal/100 g. This ration contains 1.32 mg of DL-methionine and 0.42 mg of choline/kcal. The same stock ration fed ad libitum to rats receiving 30% of their calories as ethanol in the drinking fluid for up to 228 days was shown by Scheig et al. (14) to be adequate in terms of lipotropic agents. The six control rats were pair-fed in terms of both stock ration and drinking fluid. The fluid consumed daily by each control rat contained an amount of glucose which was isocaloric with the ethanol consumed daily by its experimental paired counterpart. All 12 rats were given one-half of their next day's allotment of food and drinking fluid 4 hours before they were killed in the morning. Immediately following exsanguination by decapitation, the livers were excised, weighed and sampled for frozen-dried sections, homogenates and fixed histologic stained sections. The average body and liver weights are presented in table 1.

Homogenates. Samples of fresh liver weighing 300 to 500 mg were homogenized by hand at 0° in all-glass Potter-Elvehjem homogenizers with 10 vol of 0.1 M potassium phosphate buffer, pH 7.3. Immediately before the enzyme assays, 10-µliter aliquots of these homogenates were diluted with 1 ml of 0.1 M potassium phosphate buffer, pH 7.3, containing 0.05% bovine serum albumin. Triplicate assays of each liver were performed to determine the activity of alcohol dehydrogenase, the total protein content and the total lipid content.

Frozen-dried sections. A small biopsy from the periphery of the left lateral lobe of the liver was immediately frozen in liquid nitrogen. Sections were cut with a thickness of 22 µ in a cryostat at −20° and dehydrated in vacuo at −35°. These lyophilized sections were stored for up to 4 weeks at −70° in evacuated tubes, a method of storage which did not impair the activity of alcohol dehydrogenase. Dissection of lyophilized sections under a microscope at a magnification of × 72 and weighing of dissected segments on a quartz beam balance were carried out in a room with constant low humidity at 18°. During microdissection, the area of each lobule lying along a radius between portal triads and central veins was divided into 4 approximately equal segments. The periportal and central quarters of the lobular segments weighed approximately 0.14

[5] Purina Rat Chow, Ralston Purina Company, St. Louis.

TABLE 1

Mean body and liver weights of rats fed stock ration and either 25% ethanol or isocaloric glucose solutions for 3 to 4 months [1]

	Experimental rats	Pair-fed control rats
	Stock ration [2] + 25% ethanol ad lib.	Stock ration + isocaloric glucose solutions
Mean initial body wt, g	160 [3]	157 [3]
Mean final body wt, g	226	230
Mean final liver wt, g	7.61	7.49
Mean ratio of liver wt to body wt, g/g	0.0337	0.0325

[1] Six rats/group.
[2] Purina Rat Chow, Ralston Purina Company, St. Louis.
[3] Body weight after the 6 weeks during which rats consumed 10, 15 and 20% ethanol or isocaloric amounts of glucose solutions as the only source of drinking fluid.

µg. After weighing they were placed in microtest tubes for immediate enzyme assay. In each liver, periportal and central areas of 6 lobules were assayed to determine the activity of alcohol dehydrogenase, the total protein content and the total lipid content.

Alcohol dehydrogenase assay. The reaction mixture for blanks, standards and samples contained in the following final concentrations: potassium phosphate buffer, pH 7.3, 0.1 M; DPNH, 0.8 mM; acetaldehyde, 10 mM; sodium amytal, 1 mM; nicotinamide, 20 mM; and bovine serum albumin, 0.05. In measuring homogenates, 4 µliters of dilute homogenate, representing 3.6 µg of fresh liver, were added to tubes containing 25 µliters of ice-cold reaction mixture. In the assay of microdissected segments of the lobule, 3 µliters of ice-cold reaction mixture were added to microtest tubes containing a segment of dried liver weighing approximately 0.14 µg. Blanks consisted of 4 µliters of potassium phosphate buffer mixed with bovine serum albumin added to 25 µliters of reaction mixture for homogenate measurements or merely 3 µliters of reaction mixture for measurements of microdissected segments. If amytal is not added to the reaction mixture, tissue blanks are necessary. Standards consisted of 4 µliters of potassium phosphate buffer with bovine serum albumin and 1 mM DPN^+ added to 25 µliters of reaction mixture for homogenates or 3 µliters of reaction mixture containing 0.14 mM DPN^+ for microdissected segments. After mixing, the tubes were transferred from the ice bath to a water bath at 38° and incubated for 30 minutes. Following incubation, the reaction was stopped by returning tubes to the ice bath and adding a volume of 0.5 N HCl equal to that of the reaction mixture to remove the fluorescence of the remaining DPNH. The fluorescent alkaline derivative of the DPN^+ generated during incubation was developed by mixing 5-µliter aliquots of the acidified solution with 50 µliters of 6.6 N NaOH in a fluorometer tube and heating this solution at 38° for 30 minutes. Thereafter, 1 ml of water was added and the fluorescence read on a photomultiplier-type fluorometer with the primary Corning filter no. 5860 and secondary Corning filters nos. 3387, 4308, and 5562.

The activity of alcohol dehydrogenase with this method was found to be proportional to the amount of liver present between a range of 1 to 5 µg of fresh liver per 25 µliters of reaction mixture. Using 9.7 µg of fresh liver per 25 µliters of reaction mixture, the activity of alcohol dehydrogenase increased linearly throughout the 30-minute incubation period at 38°.

Analysis of chemical constituents. Total protein was measured in homogenates and microdissected segments of lyophilized tissue by the Folin phenol method (15). The total lipid content of microdissected segments of lyophilized tissue was determined gravimetrically by difference after extraction with anhydrous ethanol and hexane according to the procedure of Lowry (16).

Statistical analyses. In all experiments, results of each experimental rat liver were compared with those for the livers of their pair-fed controls. The mean of the individual differences was tested by Student's *t* test (17).

RESULTS

The process of lyophilization does not alter the activity of alcohol dehydrogenase. The average activity of alcohol dehydrogenase in homogenates of the 4 normal rat livers was 1.85 mmoles/g fresh weight/hour. Since the mean moisture content of these livers was determined to be 66% of their fresh weight, this activity represents 5.44 mµmoles/µg dry weight/hour. This value compares favorably with the average of the sum of the periportal and centrolobular activities from lyophilized sections of these livers, 5.28 mµmoles/µg dry weight/hour.

The growth of the 6 ethanol-fed rats closely paralleled that of the 6 rats fed isocaloric amounts of glucose not only during the 6 weeks while the concentration of ethanol in the drinking fluid was increased from 10 to 25% but also during the 3 to 4 months thereafter when the only source of drinking fluid was 25% ethanol (fig. 1). After the ethanol concentration in the drinking fluid was increased to 25%, the 6 experimental rats consumed an average of 11.97 g of ethanol/kg body weight/day, an amount which is twice that necessary to alter the $DPNH/DPN^+$ ratio in normal
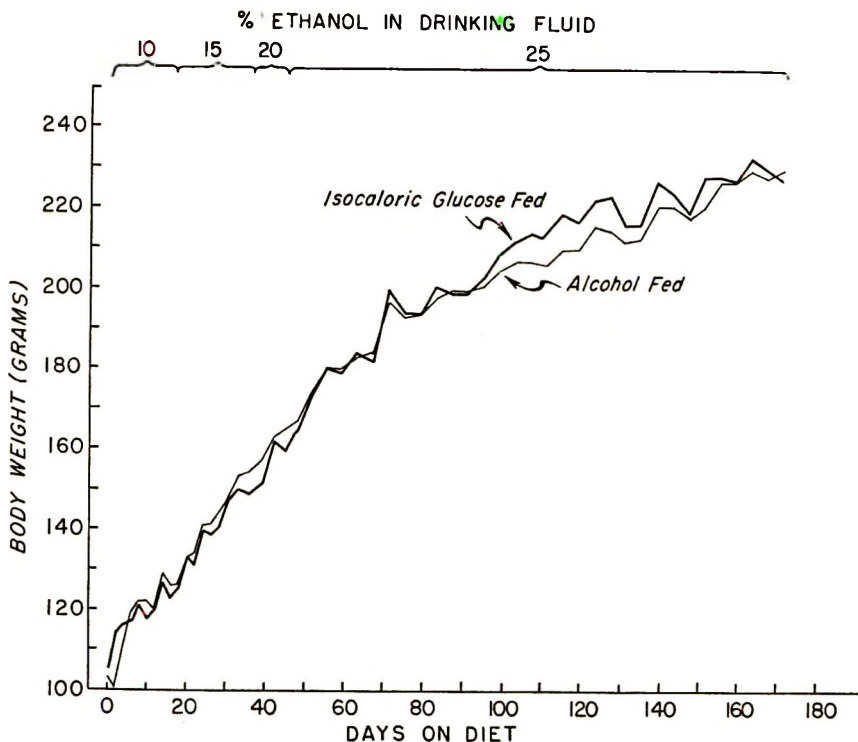
Fig. 1   Mean body weights of the groups of rats consuming either increasing amounts of ethanol (as noted above) or isocaloric amounts of glucose in their drinking fluid over a period of 172 days.

rats (18). This amounted to 37% of their total dietary calories. The 6 pair-fed control rats consumed an average of 20.94 g of glucose/kg body weight/day in their drinking fluid which amounted to 37% of their total dietary calories. It should be noted that each group of rats was given ration and either ethanol or glucose in the late afternoon and that whereas the experimental group consumed their ethanol drinking fluid over a span of 24 hours daily, the control group as a rule consumed their daily allotment of glucose drinking fluid during the first 12 hours after it was given. When killed, the average ratio of liver weight to body weight (g per g) in the ethanol-fed rats and the 6 control glucose-fed rats differed by only 3.5% (table 1).

In homogenates of livers from rats receiving 37% of their calories as ethanol for 3 to 4 months, the activity of alcohol dehydrogenase on a protein basis was 83% ($P < 0.05$) of the activity in livers of the pair-fed control rats receiving isocaloric amounts of glucose. In lyophilized segments of lobules from livers of ethanol-fed rats, the periportal and centrolobular activities of alcohol dehydrogenase on a protein basis were both 80% ($P < 0.05$ for each) of the activity in corresponding areas of lobules from livers of glucose-fed control rats (table 2).

The analysis of lipid in lyophilized segments of liver lobules showed that the total lipid content on a dry-weight basis was increased in the livers of the ethanol-fed rats over that in the livers of the control glucose-fed rats. However, this increase was only significant in the central area of the lobule. In livers from ethanol-fed rats, the centrolobular total lipid content was 134% ($P < 0.01$) of that in the corresponding area of livers from control glucose-fed rats (table 3).

The histologic stained sections of the livers from the 6 control glucose-fed rats were normal but each of the livers from

TABLE 2

Alcohol dehydrogenase activity within the lobules of livers from rats fed 37% of calories as ethanol or glucose for 3 to 4 months [1,2]

Treatment group	Alcohol dehydrogenase activity		P value [3]
	Area of lobule of liver		
	Periportal	Central	
	mμmoles/μg protein/hour		
Fed 25% ethanol	5.33 ± 0.38	6.65 ± 0.23	P < 0.05
Fed isocaloric glucose	6.66 ± 0.41	8.30 ± 0.54	P < 0.01
P value [4]	P < 0.05	P < 0.05	

[1] All values are expressed as means of determinations ± SE; probability values are derived by Student's *t* test.
[2] Six livers/group assayed.
[3] Probability that the portal-central difference is due to chance.
[4] Probability that difference between the 2 groups is due to chance.

TABLE 3

Total lipids within the lobules of livers from rats fed 37% of calories as ethanol or glucose for 3 to 4 months [1,2]

	Total liver lipids		P value [3]
	Area of lobule		
	Periportal	Central	
	μg total lipid/μg dry wt		
Fed 25% ethanol	0.163 ± 0.006	0.206 ± 0.009	P < 0.01
Fed isocaloric glucose	0.154 ± 0.005	0.154 ± 0.006	ns
P value [4]	ns	P < 0.01	

[1] All values are expressed as means of determinations ± SE; probability values are derived by Student's *t* test; ns, not significant.
[2] Six livers/group assayed.
[3] Probability that the portal-central difference is due to chance.
[4] Probability that difference between the 2 groups is due to chance.

the 6 ethanol-fed rats revealed slight fatty metamorphosis with an occasional cell in the central area of the lobule which was laden with fat in a globule displacing the nucleus.

DISCUSSION

In mammals, the liver is the richest source of alcohol dehydrogenase and an estimated 90 to 98% of the ethanol oxidized by the body takes place in the liver (19). In the oxidation of ethanol, alcohol dehydrogenase is probably the rate-limiting enzyme among animals on an adequate diet (7–9) although the DPNH/DPN+ ratio may be the actual limiting factor for the rate of ethanol metabolism in fasting animals (20, 21).

In the experiment reported here, the diet of the ethanol-fed group of rats was adequate enough to permit continued growth of the rats. Furthermore, there are 4 reasons for justifying the acceptance of the glucose-fed rats as satisfactory controls for the ethanol-fed rats. The glucose-fed group of rats received daily quantities of glucose which were isocaloric with the ethanol consumed by the experimental group of rats, they were carefully pair-fed both ration and drinking fluid, they grew at the same rate as the experimental group of rats, and they had a liver weight-to-body weight ratio when killed which was identical to that of the experimental group.

Our data indicate that the activity of hepatic alcohol dehydrogenase on a protein basis is significantly decreased by feeding large amounts of ethanol along with an adequate diet for 3 to 4 months. Alcohol dehydrogenase activity has been reported to be decreased in both man (18, 22–24) and the experimental animal (18, 25) with liver disease which is severe. In such cases, the decreased enzyme activity may very well be related to the severity of the liver disease and thus be secondary to a decreased capacity of hepatic parenchymal cells to synthesize proteins. Other investigators using experimental animals, however, have measured alcohol dehydrogenase activity in livers showing little in

the way of pathologic disease following the chronic ingestion of ethanol. Homogenates of livers from rats fed 20% ethanol as the sole source of drinking fluid (with or without extra ethanol added by gastric tube) for 6 to 36 weeks have showed either no change (26), a decrease (10, 12) or a temporary increase (11) in the activity of alcohol dehydrogenase. The latter investigators observed that during the chronic consumption of ethanol, the activity of this enzyme increased steadily to a peak after 26 weeks and over the subsequent 10 weeks returned to the level which had been maintained during the entire 36 weeks by the livers of the control rats. Because of this, we chose to measure the hepatic activity of alcohol dehydrogenase 20 and 25 weeks after starting the rats on ethanol. A comparison of our results with these reported studies presents certain problems because their experimental method differs from ours. Stock ration (10, 11, 26) or drinking fluid (11–12, 26) were not pair-fed to control rats (11, 26) or were only roughly isocaloric (10, 12) with the ethanol consumed by the experimental animals. Furthermore, unlike the present experiment, in these reported studies the body weights of the experimental group of rats either decreased or increased much more slowly than that of the control group during the ingestion of ethanol.

In the present study, the pathologic changes (an occasional centrolobular cell showing marked fatty infiltration) in the livers of the ethanol-fed rats were so minimal, it appears reasonable to conclude that the decrease in alcohol dehydrogenase activity following the chronic ingestion of ethanol is not related to an impaired capacity of the liver to synthesize proteins. Ethanol, therefore, must not induce alcohol dehydrogenase. Such a conclusion raises the possibility that ethanol may not be the natural substrate for alcohol dehydrogenase in the liver of the rat. Although there is a small amount of endogenously synthesized ethanol (27, 28), alcohol dehydrogenase in the rat and in man has a wide specificity for alcoholic compounds (29) and Theorell may be correct in suggesting that cholesterol may be the natural substrate for this enzyme.[6]

The quantitative microchemical assays of the lobule show that following the chronic ingestion of large amounts of ethanol, the activity of alcohol dehydrogenase is still significantly higher in the centrolobular area and that the total lipid content increases significantly only in the centrolobular area. While it is tempting to conclude that this centrolobular accumulation of lipid is more related to the metabolic effects of the products of ethanol oxidation than to the toxic effects of ethanol, such a conclusion must await future investigations because of structural and metabolic differences between periportal and centrolobular cells (30). The metabolic potential of several biochemical pathways in periportal hepatic cells differs from that of centrolobular hepatic cells in both the rat (31, 32) and in man (33). It is such intralobular differences in the metabolic potential of hepatic cells which undoubtedly account for the observation by Hartroft et al. (34) that when rats are fed a lipotropic adequate diet with 36% of the calories as ethanol for 3 months, moderate centrolobular fatty infiltration develops if the diet is normal in protein and high in fat content but will produce severe periportal fatty infiltration when the diet is low in protein and high in carbohydrate content.

In future studies to evaluate the effects of chronic ethanol ingestion upon the hepatic accumulation of fat, careful attention should, therefore, be given to the diet but also to determining whether a diet truly is adequate in lipotropic agents. An increased ethanol content in a diet is known to increase the choline requirement of the rat (13) and an increased fat content in a diet is also known to increase the choline requirement in rats (35). A comparison of the present experiment with other relatively recent studies (3, 5, 14) appears to support these 2 conclusions and has brought us to appreciate that the amount of lipotropic agent necessary to protect livers against the centrolobular fatty infiltration of a relative choline deficiency has been established for only a few diets with only a few levels of in-

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[6] Hugo Theorell, personal communication.

gested ethanol in only a few experimental animals.

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