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AXEL HOLST

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(September 6, 1860–April 26, 1931)

In the spring of 1902 a young Norwegian physician, making a world tour to study tropical diseases in their native places, visited the laboratory of Eijkman and Grijns at Batavia (now Jakarta) on the island of Java in the Dutch Indies, in order personally to acquaint himself with the investigations going on there on beri-beri. This physician was Dr. Axel Holst who, at that time, was Professor of Hygiene at the University of Christiania (now Oslo). Holst was particularly interested in "ship beri-beri," a disease which had long been haunting Norwegian sailing vessels. He discussed with Grijns (1, 2) the experimental work going on at Batavia and its bearing on his own interests. After his return home, Holst devoted himself eagerly to the study of "ship beri-beri" or scurvy on an experimental basis. He soon realized that the polyneuritis produced in poultry resembled tropical beri-beri much more than it did "ship beri-beri," a fact which led him to discontinue his experiments on poultry and pass over to investigations on mammalia. It was, indeed, fortunate that Holst and his coworker, Frølich, chose guinea pigs in their experiments because it was the successful outcome of these investigations which led to the recognition of the cause of scurvy, an achievement which has ranked Axel Holst as one of the pioneers in the field of vitamin research.

Axel Holst was born in Christiania, Norway, September 6, 1860. His family was one of long-standing medical traditions, both his father and his grandfather having been physicians before him. His grandfather was Professor of Hygiene at the University of Christiania for almost 40 years while his father, Dr. Axel Holst, Sr., was a military surgeon of some renown.

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Holst matriculated from Christiania Cathedral School in 1877 and passed the final medical examination in 1884. His interest in medical science was deeply rooted, and after receiving his medical degree (M.B.), he was appointed second assistant and then first assistant at the Institute of Pathology and Anatomy of the Government Hospital ("Rikshospitalet") in Christiania, working under the distinguished professor, Hjalmer Heiberg. In 1866, while he was an assistant at the Institute, Holst married Anna Midelfart, the daughter of a logging manager. At this time, Holst seized the opportunity to devote himself to the study of bacteriology, a field which had been opened up in the previous 15 years by the work of Pasteur, Koch and others, and which played a dominant role at that time in formulating our modern concept of hygiene. In 1887 Axel Holst was appointed Fellow in Bacteriology at the University of Christiania. In 1890, on the basis of his training and interest in bacteriology, Holst wrote a handbook of bacteriology, "Oversigt over Bacteriologien," which was translated into German in 1891. A second edition appeared in 1901. This book was for many years a guide for doctors and students of medicine.

In order to advance his bacteriological training, Holst obtained a traveling scholarship; and from September, 1890 until June, 1892 he studied at various European laboratories including: Kiel, Berlin, Wiesbaden, Munich, Paris (Pasteur Institute) and London. Holst was in Berlin studying with Koch when the announcement by Koch of tuberculin caused a sensation throughout the medical world. Following his return in 1892, Holst was awarded his doctor's degree (M.D.) from the University of Christiania. The title of his thesis was: "New Experiments with *Streptococcus* from Human Infections."

With this background of training Holst, at the age of 33, was made Professor of Hygiene and Bacteriology at Christiania succeeding Professor Lochmann, whose field of subjects, hygiene and pharmacology, was divided. Holst held this office until his retirement in 1930. Axel Holst was not only concerned with furnishing Norwegian doctors with the best pos-

sible training in hygiene, but he also felt strongly and was intensely interested in the importance of the practical aspects of the study of hygiene. In his inaugural lecture the new professor emphasized strongly the importance of the study of hygiene at the practical level and while he was the first professor of bacteriology at Christiania, he applied his knowledge of bacteriology chiefly in the field of applied hygiene. He was acting Medical Officer of Health in Christiania in 1893, a member of the Municipal Board of Health, and for two years School Medical Officer at Christiania Cathedral School.

Very soon after entering into his new office he demonstrated further his interest in practical hygiene by zealously setting to work on two questions which were of immediate practical importance at that time; namely, the housing conditions of the working classes of Christiania, and the pollution of the harbor waters of the capital.

The housing investigations were initiated at the request of the Labor Party in Christiania and were published in the ''Christiania Municipal Records'' in 1895, no. 29 (see also 3). In a lecture which he delivered before the Norwegian Medical Association on May 15, 1895, Holst stated that not less than 3,000 flats were overcrowded to such an extent as to be injurious to health. His work on the pollution of the water in the Christiania basin was printed as a supplement to ''Christiania Municipal Records,'' 1901 and was also published in German (4).

During the tenure of his professorship, Holst investigated many other problems in the field of practical hygiene. In 1898 very extensive discussions were going on in the Norwegian Medical Society on the problem of prostitution control. Holst was decidedly in favor of regulation. In 1888 the control of prostitution had been suspended, and in Holst's opinion the experiences of the intervening years had shown that regulation needed to be reintroduced. His point of view, however, met with strong opposition and was not accepted.

He had been acting School Medical Officer for some years and carried out many studies in school hygiene, publishing in

1909 a brief handbook on the subject entitled: "School Hygiene
— A Survey for Teachers." A study of milk control was
undertaken by Holst and in 1908 he took part in heated discus-
sions in the Norwegian Medical Society over a very serious
epidemic of "milk-disease" which was prevalent in Chris-
tiania. At a still later date, September 23, 1910, Holst was
appointed chairman of a combined ministerial and parliamen-
tary commission to deal with the alcohol question. This duty
he performed until 1915, making extensive inquiries and ac-
cumulating statistics in connection with this problem. As
usual he gave the matter great care and attention notwith-
standing the fact that it often became a burden to him because
he felt it so futile. He, himself, took a strict stand against
prohibition.

From a scientific point of view, however, the most outstand-
ing contribution of Holst was in the field of experimental
nutrition research. The factor which was of greatest signifi-
cance in stimulating his interest in experimental work was his
visit with Dr. Grijns in Batavia.

The prevalence of the disease "ship beri-beri" on Norwegian
sailing vessels together with the epidemic character of the
beri-beri which occurred in tropical regions was the occasion
for Holst's long study tour (October, 1901 to the summer of
1902), which was referred to earlier. The disease, "ship beri-
beri," was under active investigation at that time by Nocht in
Hamburg and also by a Norwegian Ship Beri-Beri Committee.
However, it appears from the records that medical terminology
of the time was so confused as to have led Holst and others
to believe that "ship beri-beri" and tropical beri-beri were
essentially similar and could be studied together. In his earlier
work, Holst apparently did not accept the possible identity of
"ship beri-beri" with scurvy, and even as late as 1918 he pub-
lished a paper on beri-beri on Norwegian ships (5).

The early work of Holst, using chickens and pigeons as ex-
perimental animals, was reported first in the "Norwegian
Magazine of Medical Science" ("Norsk Magazin for Laege-
videnskaben") in 1907 and almost at the same time in "The

Journal of Hygiene'' (6). In this report, Holst points out
clearly the clinical differences between tropical beri-beri with
its concomitant neuritis and ''ship beri-beri'' in which neuritis
was comparatively rare. He states that ''the recovery of pa-
tients suffering from 'ship beri-beri' is due to *fresh food,''*
and that ''above all, fresh vegetables or potatoes were cura-
tive.'' Apparently the long history of scurvy on shipboard
during the era of extended sea voyages was unfamiliar to the
Norwegian investigators since Holst states, ''Dr. Stian Erick-
sen of Norway in 1899 first observed that the disease appears
almost solely on-board sailing ships on long voyages.'' Or, it
may be that the appearance of the disease as it occurred on
Norwegian and other northern sailing vessels was sufficiently
different from classical scurvy as to warrant the designation
applied (''ship beri-beri''). It is quite probable that the diets
on ship were low in both vitamin C and thiamine as well as
other nutrients and that a multiple deficiency disease existed.
However, Holst does express the opinion in the introduction to
his first papers that it seems probable that *''Ship beri-beri'' is
''a food disease showing a marked congruence with scurvy.''*

Holst soon gave up his work with chickens and pigeons since
it gave no information concerning ''ship beri-beri,'' a fact
which, of course, would be expected in the light of our present
knowledge on species requirements for vitamin C. These ex-
periments did show, however, that foods other than those used
by Eijkman and Grijns would cause *polyneuritis gallinarum,*
analogous to tropical beri-beri. Holst did accept the results of
Eijkman and Grijns that there were ''nutritive constituents,
the presence of which prevent and conversely, the absence of
which produce the disease.'' He states ''because the experi-
ments . . . have not thrown any clear light upon the question
which has been to me the principal one, i.e. the etiology of 'ship
beri-beri' . . . I, therefore, discontinued the experiments on
poultry and passed over to investigations on mammalia.''
*These experiments were carried out together with Professor
Theodor Frølich and later Chief-Physician Valentin Furst.*
Since Dr. Frølich contributed so much to this phase of Holst's

work, it would seem appropriate to refer briefly to this out-
standing pediatrician.

Theodor Frølich (1870–1947) worked for a number of years
in the pediatric department of the "Rickshospitalet" and was
also an assistant at the Institute of Hygiene. From 1922 until
his retirement in 1941, Frølich was Professor of Pediatrics at
the University. As stated by Dr. Ragnar Nicolaysen (7), at
the time of Frølich's death (August 14, 1947), Frølich at his
retirement in 1941 could look back upon a long and active life
in the service of science and pediatrics. He had trained most
of Norway's present (1947) specialists in pediatrics and more
than half of their practising physicians. There was no one
who did not have kind memories of him. His speaking ability,
his loving nature, and the intensity with which he performed
his duties as a professor and doctor made him a most attrac-
tive personality. Influenced by his work on scurvy with Holst,
he took a particular interest in infant nutrition and his in-
fluence on it was great in Norway.

Frølich had been especially interested in studying Barlow's
disease (infantile scurvy). After Holst's preliminary investi-
gations upon his return from the East, the evident relation-
ship of "ship beri-beri" with scurvy and Barlow's disease
made the cooperation between Holst and Frølich particularly
fruitful. It is, perhaps, to be regretted from a research point
of view that Frølich, a man of clear vision and originality, did
not continue his experimental work. However, making clinical
pediatrics his choice, influenced perhaps by the greater finan-
cial return to the practising physician than to the research
professor, he deserves no less to be remembered.

Holst and Frølich's paper "On the etiology of scurvy" was
published in the next issue of the Norwegian Magazine of
Medical Science and as part II of the paper in "The Journal
of Hygiene" (8). It is subtitled: "On the Macroscopical Al-
terations in the Tissues of Guinea Pigs which Had Been Fed
Exclusively on Bread, Groats and Unpeeled Grain." The
paper goes on to say, "By experimenting with the 'one-sided'
diets which were used in the experiments . . . on polyneuritis

in poultry, we found that guinea pigs also contract a disease and that this disease is accompanied by very characteristic changes . . . These alterations, however, do not as a rule develop until from three to four weeks after the beginning of the experiment . . . The present section of our paper deals with the 64 animals that died in 18 days or more when fed on ground or unground oats, barley, rye or wheat and water . . . each animal received one single nutriment only. (This latter is reminiscent of the Wisconsin single grain experiments of the same era.) In the 64 animals death occurred on an average after 30 days." Holst and Frølich continued with a precise description of the characteristic symptoms involved: hemorrhages in the musculature, of the extremities, in the intercostal muscles, around numerous junctions between the ribs and their cartilages, and, less frequently, subcutaneous hemorrhages. A description is also given of hemorrhages in the stomach, kidneys, lungs, etc. Then the characteristic bone injuries are described; bone fragility that went so far that repeatedly when the tubular bones were removed from the body the epiphyses of the humeri, tibia, and femur separated from the shaft. In many cases the hind part of the lower maxilla actually crumbled between the fingers during dissection. Further, without a single exception the back-teeth were found to be loose, whereas the front-teeth proved to be loose only in some particular animals. The gums often displayed a greyish-green discoloring. Occasionally bleedings beneath the mucous membrane of the gums were detected but never wound formations.

Hemorrhages, loose teeth, and bone fragility — all these symptoms led Holst and Frølich to write, "From these observations we were led to assume that the disease might possibly be *scurvy*."

Obviously, neither Holst nor Frølich seemed to have had any particular personal experience with scurvy symptomatology in the human adult, for they compare their observations on the guinea pigs to descriptions of scurvy epidemics during the siege of Paris, in St. Petersburg, and elsewhere, going back as

far as Harvey's book "The Disease of London or a New Discovery of Scurvy" published in 1675.

In addition to the gross symptoms noted, Holst and Frølich also studied the histological changes which occurred in the guinea pigs. They report some muscle fiber changes, but in general confined their observations to the bones where they found alterations which "are, in all essentials, wholly identical with those found in human scurvy." This was particularly notable in the bone-marrow. They studied the effect of starvation (cf. modern inanition controls and pair-feeding techniques) and showed that starvation-marrow in no way resembled the bone-marrow of their scorbutic guinea pigs. They state, "scurvy *cannot* be caused in guinea pigs either by simple starvation or by diets of *any* kind; *on the contrary the disease originates in these animals as well as in man as a result only of a certain special diet.*" In order to obtain further evidence as to whether the disease in their guinea pigs was identical with scurvy, Holst and Frølich tried the preventive effect of some foods known as "antiscorbutics" from human experience and found that apples, lemon juice, and particularly cabbage protected guinea pigs from the typical lesions.

Of major importance in considering the contribution to nutrition made by Holst and Frølich was the thorough study of the nutritional disease which they had produced so as to be able to positively identify it and thus make available to future workers this experimental tool.

At the time of Holst's work there were three rather widely held theories as to the cause of scurvy. These existed despite the classic work of Lind and many others showing the nutritional nature of the disease and the curative value of certain fresh foods.

The first theory was that the malady was infectious. Although this idea prevailed rather widely, it had no real evidence in its support. A second and more popular theory was that scurvy was caused by the ingestion of damaged food. The third theory supposed that the disease was caused by a deficient diet — a diet containing too little fresh food. With re-

spect to the latter theory, Holst states: "It seems to us that the facts by which Lind, Hirsch and many other writers have supported this theory are in every respect convincing, and they agree with our own experiments as to the influence of fresh cabbage, apples, potatoes, and so on . . . We may . . . draw attention to the fact scurvy has repeatedly arisen where the food consisted of the same or about the same nutriments as we have used in our experiments with guinea pigs." He concludes: "Thus, also **epidemiological facts speak in favor of the opinion that the described disease in guinea pigs is identical with human scurvy.**"

In an addendum to this paper the authors report experiments with dogs on similar diets. As would be expected in light of present knowledge, the dogs failed to come down with the symptoms characteristic of scurvy. How fortunate it was that Holst chose the guinea pig as the mammal with which to study "ship beri-beri"!

In view of the fact that it is now recognized that there were other workers who had produced scurvy-like symptoms in guinea pigs it should be mentioned, in justice to Holst, that he did refer to the earlier work of Bartenstein (9) although not that of Bolle (10) nor Smith (11). Holst stated, however, that he thought the "interesting disease of Bartenstein must . . . be placed in a class by itself."

Holst lectured on his investigations before many groups including: the Norwegian Medical Society (1907); the Epidemiological Society of London (1907); the Annual Meeting of the British Medical Association, Sheffield (July, 1908); the Society of Tropical Medicine and Hygiene, London (1911); the International Congress of Hygiene, Washington (1912); and elsewhere.

Because of the varied opinions held at the time, it was only natural that a heated discussion arose when Holst first reported his work before the Norwegian Medical Society. On this occasion his principal opponent was Dr. Torup who had already completed his elaborate preparation for Nansen's arctic ex-

peditions and who believed scurvy to be a result of the in-
gestion of "spoiled" foods. Even Hess, the American pediatri-
cian and authority on scurvy, as late as 1917 stated (12) that
infantile scurvy was due to the toxins produced by intestinal
bacteria as a result of "malnutrition." While Holst was very
much interested in continuing his experimental work, the lack
of support and the pressure of administrative duties prevented
his doing so. This is evidenced by a statement made by Frølich
in his memorial address on Professor Holst (13) "It is much
to be regretted — as a matter of fact it engendered much
bitterness in Holst's mind — that the wretched economic con-
ditions under which Norwegian science had to work in the
pre-war years, made it impossible to carry on a continuous
program within this most significant field of study, a field in
which Norwegian science had played such a leading part."
From the writings of Holst (14) we quote, "As we have had
nobody to assist us, we regret not to have been able to apply
the method for measuring the antiscorbutic value . . ., etc."
It is in this paper also that Holst first uses the term "vita-
mins." Other papers on scurvy were published by Holst and
his co-workers (14–18). These included studies on the effect
of processing on the antiscorbutic value of foods (15); on the
comparative symptomatology of guinea pig scurvy and infan-
tile scurvy (by Dr. Frølich) (17); on the conservation and
extraction of the specific components from antiscorbutic foods
(18); and after the war one paper on the preservation of the
antiscorbic properties of cabbage by dry storage (19). In ad-
dition, Furst in his laboratory (16) found that "dry peas and
grain, which do not prevent the experimental disease, acquired
pronounced antiscorbutic properties when moistened and al-
lowed to germinate. This process converts them into 'fresh
vegetables'."

The work which they did on the experimental production of
scurvy in the guinea pig undoubtedly ranks Holst and Frølich
with those other pioneers of vitamin research: Lunin and von
Bunge, Eijkman and Grijns and Hopkins. On looking back
over the work of Holst one wonders what other important dis-

coveries he might have made if he had been given as much encouragement financially and otherwise in his fundamental research as he was given in carrying out the practical aspects of his position as Professor of Hygiene. In the writings of Holst, himself, one notes this same "is it practical" point of view. Yet, in the light of present day knowledge we know that the basic work he did was the most practical and the so-called practical aspects were of little lasting importance. For example, Holst states (6), "In these experiments, however, the beef was more strongly heated than in the manufacture of tinned meat." The chickens fed this meat all died of neuritis, because of the heat lability of thiamine — Holst felt that the results were not of practical importance because the meat had not been processed in a conventional manner.

Let us next look at the character and personality of the man Holst, insofar as we can visualize it from the reports of co-workers that have come down to us (13, 20, 21). The painting of Professor Holst gives a fine impression of the colorful personality which he seems to have been. de Besche characterizes him as highly gifted and filled with a great interest in the tasks ahead. Considering his natural endowments and personality, it was only natural and to be expected that he would make a name for himself. He was a full professor at 33; and became president of the Norwegian Medical Association at the early age of 38 illustrating the high regard in which he was held by his professional colleagues.

He set about each task with great enthusiasm and "whole-heartedness," and by his forcefulness saw the realization of many of his ideas. He was an exceptionally gifted speaker of remarkable and distinctive eloquence and also a lively and witty debater. During a debate his energetic sallies against his opponents at times provoked criticism and spirited counter-attacks. Quoting again from de Besche, "he always spoke easily, concisely, and pointedly. And, his excellent oral presentment of a case lent him great strength. As a debater he was striking and aggressive, and we could not fail to see the merry fighting spirit beaming from his lively eyes. Often he

displayed a refreshing sense of humor, his discourse being seasoned by numerous barbs. When he was at his best, it was a treat to listen to him . . . The same qualities distinguished him as a public speaker, and it may well be understood why on occasion, he has been named the best orator in our country.''

As a research worker he much preferred to pursue his goal uninhibited by the ideas of others and to work out his problems independently. At times this characteristic led him to suggest that he would rather not read what others had done on the subject on which he himself was working lest he be influenced by the opinions of others. However, in his classic papers already discussed he showed an excellent knowledge of most of the prior work.

He was, in general, a man of reality, holding scientific views in line with the scientific thought of his time. Occasionally his fervent interest in his subject might obscure his sense of criticism, but more important was the fact that he approached each new problem with vigor and enthusiasm. Frølich (13) commented thus, ''A new experiment often founded on a passing idea would captivate him entirely for days and weeks at a time. It might then well happen that after long working hours his disappointment would be great. On the other hand, how great was his joy when his experiment had successfully fulfilled his expectations.''

In summing up his personality, de Besche says, ''When we think of Axel Holst as we met him, whether in every day life, fully occupied with the work and questions of the day, and indeed with all the activities of human life, lively, keen on discussing any problem, or as we met him on festive occasions, this phrase comes readily to our lips — a brilliant personality.''

With the passing years Holst was entrusted with many administrative tasks. He was an able administrator, becoming completely engrossed in every task, even those thrust upon him. He was president of the Norwegian Medical Association in 1898–99 and again in 1902–03; as well as chairman of the Association, 1908–10. He was Norwegian deputy to a number

of international congresses including Brussels in 1899, Berlin in 1907, and Berlin again in 1910 for the Koch memorial. Finally in 1919–21 he was Rector of the University of Christiania.

Because of his interest in the improvement of public hygiene, Holst began to work as early as 1893 for the establishment of a postgraduate course in social medicine for physicians in the state's employ. This measure he managed to put into effect in 1929 a year before his retirement.

Holst was a member of the Norwegian Academy of Science, the Society of Tropical Medicine and Hygiene of London and other learned societies.

On April 26, 1931, at the age of 70, Professor Holst died in Oslo.

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                                               B. CONNOR JOHNSON

DENTAL CARIES IN THE COTTON RAT

XIV. FURTHER STUDIES OF CARIES PRODUCTION BY NATURAL DIETS
WITH ESPECIAL REFERENCE TO THE ROLE OF MINERALS,
FAT, AND THE STAGE OF REFINEMENT OF CEREALS [1]

MARGUERITE A. CONSTANT, H. WILLIAM SIEVERT,
PAUL H. PHILLIPS AND C. A. ELVEHJEM
Department of Biochemistry, University of Wisconsin, Madison

ONE FIGURE

INTRODUCTION

Many dietary ingredients have been shown to affect the production of caries in laboratory animals. A decrease in caries with an increase in the dietary level of protein or fat has been found repeatedly. The increased tooth decay which occurs with increased sugar consumption is well known. Little attention has been paid to the mineral composition of the diet. In a review of the effect of minerals and vitamins on caries, Robinson ('49) noted that the importance of minerals was primarily concerned with the development of sound teeth. In this regard the teeth of rats are fully erupted at weaning and thus dietary minerals have little effect in the experimental production of caries. However, previous studies (Constant et al., '52) showed that the level of refined sugar did not represent a reliable criterion for predicting the cariogenicity of the diet. Natural diets which consisted of dried whole milk, processed cereals and 18% sucrose were found to be more cariogenic than

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a partially purified diet containing 67% sucrose. The data from these studies suggested that the mineral component of the diet influenced the production of caries in the fully erupted teeth of cotton rats. Wynn et al. ('53) have reported a difference in the cariogenicity of two semi-synthetic diets which contained identical amounts (64%) of sucrose. The major differences in the two diets were the composition of the mineral mixtures and of the vitamin supplements. It is the purpose of this report to present data on the effect of certain minerals, dietary fat, tooth maturity and processed cereals on the production of dental caries in the teeth of cotton rats.

TABLE 1

Composition of cereal diets and salt mixtures

INGREDIENTS	CEREAL-MILK-SUCROSE (CMS)	CEREAL-DEXTRIN (CD)	CEREAL-DEXTRIN-SUCROSE-FAT (CDS + F)	CEREAL-DEXTRIN-SUCROSE (CDS)
	%	%	%	%
Oatmeal, finely ground [1]	50	50	50	50
Dried whole milk [2]	32	. .	. .	. .
Casein, vitaminized [3]	. .	8.5	8.5	8.5
Sucrose	18	. .	18	18
Dextrin, powdered	. .	41.5	14.5	23.5
Corn oil	. .	. .	9	. .

BASIC SALT MIXTURE		SALTS IV	
	gm		gm
CaO [4]	154.0	$CaCO_3$	240.0
K_2HPO_4	185.6	$CaHPO_4 \cdot 3H_2O$	60.0
KCl	61.9	K_2HPO_4	258.0
MgO	13.3	$MgSO_4 \cdot 7H_2O$	81.6
$Na_3PO_4 \cdot 12H_2O$	290.5	NaCl	134.0

$FeC_6H_5O_7 \cdot 6H_2O$, 22.0 gm; KI, 0.64 gm; $MnSO_4 \cdot 4H_2O$, 4.0 gm; $ZnCl_2$, 0.2 gm; and $CuSO_4 \cdot 5H_2O$, 0.24 gm added to each mixture. The ratios of essential cations and phosphate were kept as similar as possible in the two mixtures.

[1] Reported in Vitamin Survey, The Quaker Oats Co., 1947, to contain approximately 50 mg Ca and 441 mg P/100 gm.

[2] Klim — stated to contain 38 gm lactose, 28 gm fat and approximately 0.95 gm Ca and 0.73 gm P/100 gm.

[3] Shaw et al., '44; contains approximately 0.06 gm Ca and 0.8 gm P/100 gm.

[4] Cellu flour was substituted for CaO in the calcium-free salts.

EXPERIMENTAL

Weanling cotton rats (19 to 21 days of age) were used. Litter-mates were distributed among the control and experimental groups because of the heredity differences observed by Schweigert et al. ('45). Carious lesions were evaluated according to the method of Shaw et al. ('44).

The basal diets and salt mixtures used in these experiments are given in table 1. Cereal products were ground in a Wiley mill to pass through a 40 mesh screen. All animals received food and water ad libitum and were given two drops of haliver oil each week.

RESULTS AND DISCUSSION

In the first experiment a study was made of the effect of replacing the milk in a cereal-milk-sucrose (CMS) diet with casein and dextrin (cereal-dextrin-sucrose, CDS), with casein, dextrin, and fat (CDS + F), or by casein and dextrin (CD, no "added" sugar). Previous experiments (Constant et al., '51) showed that decreasing the milk content of the diet resulted in increased caries, and the substitution of casein and dextrin for the milk resulted in severe tooth decay. Similar results were obtained in the present experiment. The substitution of casein and dextrin for milk (CDS diet) resulted in an increase in the number of lesions per capita from 12 to 23 and the extent was increased from 26 + to 62 +. The inclusion of corn oil in the substitutions for milk (CDS + F) did not afford protection against the cariogenicity of the milk-free diet (CDS diet). It was also observed in this experiment that the animals showed extensive caries despite the omission of sucrose from the diet (CD diet). The average number of lesions per capita was 15.

Because the substitutions for milk lowered the sugar content of the diet (lactose), a decrease in caries rather than an increase would be expected. The second experiment, therefore, was organized to determine whether dietary minerals would affect the production of caries. Calcium carbonate was added to the CDS ration with fat (CDS + F). Haliver oil was mixed in the corn oil at a level of 0.1% to provide ample vitamin D.

TABLE 2

The effect of various supplements to cereal diets on dental caries production in the cotton rat

| RATION | NO. OF RATS | CARIES SCORES [1] | | DIET [2] pH | FEMUR [3] ASH % |
| | | Incidence [4] | Extent | | |
		Av. ± S.D.	Av. ± S.D.		Av. ± A.D.
Experiment 1. Effect of milk or sugar (6 wk.)					
CMS Basal	5	12 ± 3	26 ± 7	6.2	(59)
CDS Basal	4	23 ± 4	62 ± 13	5.4	(51)
CDSF Basal	4	25 ± 4	69 ± 17	5.4	(51)
CD Basal	5	15 ± 9	40 ± 29	5.4	(46)
Experiment 2A. Caries inhibition by $CaCO_3$ (5 to 7 wk.)					
CDSF Basal	3	17 ± 4	38 ± 12	5.4	53 ± 1
+ 1% $CaCO_3$	4	4 ± 5 [5]	7 ± 9	5.9	62 ± 1
+ 3% $CaCO_3$	4	2 ± 2 [6]	2 ± 2	6.4	58 ± 2
Experiment 2B. Caries inhibition by $CaCO_3$ or Na_2HPO_4 (6 wk.)					
CDSF Basal	5	14 ± 5	29 ± 12	5.4	52 ± 2
+ 1% $CaCO_3$	5	6 ± 2	13 ± 4	5.9	65 ± 1
+ 2% $CaCO_3$	5	2 ± 2 [7]	6 ± 4	6.2	62 ± 2
+ 2% Na_2HPO_4	5	3 ± 2	6 ± 4	7.0	57 ± 2
Experiment 3. Caries inhibition by basic salts and calcium (8 wk.)					
CD Basal	5	22 ± 4	67 ± 20	5.4	59 ± 1
+ 1.5% $CaCO_3$	5	18 ± 6	46 ± 18	6.1	65 ± 2
+ 4% basic salts (minus Ca)	5	12 ± 5	25 ± 12	7.1	57 ± 2
+ 4% basic salts (plus Ca)	4	4 ± 1	10 ± 5	8.8	67 ± 1
+ 18% sucrose + 4% basic salts + Ca	4	10 ± 5	23 ± 12	8.8	67 ± 1
Experiment 4. Effect of neutral salts, protein, fat or delayed feeding (6 wk.)					
CDSF Basal	5	22 ± 6	58 ± 26	5.4	56 ± 2
Delayed feeding (1 month past weaning)	5	8 ± 5	13 ± 9	5.4	64 ± 1
+ 4% Salts IV	5	10 ± 3	18 ± 7	6.4	69 ± 1
+ 8.5% protein (— 8.5% sucrose)	3	16 ± 6	42 ± 13	5.0	57 ± 3
With extr'd oats + 3.5% corn oil	5	16 ± 7	39 ± 22	5.4	55 ± 2
With extr'd oats + corn oil and carvone	5	15 ± 6	35 ± 22	5.4	56 ± 3

TABLE 2 *(continued)*

The effect of various supplements to cereal diets on dental caries
production in the cotton rat

RATION	NO. OF RATS	CARIES SCORES [1]		DIET [2] pH	FEMUR [3] ASH %
		Incidence [4]	Extent		
		Av. ± S.D.	Av. ± S.D.		Av. ± A.D.
Experiment 5. Effect of ''natural'' fat or ''added'' oil (11 to 14 wk.)					
Milk (dried)					
— oatmeal basal	5	15 ± 4	26 ± 11		
With 7% corn oil	5	7 ± 4	9 ± 6		
With 14% corn oil	4	5 ± 3	6 ± 4		
With 14% butterfat	5	3 ± 2	3 ± 2		
Experiment 6. Caries activity of oat products (14 wk.)					
Whole oats	7	10 ± 5	14 ± 8		
Groats (unsteamed)	7	20 ± 3	36 ± 9		
Groats (steamed)	7	20 ± 3	37 ± 11		
Rolled oats	7	19 ± 4	31 ± 9		
Experiment 7. The effect of oat hulls (14 wk.)					
Groats	4	26 ± 4	49 ± 16		
Whole oats	4	17 ± 5	21 ± 5		
1: 1 Groats and hulls	4	13 ± 2	15 ± 3		
Groats plus 0.9% ash	4	22 ± 5	37 ± 14		

[1] Incidence equals average number of carious fissures per capita. (The cotton rat has a total of 40 fissures per head, 10 in each quadrant.) Extent is an expression of the average total caries involvement per capita. Individual lesions are evaluated 1 + to 5 + with increasing severity of lesions; 1 + and 2 + = lesions involving primarily the enamel; 3 + = enamel and a considerable amount of dentine; 4 + and 5 + = lesions resulting in fractures.

[2] Diet pH = pH of aqueous, saturated solution of diet.

[3] Fat and moisture free basis except data in parentheses which are based on moisture-free weight.

[4] Animals with caries = 100%, except for three groups.

[5] Animals with caries = 50%.

[6] Animals with caries = 75%.

[7] Animals with caries = 80%.

The caries scores in table 2, experiment 2A, show that the addition of 1% or more of $CaCO_3$ markedly reduced tooth decay. Caries incidence was reduced from an average of 17 (control group) to 4 ($CaCO_3$ group) and the extent from 38 + to 7 +. In a subsequent study a 4th group was fed the CDS + F ration plus 2% Na_2HPO_4. With this group of litters (table 2, ex-

periment 2B) it was again observed that the teeth of animals fed $CaCO_3$ showed little tooth decay compared to their litter-mates which were fed the CDS + F basal. These data also showed that feeding dibasic sodium phosphate reduced tooth decay. The average caries incidence of these litter-mates was 3.

This experiment was repeated in a slightly modified form in order to determine whether other basic salts would retard tooth decay and whether this retardation effect would occur when another diet was used. Basic salt mixtures (with or without calcium) or calcium carbonate were added to the CD ration. In this experiment the reduction in tooth decay was less when calcium carbonate was added to the CD ration. The addition of 4% basic salt mixture minus calcium (table 2, experiment 3) resulted in approximately 50% less caries. The inclusion of calcium reduced the number of lesions from 22 per capita for the animals consuming the basal ration to 4 per capita for those fed the basal plus 4% basic salts plus calcium. The basic salts plus calcium were not as effective when the ration contained 18% sucrose (substituted for a like amount of dextrin). The number of lesions increased from 4 to 10 per capita. Basic salt mixtures in semi-synthetic diets have also been observed to reduce caries production (Constant et al., '54).

The reduction in caries brought about by the addition of dibasic sodium phosphate or a basic salt mixture minus calcium occurred despite the fact that these animals showed a decreased femur ash comparable to that of animals fed the basal (milk-free) rations. Leicester ('49) cited several in vitro studies which showed that a decrease in pH, calcium, or phosphate caused an increase in the solubility of enamel. The increase in the pH of the diet which occurred when the basic minerals were added suggests that the pH of the diet influences its cariogenicity. However, the increased dietary level of phosphate provided by these supplements may also be a factor.

In the 4th experiment the mineral deficient CDS + F basal diet was not fed until the animals were 7 weeks old. A non-cariogenic stock ration was fed in the interim. The effect of

feeding a nearly neutral salt mixture (salts IV, table 1) was also studied. The increased number of animals per litter permitted a study of whether the ether extractable portion of the oatmeal was influencing its cariogenicity. The ether extract (3.5%) was replaced by corn oil or corn oil plus carvone.

The caries scores which are given in table 2, experiment 4, show that a delay of one month in feeding the highly cariogenic basal resulted in a marked decrease in tooth decay. The average incidence was reduced from 22 to 8 lesions and the average extent was reduced from 58 + to 13 +. Similar observations have been made with hamsters by Volker ('51) and with cotton rats fed semi-synthetic diets (Constant et al., '54). A reduction in tooth decay was again observed when 4% Salts IV was added to the basal diet. The number of lesions was reduced from 22 to 10 per capita and the extent was reduced from 58 + to 18 +. The reduction in tooth decay caused by the several dietary mineral supplements used in these studies indicates that certain dietary minerals influence the development of caries in the erupted teeth of cotton rats. These data suggest that the loss of the minerals when substitutions were made for the dried whole milk was partly responsible for the greater cariogenicity of the milk-free diets. The substitution of casein for almost half of the "added" sugar in the basal diet resulted in some decrease in tooth decay. However, it was evident that the level of sugar which was still present, 9.5%, was sufficient to cause extensive caries. These animals averaged 16 lesions per capita and the extent was 42 +. The substitution of corn oil for the lipids extracted from the oatmeal resulted in a slight reduction in tooth decay. The incorporation of carvone in the corn oil did not affect the results.

The possibility that the type of fat may influence the cariogenicity of a ration, as indicated in the previous study, was checked in the 5th experiment. In previous studies on the protective effect of fat in a diet (Schweigert et al., '46), the fat was substituted isocalorically for sucrose and thus resulted in a proportionally greater reduction of sucrose as the fat content was increased. In the present experiment a basal diet was used

which consisted of 50% cereal and 50% dried whole milk (which supplied 14% fat). Dried skim milk and oily fats, such as corn oil or butter, replaced the dried whole milk in appropriate amounts to provide 7 or 14% oily fats. Liver concentrate N.F. powder was added at a level of 4% at the expense of the entire ration. Despite the fact that no sucrose was added to the basal diet and the major portion of the soluble sugars in the diet was the lactose of the milk, the teeth of these animals showed considerable caries activity. An average of 15 lesions per capita was observed and no animals were caries-free (table 2, experiment 5). The caries scores of these animals showed that the substitution of 7% oily fat for the natural fat afforded approximately maximum protection against the caries-producing activity of the other ingredients of the diet.

In the 6th experiment an attempt was made to determine at which stage of processing of cereals the increased cariogenicity occurred. A supply of whole oats, groats before steaming, groats after steaming and rolled oats from the same origin were obtained.[2] These cereal products were fed at a level of 50% in the CMS ration (table 1). Four per cent of liver concentrate N.F. powder was added at the expense of the entire ration. The caries scores of these animals (table 2, experiment 6) show that the increased cariogenicity of processed cereals occurred in the case of oat products at the stage of removal of hulls. Animals fed unsteamed groats had 20 lesions per capita and those fed the whole oats had 10 lesions per capita. Unsteamed groats were equally as cariogenic as steamed groats or rolled oats.

The next experiment, therefore, was organized to determine whether the fibrous hulls were responsible for the lesser cariogenicity of whole oats. A supply of the oats, groats and hulls was obtained from the original source. The CMS basal was used as in experiment 6. The experimental plan was as follows: (1) groats; (2) whole oats (contain approximately 25% hulls, thus supply 12.5% hulls to the ration); (3) 1:1 mixture

[2] Obtained through the courtesy of Dr. F. N. Peters of the Quaker Oats Company, Chicago, Ill.

of groats and hulls (approximately 25% in the ration); and (4) groats plus 0.9% ash obtained from dry ashing whole oats. The addition of ash from whole oats to a diet containing groats did not alter the cariogenicity of the groats (table 2, experiment 7). The animals fed groats had an average of 26 lesions and an extent of 49 + and those fed the ash in addition to the groats had 22 lesions and an extent of 37 + per capita. The animals fed whole oats again had less tooth decay (incidence reduced from 26 to 17 and the extent from 49 + to 21 +). The feeding of oat hulls in a 1 : 1 mixture of groats and hulls (lot 3) decreased the tooth decay to an average number of 13 lesions
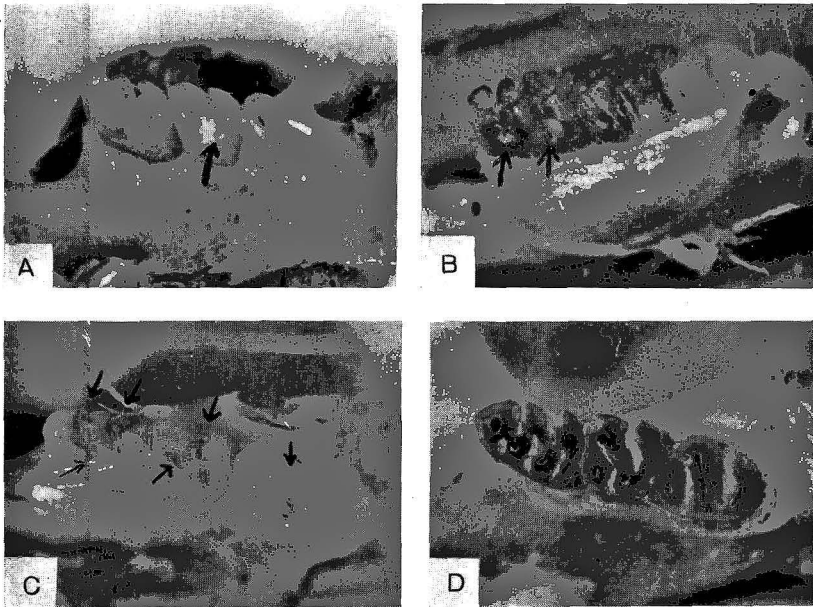
Figure 1

A   Teeth of cotton rat fed a low mineral oatmeal diet. The thickest portion of enamel was easily penetrated with a fine wire no. 42 dental probe.

B   The oatmeal diet contained 4% liver concentrate N.F. powder. Note the deep pigmentation of the lesions and bleaching at the fissure surface.

C   Same as B but the teeth were ground parallel to the occlusal surface. Arrows indicate carious lesions.

D   Teeth of animal fed the CDS + F basal plus 2% CaCO$_3$. Note the differences in the appearance of the enamel and dentine in contrast to C.

per capita with an extent of 15 +. Although the animals fed the 1:1 mixture of groats and hulls had fewer carious lesions than those fed groats alone, they consumed more food, 10.3 gm, per day compared to 9.0 gm. These observations are being studied further in order to eliminate the possibility that the protective effect of hulls may be a dilution effect.

The marked whitening and softening of the teeth which occurred frequently when the dried whole milk was omitted from the diet is shown in figure 1A. These lesions were readily penetrated with a no. 42 dental probe and were non-carious, unpigmented, and opaque without grossly observable loss of enamel. When large litters of cotton rats were available, the extra siblings were fed the basal diets plus 4% N.F. liver powder. The failure of the liver extract to modify the cariogenicity of the diet as noted by Shaw et al. ('44) was thus confirmed. However, the addition of liver extract caused deeply pigmented, yellow-brown lesions (figure 1B and in cross section 1C) which frequently appeared bleached at the fissure surface (figure 1B). The teeth of animals fed the CDS + F ration plus $CaCO_3$ were the healthiest appearing teeth that have been observed in our experiments. The enamel was blue-white and very hard. The dentin showed capillary distribution throughout (figure 1D).

SUMMARY

Experiments have been conducted to study the effect of minerals, especially calcium and basic mineral mixtures, fat, tooth maturity and the stage of refinement of cereals on the cariogenicity of natural diets fed to cotton rats.

Calcium carbonate, disodium phosphate, basic salt mixtures with and without calcium and near neutral salt mixtures containing calcium retarded the development of caries in the erupted tooth.

A low-mineral natural diet which produced extensive tooth decay when fed to weanlings was relatively non-cariogenic if fed to older animals. Oily fats were found to give partial protection against tooth decay when substituted for the "natural" fat of a cereal-milk diet.

Unsteamed oat groats were found to be more cariogenic than whole oats. The increased cariogenicity of processed oats occurred at the de-hulling step.

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DENTAL CARIES IN THE COTTON RAT

XV. THE EFFECT OF TOOTH MATURITY AND MINERALS ON CARIES PRODUCTION BY SEMI-SYNTHETIC DIETS [1]

MARGUERITE A. CONSTANT, H. WILLIAM SIEVERT, PAUL H. PHILLIPS
AND C. A. ELVEHJEM

Department of Biochemistry, University of Wisconsin, Madison

INTRODUCTION

During the course of studies on the experimental production of dental caries in cotton rats, certain mineral supplements were found to reduce the cariogenicity of natural diets (Constant et al., '54). In addition, low-mineral natural diets which produced extensive tooth decay when fed to weanlings were found to be relatively non-cariogenic if fed to older animals. It was of interest to determine whether similar results would be obtained with the use of high-sucrose, partially purified diets, as the composition of these diets facilitated their modification with regard to single constituents. The purpose of this paper is to present the results of experiments which were conducted to study the effects of dietary calcium, tooth maturity, acidic and basic salts on caries and to attempt to modify the cariogenicity of low calcium diets.

EXPERIMENTAL

The basal diet consisted of casein (24%), sucrose (67%), Salts IV (4%), corn oil (5%), adequate levels of B-vitamins

TABLE 1

Composition of mineral mixtures

SALTS IV		OXIDES		ACID	
	gm		gm		gm
CaCO$_3$	240.0	CaO	154.0	CaH$_4$(PO$_4$)$_2$·H$_2$O	230.7
CaHPO$_4$·3H$_2$O	60.0	K$_2$HPO$_4$	185.6	CaSO$_4$·2H$_2$O	315.4
K$_2$HPO$_4$	258.0	KCl	61.9	KCl	220.8
MgSO$_4$·7H$_2$O	81.6	MgO	13.3	MgCl$_2$·6H$_2$O	33.6
NaCl	134.0	Na$_3$PO$_4$·12H$_2$O	290.5	MgSO$_4$·7H$_2$O	40.8
Trace minerals[1]	27.08	Trace minerals[1]	27.08	NaCl	134.0
				Trace minerals[1]	27.08
	800.08		732.38		1002.08
pH[2]	7.8–8.0		12 +		4.4

LACTATES AND ACETATES		CITRATES	
	gm		gm
Ca(C$_3$H$_5$O$_3$)$_2$·5H$_2$O	448.7	CaCO$_3$	91.7
CaHPO$_4$	175.7	CaHPO$_4$	248.9
K$_2$HPO$_4$	94.9	K$_2$C$_6$H$_5$O$_7$·H$_2$O	320.2
K$_2$C$_2$H$_3$O$_2$	184.9	Mg$_3$(C$_6$H$_5$O$_7$)$_2$·12H$_2$O	77.6
Mg(C$_2$H$_3$O$_2$)$_2$·4H$_2$O	70.9	NaCl	67.0
NaCl	67.0	Na$_3$C$_6$H$_5$O$_7$·2H$_2$O	112.3
NaC$_2$H$_3$O$_2$	94.0	Trace minerals[1]	27.08
Trace minerals[1]	27.08		
	1063.18		944.78
pH[2]	6.5		8.3

[1] Fe C$_6$H$_5$O$_7$·6H$_2$O, 22.0 gm; KI, 0.64 gm; MnSO$_4$·4H$_2$O, 4.0 gm; ZnCl$_2$, 0.2 gm; CuSO$_4$·5H$_2$O, 0.24 gm. Celluflour was substituted for the calcium salts in calcium-free mixes of salts IV or salts IV (oxides).

[2] Four-tenths to 0.5 gm/100 ml water.

and 4% liver concentrate N.F. powder added at the expense of the entire ration. The compositions of the mineral mixtures used in these experiments are given in table 1. Since the dietary changes were concerned primarily with the level or type of minerals in the diet, this information is given in the tables together with the caries scores. The young were allotted to control and experimental groups by litter-mates. Lesions were evaluated according to the method of Shaw et al. ('44). Animals were given two drops of haliver oil weekly and received food and water ad libitum.

RESULTS AND DISCUSSION

These studies which have been conducted over a period of several years are summarized as three series of experiments on (1) dietary calcium and tooth maturity; (2) acidic and basic minerals; and (3) attempts to modify the cariogenicity of low calcium, high sucrose diets.

Dietary calcium and tooth maturity

The first experiment of this series was a study of dietary calcium, added to the mineral mixture as $CaCO_3$. All the other minerals were kept constant. The caries scores (table 2, experiment 1A) showed that a decrease in the level of calcium from 0.56 to 0% caused an increase in incidence from 5 (0.56% Ca) to 15 (0% Ca) and extent from 6 + to 21 +. Subsequent availability of three siblings per litter made it possible to make litter-mate comparisons on the effect of an intermediate level of calcium (0.28%). Although caries production by the basal diet with calcium was greater in this series of animals, the omission of calcium from the mineral mix was again observed to increase caries. The incidence increased from 11 (0.56% Ca) to 21 (0% Ca) and the extent from 13 + to 44 +. The tooth decay of animals fed the intermediate level of calcium was slightly but not significantly decreased.

In the second experiment the feeding of the low calcium diet was started when the animals were two, 5 or 8 weeks old. The

TABLE 2

The effect of dietary calcium and tooth maturity on caries

RATION	SALTS IV		CALCIUM [1]	NO. OF RATS	AGE AT START OF EXP.	CARIES SCORES [2]	
	With calcium	Calcium-free				Incidence Av. ± S.D.	Extent Av. ± S.D.
	%	%	%		weeks		
Experiment 1A (7 to 10 wks.)							
Basal (high minerals)	4	.	0.56	4	3	5 ± 2	6 ± 3
Low calcium	.	4	0.0	4	3	15 ± 5	21 ± 8
Experiment 1B (8 wks.)							
Basal (high minerals)	4	.	0.56	3	3	11 ± 3	13 ± 4
Intermediate calcium	2	2	0.28	4	3	19 ± 5	34 ± 14
Low calcium	.	4	0.0	4	3	21 ± 5	44 ± 17
Experiment 2 (11 wks.)							
Basal (high min.), low Ca	.	4	0	4	2	25 ± 5	51 ± 13
Basal, low Ca	.	4	0	5	5	22 ± 6	37 ± 24
Basal, low Ca	.	4	0	5	8	16 ± 1	19 ± 1
Experiment 3 (11 wks.)							
Basal (high min.), low Ca	.	4	0	4	3	20 ± 6	31 ± 12
Basal, low Ca	.	4	0	4	6	10 ± 5	13 ± 9
Basal, intermediate Ca.	2	2	0.28	4	3	17 ± 7	24 ± 13
Basal, intermediate Ca.	2	2	0.28	4	6	8 ± 3	9 ± 3

[1] Provided by the mineral mixes.
[2] One hundred per cent of the animals had caries.

stock diet was fed during the interim. The caries scores (table 2, experiment 2) showed that the delay in the feeding of the cariogenic diet until the animals were 5 weeks old decreased the tooth decay of some animals, but all animals had less tooth decay if the cariogenic diet was withheld until the animals were 8 weeks old. The number of carious lesions decreased from 25 to 16 per capita and the extent from 51 + to 19 +.

The third experiment was a study of the effect of tooth maturity (experiment 2) as well as of low and intermediate levels of calcium (experiment 1B). The stock diet was fed to the experimental groups for three weeks prior to feeding the cariogenic diet, while the latter was fed to the control groups from weaning. It was again observed that feeding the intermediate level of calcium (0.28%) gave little protection against the cariogenicity of a high sucrose diet. However, if the cariogenic diet was withheld until the animals were 6 weeks old, caries incidence was reduced from 20 to 10 and the extent from 31 + to 13 + (low calcium groups). The caries incidence was reduced from 17 to 8 and the extent from 24 + to 9 + in the case of animals fed the intermediate level of calcium.

Acidic and basic minerals

The basal diet contained floured dextrin and 18% sucrose in place of the 67% sucrose in the first, second and 5th experiments of this series. The mineral portion of the diet was adjusted as indicated by substitutions for the dextrin. The composition of the salt mixtures was such that the ratios of essential elements were maintained similarly from one mixture to another. The first experiment was a study of the effect of acidic, neutral and basic inorganic mineral mixtures on caries. The salt mixtures were fed at a level of 8%. The caries data in table 3, experiment 1, show that there was no difference in the effect of acidic or neutral (Salts IV) mineral mixtures on caries incidence. However, the basic mineral mixture (oxides) protected against tooth decay. The incidence was reduced from 18 to 4 and the extent from 47 + to 5 +. The growth rates of the animals were 8.8, 8.3, and 6.9 gm per week for the acidic,

TABLE 3

The effect of acidic and basic minerals on caries

| RATION | SALTS IV | | CALCIUM | NO. OF RATS | CARIES SCORES | | DIET pH |
	With calcium	Calcium-free			Incidence Av. ± S.D.	Extent Av. ± S.D.	
	%	%	%				
Experiment 1 (14 wks.)							
Basal (8% minerals)	8		1.12	9	18 ± 8	47 ± 24	6.3
Acid minerals	(8)		0.86	10	15 ± 6	30 ± 15	4.5
Oxide minerals	(8)		1.17	10	4 ± 3 [1]	5 ± 4	7.5
Experiment 2 (14 wks.)							
Basal (10% minerals)	10		1.09	9	14 ± 8	35 ± 26	6.3
Citrate minerals	(10)		1.02	9	12 ± 7	32 ± 21	5.4
Lactate and acetate minerals	(10)		1.02	9	14 ± 4	25 ± 11	6.2
Experiment 3 (14 wks.)							
Basal (high minerals)	4		0.56	7	8 ± 4	11 ± 5	5.8
Basal low Ca.		4	0	7	14 ± 4	25 ± 11	5.4
Basal (high minerals — oxides)	(4)		0.56	6	5 ± 3	5 ± 4	6.4
Basal (high minerals, low Ca.		(4)	0	6	10 ± 4	16 ± 8	7.0
Experiment 4 (10 wks.)							
Basal (low min.), low Ca.		1	0	5	20 ± 4	37 ± 14	
+ 1.1% $CaCl_2 \cdot 2H_2O$		1	0.30	5	16 ± 4	32 ± 15	
+ 0.8% $CaCO_3$		1	0.32	5	14 ± 5	21 ± 9	
+ 0.8% $CaCO_3$ + 1.6% NaCl		1	0.32	4	21 ± 2	45 ± 6	
Experiment 5 (14 wks.)							
Basal (high minerals)	4		0.56	5	14 ± 7	35 ± 21	5.8
+ 3% NH_4Cl	4		0.56	5	13 ± 8	31 ± 21	5.8
+ 2.8% $(NH_4)_2CO_3$	4		0.56	4	7 ± 6	14 ± 14	7.1
+ 1.8% Urea	4		0.56	4	6 ± 5	11 ± 13	5.8
+ Urea + NH_4Cl injections	4		0.56	5	3 ± 4	6 ± 8	5.8

[1] All animals in these series of experiments had caries with the exceptions of two animals in this group, and one animal each in groups 4 and 5 of experiment 5.

basic, and Salts IV groups, respectively. It was observed that the teeth of animals fed the high level of basic minerals were very white and devoid of plaque material in contrast to the usually moderate to heavy coating of deep yellow-brown plaque material on the teeth of animals fed the basal diet with Salts IV.

In the second experiment the effect of organic salts was compared to that of Salts IV. The salts were fed at a level of 10%. The total Salts IV mixture was made up to 1,000 gm with dextrin in order to more nearly equalize the level of minerals in the three diets. The caries data in table 3, experiment 2, show that there was no difference in the effect of these various mineral mixtures on caries incidence. The dissolution of the tooth by citrate salts to form deep cups as noted by McClure and Ruzicka ('46) was observed but this defect did not increase the caries experience of these animals.

The level of minerals used in the first two experiments was twice as high as that usually used in nutrition experiments and the unequal total weights of the salt mixtures resulted in slightly variable amounts of essential elements in the diets. This was equalized in the third experiment by adding celluflour to the Salts IV. Complete, calcium-free Salts IV or oxides were fed at a level of 4% in the diet to weanling cotton rats. The data on caries in table 3, experiment 3, again show the protective effect of calcium that was observed in the first series of experiments. In the case of the Salts IV groups, the inclusion of calcium reduced the incidence from 14 to 8 and the extent from 25 + to 11 +. The addition of calcium to the oxide mineral mix reduced the incidence from 10 to 5 and the extent from 16 + to 5 +. There was a definite tendency for the oxides to reduce tooth decay more than Salts IV.

In the 4th experiment the basal diet contained 1% calcium-free Salts IV and $CaCl_2$, $CaCO_3$, or $CaCO_3$ and NaCl were added to provide approximately 0.3% calcium in the diet. The data on caries are presented in table 3, experiment 4, and show that $CaCl_2$ did not give any protection against caries development. There was a tendency for $CaCO_3$ to afford some pro-

tection but as discovered in the first series of experiments (table 2) this level of calcium was not sufficient to give consistent protection to all animals under these conditions. It was observed that a significant increase in caries developed when a high level of salt was added to the diet. The animals fed only $CaCO_3$ in the diet in addition to Salts IV showed the presence of 14 carious lesions per capita with an extent of 21 +, while those fed $CaCO_3$ and NaCl.had 21 lesions per capita with an extent of 45 +.

The 5th and last experiment of the second series was a study of the effect of acidic, basic and neutral ammonium salts on caries incidence. In addition, the animals of a 4th group were injected subcutaneously with a 3% solution of NH_4Cl in increasing doses starting at 0.25 ml every other day the second week, every day the third week, 0.5 ml every other day for the next week and a half, etc. until by the 8th week they were receiving 1.0 ml every other day and received a total of 29 ml by the end of 13 weeks. The growth of these animals was comparable to that of animals in the other 4 groups. The caries data are summarized in table 3, experiment 5, and show that there was no difference in the caries experience of animals fed the basal diet and those fed the basal diet plus NH_4Cl. The addition of $(NH_4)_2CO_3$ to the basal food resulted in a decrease in tooth decay from 14 lesions per capita, with an extent of 35 + for the controls, to an incidence of 7 with an extent of 14 + for those receiving the diet with ammonium carbonate. The addition of urea to the diet resulted in a similar decrease in tooth decay. Although there was a decrease in caries experience of all animals in the urea group, the greater degree of protection was given to those animals whose teeth were partially caries resistant. The injection of NH_4Cl did not offset the anticariogenicity of the urea in the diet. Both of the groups receiving NH_4Cl either in the diet or by subcutaneous injection frequently showed the presence of small, soft, white areas on the buccal or lingual surfaces of the third molar. These areas, however, were not carious.

These data indicate that calcium has a retarding effect on caries. In almost every case the percentage decrease in extent of the lesions was greater than that of incidence when the dietary level of calcium was increased from 0 to 0.56%. However, the failure of high levels of Salts IV (8%, supplying approximately 1.1% Ca and 0.56% P) to prevent tooth decay indicates that the retarding effect of calcium was limited in these high sucrose diets. It is possible that the reduction in tooth decay observed when the level of calcium was increased from 0 to 0.56% would not be as great if the experimental period was considerably extended. An inhibition of caries occurred when the diet contained a high level (8%) of very basic minerals (oxide mineral mixture). Two of the animals were caries-free and the remainder averaged only 4 carious lesions per capita compared to 18 for animals fed Salts IV. The levels of calcium and phosphorus supplied by the basic mineral mixture were similar to those of Salts IV (approximately 1.2% Ca and 0.62% P). The organic salt mixtures failed to reduce tooth decay although they similarly supplied a high level of alkaline-ash minerals to the diet. These data indicate that the increased pH of the diet which was caused by the supplement of oxide mineral mixture was a factor in the protective effect of these minerals.

Low-calcium, high-sucrose diets

In the third series of experiments attempts were made to modify the cariogenicity of low-calcium, high-sucrose diets. In two experiments an increase in dietary fat failed to reduce tooth decay. The high-fat experimental diet in the first experiment (table 4) contained an additional 10% corn oil which replaced 4% casein and 6% sucrose of the basal diet. In the second experiment (table 4) an additional 8% corn oil was substituted for 6% celluflour and 2% sucrose of the basal diet which contained 24% casein, 62% sucrose, 10% celluflour, 2% Ca-free Salts IV, 2% corn oil, B-vitamins and liver extract. It was observed in the second experiment that an increase in casein content of the diet to 34% (the additional 10% was substituted for 6% celluflour and 4% sucrose) tended to increase

TABLE 4

Attempts to modify the caries activity of low calcium, high sucrose diets by fat, amino acids or proteins

RATION	SALTS IV With Ca (%)	SALTS IV Ca-free (%)	Ca (%)	NO. OF RATS	AGE AT START OF EXP. (weeks)	CARIES SCORES [1] Incidence Av. ± S.D.	CARIES SCORES [1] Extent Av. ± S.D.
Experiment 1 (12 wks.)							
Basal, intermediate Ca	2		0.28	6	2	24 ± 5	46 ± 21
Basal, intermediate Ca + 10% corn oil	2		0.28	6	2	21 ± 4	36 ± 18
Experiment 2 (10 wks.)							
Basal, intermediate Ca + 10% corn oil	2		0.28	6	5	18 ± 3	28 ± 12
Basal (2% fat; intermediate min.), low Ca		2	0	4	3	20 ± 4	29 ± 8
Basal + 8% corn oil		2	0	5	3	17 ± 4	24 ± 10
Basal + 8% corn oil + 0.8% CaCO3		2	0.32	5	3	20 ± 4	31 ± 11
Basal + 10% protein		2	0	4	3	27 ± 2	49 ± 9
Basal + 8% fat + wheat flour (no sucrose)		2	0	5	3	4 ± 3	4 ± 3
Experiment 3 (14 wks.)							
Basal (high min.), low Ca		4	0	6	3	9 ± 6	16 ± 14
Basal + 1% lysine		4	0	6	3	14 ± 6	24 ± 16
Basal + 1% tryptophan		4	0	6	3	14 ± 5	24 ± 9
Basal + 1% lysine + tryptophan		4	0	6	3	15 ± 5	28 ± 8
Experiment 4 (14 wks.)							
Basal (high min.), low Ca		4	0	6	3	16 ± 5	29 ± 16
with albumin		4	0	6	3	11 ± 5	13 ± 6
with fibrin		4	0	6	3	8 ± 3	11 ± 7
with 1:1 gelatin: casein		4	0	5	3	14 ± 3	27 ± 8
Experiment 5 (11 wks.)							
Basal (60% sucrose), intermediate Ca	2		0.28	6	3	19 ± 9	33 ± 24
Basal 40% sucrose, intermediate Ca	2		0.28	6	3	24 ± 4	43 ± 12
Basal 40% sucrose, low Ca		2	0	5	3	27 ± 4	64 ± 19
Basal 40% sucrose (high min.), intermediate Ca	2		0.28	6	3	26 ± 4	53 ± 11
Basal 20% sucrose, intermediate Ca	2		0.28	5	3	26 ± 3	50 ± 14

[1] One hundred per cent of animals had caries.

rather than decrease tooth decay. The incidence increased from 20 for the basal group to 27 for the high casein group and the extent from 29 + to 49 +. Although little tooth decay was observed when wheat flour was substituted for the sucrose, the animals were not caries free.

A study of the effect of amino acids was made in the third experiment in which the low calcium basal diet was supplemented with 1% lysine, 1% tryptophan, or 1% lysine and 1% tryptophan. Although there is some evidence of a beneficial effect of tryptophan (Turner, '47, '48), Shaw ('49) found no protection when 0.5% tryptophan was included in a complete diet and fed to cotton rats. The data on caries summarized in table 4, experiment 3, show that these amino acid supplements had no effect on the cariogenicity of a low calcium diet.

Shaw ('49) found that various proteins in a complete ration did not alter the cariogenicity of the diet. The observation that an increase in casein content of a low-calcium, low-mineral diet tended to increase tooth decay suggests that the type of protein may effect the cariogenicity of low mineral diets. In the 4th experiment, albumin, fibrin or a 1:1 mixture of gelatin and casein were substituted for the casein of the basal diet. The caries scores (table 4, experiment 4) showed that albumin tended to produce less tooth decay especially with regard to the extent of the lesions. A definite reduction occurred when fibrin was used. The incidence was reduced from 16 (casein) to 8 (fibrin) and the extent from 29 + to 11 +. A combination of gelatin and casein had no effect. It was observed that an increasing pH of a saturated aqueous solution of the various diets tended to follow their anticariogenic properties. The possible influence of the minerals associated with the proteins on the caries activity of the proteins cannot be ascertained at the present time.

Previous studies of natural diets indicated that under certain conditions mixed carbohydrates were equally as cariogenic as sucrose (Constant et al., '52). Therefore, in the 5th experiment a study was made of the effect of substituting finely

ground dextrin for sucrose in a low mineral diet. When the dextrin was screened to determine the particle size, it was found that 26% was retained on a no. 40 mesh screen and 46% passed through a no. 60 mesh screen. The basal diet contained 60% sucrose, 9% dextrin and 2% Salts IV in place of 67% sucrose and 4% Salts IV of the basal diet. Dextrin replaced sucrose as the level of sucrose was decreased. The data on caries (see table 4, experiment 5) show that a reduction of the sucrose content of the basal from 60 to 20% did not result in a decrease in caries. On the contrary there was a tendency towards increased caries development. The severest tooth decay occurred when the diet contained 40% sucrose and was deficient in calcium. The teeth of 4 of the 5 animals in this group showed the presence of three to 8 fractured cusps per animal whereas only two animals in each of the 60% sucrose and the 20% sucrose groups showed evidence of fractured teeth.

SUMMARY

Three series of experiments have been conducted to study the effect on caries of (1) tooth maturity and dietary calcium, (2) acidic and basic minerals and (3) other nutrients in a low calcium diet when semi-purified diets were fed to cotton rats.

Decreasing the calcium level resulted in increased tooth decay in the erupted tooth. Withholding the cariogenic diets until the teeth were more mature decreased the caries susceptibility of the teeth.

Acidic inorganic, basic or acidic organic salt mixtures gave no protection against tooth decay. A high level of basic inorganic salts resulted in a marked decrease in tooth decay.

Increasing the fat content to 15% did not protect against the cariogenicity of a low mineral synthetic diet. The addition of lysine or of tryptophan was without effect upon caries production obtained by feeding a low calcium diet. The type of protein tended to modify the cariogenicity of a low calcium diet. The substitution of 66% of the sucrose by mixed carbohydrate did not decrease the caries production caused by a low mineral diet.

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SOME EFFECTS OF EXCESS MOLYBDENUM ON THE NUTRITION OF THE RAT

LOUISE F. GRAY AND LOUISE J. DANIEL

U. S. Plant, Soil and Nutrition Laboratory, Bureau of Plant Industry, Soils and
Agricultural Engineering, A.R.A., U. S. Department of Agriculture,
Ithaca, New York, and the Department of Biochemistry
and Nutrition, Cornell University, Ithaca

ONE FIGURE

An excessive or inadequate amount of one nutrient may increase or decrease the animal's need for another. High levels of molybdenum have been found to be toxic for cattle (Ferguson, Lewis and Watson, '43; Britten and Goss, '46; Comar, Davis and Singer, '48), for rats (Neilands, Strong and Elvehjem, '48; Comar, Singer and Davis, '49; Gray and Ellis, '50) and for rabbits (Arrington and Davis, '52). Molybdenum toxicity has been corrected or alleviated in all of these species by the administration of copper. Thus, molybdenum interferes in some way with the copper metabolism of the animal, its presence in the diet increasing the dietary requirement for copper. Neilands, Strong and Elvehjem ('48) also observed a correction of molybdenum toxicity in rats by the feeding of whole liver, a correction which was not attributed to the copper content of the liver. This paper describes experiments in which several dietary interrelationships with molybdenum were studied.

METHODS

Weanling rats of the Sprague-Dawley strain were used in these studies. They ranged in age from 21 to 26 days when placed on experiment. The animals were randomized into

treatments and then into battery blocks with one rat from each treatment per block. They were housed individually on wire screens in an air-conditioned animal room with food and water given ad libitum. The mineralized whole milk powder [1] basal diet and the level of molybdenum used throughout the experiments were the same as those previously reported (Gray and Ellis, '50). The mineral supplementation adopted

TABLE 1

Effect of vitamin B_{12} on growth and hemoglobin formation of rats fed a toxic level of molybdenum [1]

DIET [2]	MEAN WEIGHT CHANGE [3]	MEAN HEMOGLOBIN
	gm	*gm/100 ml*
1. Basal	152	14.49
2. Basal + B_{12}	161	14.50
3. Basal + Mo	94	14.28
4. Basal + Mo + B_{12}	125	14.41

[1] Experimental period was 6 weeks. All values are the means for 5 rats (three males and two females).

[2] The basal diet was mineralized whole milk powder. The following supplements were added as indicated above: B_{12} = cobione injected subcutaneously at 3.75 µg per week. Mo = 0.08% molybdenum fed as $Na_2MoO_4 \cdot 2H_2O$.

[3] The least difference between two means required for significance ("t" test): $1\% = 34$ gm; $5\% = 24$ gm.

was one which insured a basal diet adequate with respect to copper (6.3 µg of copper per gram of whole milk powder). The molybdenum level used (0.08%) was shown to cause a highly significant growth retardation in this strain of rats under these dietary conditions, and yet it was well below the level at which mortality occurred. A-D Percomorph Liver Oil [2] was administered to the animals once a week throughout the experimental period of 6 weeks.

Analyses of variance were made and the "t" test for significance between treatments applied to all data (Snedecor, '46).

EXPERIMENTAL AND RESULTS

Experiment 1 — Molybdenum and vitamin B_{12}

Since it had been demonstrated that whole liver prevented molybdenum toxicity in rats (Neilands, Strong and Elvehjem, '48), this experiment was planned to determine if vitamin B_{12} would influence the toxic effects of molybdenum. The treatments compared were basal, basal + B_{12}, basal + Mo, and basal + Mo + B_{12}. Vitamin B_{12} (Cobione)[3] was injected subcutaneously once a week at a level of 3.75 µg. Five animals (three males and two females) were placed on each treatment.

Weekly weights were recorded and hemoglobin determinations were made at the end of the 4th and 6th weeks of the experimental period. The 6-week results are presented in table 1. Molybdenum caused a marked retardation in growth, and vitamin B_{12} significantly reduced this toxic effect ($P < 0.05$). Vitamin B_{12} when added to the basal diet did not significantly improve growth. As observed previously (Neilands, Strong and Elvehjem, '48; Gray and Ellis, '50), molybdenum had no effect on hemoglobin formation in the rat.

Experiment 2 — Molybdenum and methionine

In view of the observations made in experiment 1 that vitamin B_{12} partially prevented the toxic effects of molybdenum, Daniel and Gray ('53) studied the effect of molybdenum on the growth of *Lactobacillus leichmannii*, a microorganism requiring vitamin B_{12}. Molybdenum was found to be toxic for this organism, a toxicity which was prevented by vitamin B_{12}, British anti-lewisite (BAL), cysteine or reduced glutathione. With these results in mind, an animal experiment was planned to study the effect of methionine, the essential sulfur-amino acid for the rat, and a precursor of cysteine in the animal body.

Six male rats were placed on each of the following treatments: basal, basal + Mo, and basal + Mo + 1.2% DL-methi-

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[3] Cobione — Saline solution of crystalline vitamin B_{12}, 15 µg per cm^3. Merck & Co., Inc., Rahway, N. J.

onine. Microbiological assay of the basal whole milk powder showed it to contain 0.7% methionine and 0.19% cysteine, thereby meeting the requirement for the rat for the sulfur-amino acids (Rose et al., '49). In addition, a group of rats received vitamin B_{12} as before, but instead of one injection of 3.75 µg, two injections of the same amount were made weekly. The effectiveness of methionine in preventing molyb-denosis in the rat ($P < 0.05$) is shown in figure 1. Methionine prevented 45% of the growth retardation caused by molyb-
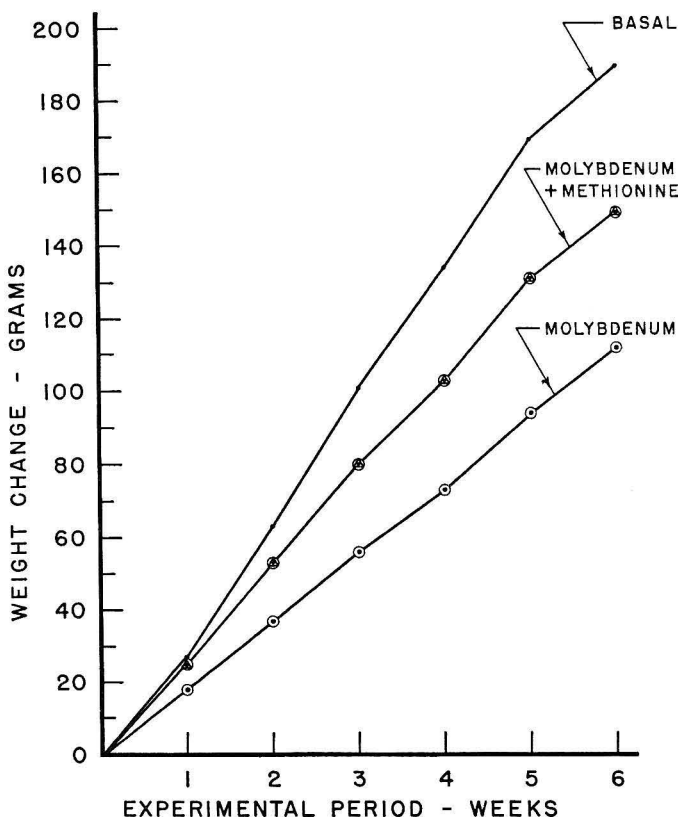
Fig. 1 Alleviation of molybdenum toxicity by methionine. Molybdenum was fed at 0.08% and DL-methionine at 1.2% of the diet. Six male rats were used per treatment. The least difference between two means required for significance ("t" test) at the end of the experimental period of 6 weeks is: 1% = 42 gm; 5% = 30 gm.

denum. In this experiment, no response was obtained with vitamin B_{12}.

Experiment 3 — Molybdenum, methionine and copper

It has now been demonstrated that both methionine (experiment 2) and copper (Neilands et al., '48) are concerned in the prevention of molybdenum toxicity in the rat. Each nutrient alone partially prevented the toxic effects. The object of this study was to verify the response to methionine

TABLE 2

Inhibition of growth by molybdenum and its correction by dietary methionine and copper

DIET [1]	MEAN WEIGHT CHANGE [2]	FEED EFFICIENCY
	gm	gm feed/gm gain
1. Basal	171	2.78
2. Basal + Mo	114	3.76
3. Basal + Mo + methionine	156	3.02
4. Basal + Mo + Cu	134	3.07
5. Basal + Mo + methionine + Cu	162	2.90

[1] The basal diet was mineralized whole milk powder with the following supplements as indicated above: Mo = 0.08% molybdenum fed as $Na_2MoO_4 \cdot 2H_2O$; methionine = 0.6% DL-methionine; Cu = 0.03% copper, fed as $CuSO_4 \cdot 5H_2O$.

[2] Experimental period was 6 weeks. All values are the means for 6 rats (three males and three females). The least difference between two means required for significance ("t" test): 1% = 24 gm; 5% = 17 gm.

and to determine if a combination of the two substances in the diet would offer greater protection from the effects of excessive molybdenum than either alone.

The results of this experiment are presented in table 2. The addition of copper to the diet protected the animal to a significant extent against molybdenum ($P < 0.05$). The beneficial effect of methionine, however, was considerably greater ($P < 0.01$). In this experiment, the mean weight change of the rats fed additional methionine was not significantly different from that of the animals on the basal diet. Those animals on the diet containing both supplementary

copper and methionine grew no better than the rats fed only the methionine supplement. Feed efficiency data are also included in table 2. These data indicate that the addition of molybdenum reduced the efficiency of the diet in every instance.

DISCUSSION

The effect of vitamin B_{12} in partially preventing the growth retardation caused by feeding excess molybdenum was observed in the first, but not the second experiment. The reason for this lack of agreement is not known, but two possible explanations are suggested. (1) There may have been a difference in the vitamin B_{12} stores of the weanling rats placed on the two experiments because of differences in dietary background of the stock females. It is known that the vitamin B_{12} stores of the young reflect the vitamin B_{12} status of the dams (Richardson, Witten and Couch, '51; Daniel, Gardiner and Ottey, '53). In the first experiment, the weanling rats were purchased from Sprague-Dawley, Inc.; in subsequent experiments, the young were offspring of our laboratory stock colony which originated from Sprague-Dawley rats. (2) The vitamin B_{12} content of the milk powder used in the two experiments may have differed. Although the milk powder was obtained from the same source, the lot used in the first experiment may have had a lower vitamin B_{12} content. In a study of the vitamin B_{12} content of milk, van Koetsveld ('53) observed that the milk from cows on pasture contained approximately twice as much vitamin B_{12} as that from the same cows fed indoors.

Methionine has been shown to alleviate cobalt toxicity in calves (Dunn, Ely and Huffman, '52), and selenium toxicity in rats (Lewis, Schultz and Gortner, '40) and in yeast (Fels and Cheldelin, '48). In the present study, it was demonstrated that methionine effectively alleviated molybdenum toxicity in the rat. Dietary copper also counteracted the growth retardation observed, but to a lesser degree than methionine. In the course of several experiments in which molybdenum toxicity in the rat has been studied, variation

in the degree of the corrective effect of both copper and methionine has been observed. However, in no instance was copper as beneficial as methionine.

The question might be raised as to whether the increase in growth when methionine was added could not have been due to an improvement of the protein quality of the basal diet. There are three pieces of evidence that refute this. (1) Microbiological assay of the whole milk powder showed it to contain 0.7% methionine and 0.19% cystine. Rose et al. ('49) reported that 0.6% methionine is adequate for maximum rat growth if the non-essential amino acids are present in abundance. In addition, one-sixth of the methionine requirement may be present as cystine (Rose, Osterling and Womack, '48). (2) Bush and Daniel [4] found that methionine added at the levels used in this experiment did not improve the basal diet; actually, in the case of the 1.2% level, growth was significantly retarded. In their study 4 male and 4 female Sprague-Dawley rats were fed the milk powder basal diet to which DL-methionine was added at levels from 0.5 to 2.5%. The mean weight change at the end of 6 weeks for the 8 rats on the basal diet was 166 gm compared with 164 gm, 138 gm and 127 gm for those receiving 0.5, 1.0 and 1.5% DL-methionine, respectively. Molybdenum and methionine must be interacting in some way in the animal body, since a significant improvement in growth of molybdenum-toxic rats resulted by the addition of a normally toxic level (1.2%) of methionine. This amount of molybdenum seems to increase the dietary requirement for methionine. (3) Similar work by the authors to be published later, in which the same basal diet was used to study the effect of methionine on zinc toxicity, showed that methionine did not counteract toxic levels of zinc, but actually caused additional growth retardation. These findings would indicate that the improvement in growth associated with dietary supplementation of a molybdenum-toxic diet with methionine resulted from an interrelationship be-

[4] Unpublished data. H. L. Bush and L. J. Daniel, Cornell University, Ithaca, N. Y.

tween molybdenum and methionine and not from an over-all improvement of the diet.

It is possible that molybdenum not only interferes with copper metabolism, but also with other metabolic systems, since methionine is more effective than copper in counteracting it. The action of methionine may be due to a direct complexing of the mineral by the amino acid, or it may function as a complexing agent after its conversion to homocysteine or cysteine, since with molybdenum toxicity in the microorganism only sulfhydryl compounds or vitamin B_{12} were effective (Daniel and Gray, '53). The demonstration of the biochemical mechanisms which are concerned in the observed relationships among methionine, copper, vitamin B_{12} and molybdenum must await further studies at the level of tissue metabolism.

SUMMARY

1. The addition of methionine to the diet markedly prevented the harmful effects of excess molybdenum.

2. The relationship between copper and molybdenum was again demonstrated, as supplementary copper prevented approximately one-third of the deleterious effect of molybdenum at the mineral levels used in these studies.

3. Supplementation of the diet with both methionine and copper was no more efficacious in counteracting the toxicity than the use of methionine only.

4. Vitamin B_{12} alleviated molybdenum toxicity in one experiment reported. This effect could not be repeated. Reasons for the differences in results obtained are suggested.

5. Possible explanations for the efficiency of methionine in counteracting the toxic effects of dietary molybdenum have been presented.

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NUTRITIONAL AVAILABILITY OF IODINE FROM SEVERAL INSOLUBLE IODINE COMPOUNDS [1]

SIDNEY MITTLER AND G. H. BENHAM

Armour Research Foundation of Illinois, Institute of Technology, Chicago

INTRODUCTION

The availability of iodine from insoluble iodine compounds is important in the selection of an iodine source to be incorporated into an animal salt block. An insoluble iodine source is desirable in that iodine would not be leeched out readily by exposure to moisture. The availability of iodine from cuprous iodide (Cu_2I_2), from diiododithymol (thymol iodide) and from 3-5-diiodosalicylic acid was compared to the availability of iodine from potassium iodide (KI), using as a criterion the prevention of the enlargement of the thyroid gland in albino rats reared on a rigorously controlled diet. Levine, Remington and von Kalnitz ('33) found that young rats develop a severe goiter within 5 weeks if fed on diets deficient in iodine. Later, Remington ('37) and Remington and Remington ('38) reported that 2 to 3 µg of iodine from KI per day prevented the enlargement of the thyroid gland in albino rats reared on a rigorously controlled diet.

AVAILABILITY OF IODINE

Four-week-old Sprague-Dawley female albino rats (35–50 gm) were reared on a low-iodine test diet (Remington, '37) which consisted of, in per cent: 18 wheat gluten, 2 Brewer's yeast powder, 78 yellow corn meal, 1 calcium carbonate and 1 sodium chloride. The control animals (group A) were reared

on this test diet. Another group of 10 animals (group B) was fed the low-iodine diet to which 265 µg of iodine (as KI) per kilogram of test diet was added. In the diet for group C, 265 µg of iodine from Cu_2I_2 was added; in D, 265 µg of iodine as diiododithymol was used; and in E 265 µg of iodine as 3-5-diiodosalicylic acid was added. Each group consisted of 10 animals housed in separate cages and fed 10 gm of food daily. At the end of 5 weeks the thyroid glands were carefully dissected and weighed wet. The results of these experiments are given in table 1. The water-insoluble iodine compounds

TABLE 1

Availability of iodine from several sources

(Female albino rats given 10 gm of food daily for 5 weeks)

GROUP	ADDITION TO IODINE-FREE DIET	IODINE SOURCE	NUMBER OF ANIMALS	AVERAGE WEIGHTS	
				Body	Thyroid gland
	µg/kg			gm	mg/100 gm of body weight
A (control)	none	none	5	111.4	15.4 ± 3.5 [1]
B	265	KI	10	105.2	10.0 ± 1.1
C	265	Cu_2I_2	10	100.1	9.8 ± 1.9
D	265	diiododithymol	10	101.6	12.5 ± 2.4
E	265	3-5-diiodosalicylic acid	10	99.0	11.9 ± 1.4

[1] Standard deviation.

used provided available iodine and protected the thyroid gland from becoming enlarged.

A second series of experiments was designed to show the influence of various amounts of Cu_2I_2 in protecting the thyroid gland of albino rats reared on Remington's iodine deficient diet. Four groups of animals were used to test the following diets during a period of 6 weeks: F, iodine-free diet (control group); G, 265 µg of iodine from Cu_2I_2/kg of iodine-free diet; H, 200 µg of iodine from Cu_2I_2/kg of iodine-free diet; and I, 150 µg of iodine from Cu_2I_2/kg of iodine-free diet. The results are presented in table 2. Cuprous iodide affords protection against enlargement of the thyroid glands. Since the rats eat about 10 gm of food per day, as little as 1.5 µg of iodine

per day from Cu_2I_2 prevents this enlargement. When the control animals were kept on the deficient diet for 6 weeks instead of 5 weeks, the average weight of the thyroid gland increased from 15.4 to 18.1 mg/100 gm of body weight.

RETENTION OF IODINE

Because the iodine was found to be available from Cu_2I_2, 3-5-diiodosalicylic acid, and thymol iodide, the question arose as to whether some of the iodides tested here underwent such a rapid clearance from the body that they were not used as efficiently as others. The intake of the iodine was reduced from

TABLE 2

Availability of iodine from Cu_2I_2

(Female albino rats allowed feed at will for 6 weeks)

GROUP	ADDITION TO IODINE-FREE DIET	NUMBER OF ANIMALS	AVERAGE WEIGHTS	
			Body	Thyroid gland
	μg/kg		*gm*	*mg/100 gm of body weight*
F (control)	none	5	138.2	18.1 ± 2.1 [1]
G	265	10	141.3	7.3 ± 1.8
H	200	10	140.1	8.5 ± 2.2
I	150	9	139.8	9.2 ± 2.4

[1] Standard deviation.

an optimum of 18.55 μg/wk. (2.65 μg/day) to 5.25 μg/wk. in order to produce a slightly goitrous condition. The 5.25 μg/wk. of iodine in the compounds to be tested was administered in two feedings (2.625 μg of iodine on Mondays and 2.625 μg on Thursdays), with 10 gm of low-iodine test diet, and the animals were allowed to eat freely as much iodine-free food as they desired for the rest of the week. Because the animals kept on the iodine-free diet for 6 weeks had larger thyroid glands than those on the diet for 5 weeks, the period of feeding on the various diets was extended to 8 weeks. The results of this series of experiments are to be found in table 3. Extending the length of the experiment from 5 to 8 weeks increased the goitrous condition of the control animals. Again

the insoluble iodine compounds tested here provided sufficient iodine to protect the thyroid gland from becoming enlarged.

DISCUSSION

Iodine is readily available from the insoluble iodine compounds, 3-5-diiodosalicylic acid, Cu_2I_2 and thymol iodide. Because of the individual fluctuations in thyroid size and in body weight, it is difficult to compare one iodine compound with another. Nevertheless, Cu_2I_2 appears to provide iodine to the thyroid gland as readily as KI, and in one series of experiments in which an amount of iodine less than optimum

TABLE 3

Retention of iodine from several iodides

(Albino rats given 5.25 µg of iodine weekly for 8 weeks)

GROUP	IODINE SOURCE	NUMBER OF ANIMALS	AVERAGE WEIGHTS	
			Body	Thyroid gland
			gm	*mg/100 gm of body weight*
J (control)	none	9	155.6	40.86 ± 5.2 [1]
K	KI	10	148.2	12.48 ± 2.47
L	Cu_2I_2	10	149.5	9.62 ± 2.06
M	diiododithymol	9	150.2	14.34 ± 2.05
N	3-5-diiodosalicylic acid	10	149.7	13.53 ± 2.6

[1] Standard deviation.

was supplied, the Cu_2I_2 appeared to afford somewhat better protection. The availability of iodine from thymol iodide agrees with the work of Baldwin, Thiessen and McInroy ('47) who found that 25 to 50% of the radioactive tagged iodine in diiododithymol was concentrated in the thyroid gland.

The weight of the thyroid gland appears to be a sensitive indicator of iodine deficiency, and it can be used as a means of evaluating iodine availability. This change in weight is indirect evidence of the utilization of iodine. The data presented agree with the work of Halverson, Shaw and Hart ('45) and of Levine, Remington and von Kalnitz ('33) who have reported that 2 µg daily of iodine from a readily available

source protects the thyroid gland of a laboratory rat from becoming enlarged. Solubility in water does not play an important role inasmuch as relatively insoluble Cu_2I_2 (0.008 gm in 100 ml of water at 18°C.) protects the thyroid gland as well as soluble KI (127.5 gm in 100 ml at 0°C.). It is also probable that some of the insoluble iodides may be absorbed slowly and thus make available a more continuous supply of iodine to the thyroid gland.

TOXICITY STUDIES

Acute and chronic toxicity studies were made of Cu_2I_2 and 3-5-diiodosalicylic acid. In acute toxicity tests with Cu_2I_2, an oral dose of 2,000 mg/kg of body weight fed to 5 laboratory rats produced diarrhea but did not kill them; with an oral dose of 500 mg of Cu_2I_2/kg 5 other animals suffered no ill effects. A dose of 125 mg of cuprous iodide fed to a 250-gm rat is 30,000 times the daily requirement of iodine to maintain normal thyroid glands and accordingly such a rat would need the low level of 0.0000039 gm/per day.

Rats were fed 1,000 mg of 3-5-diiodosalicylic acid per kilogram of body weight, mixed with meat. Watery stools were the only symptoms, and the rats recovered without any ill effects. This is about 60,000 times the daily requirement of iodine by rats. Five hundred milligrams of 3-5-diiodosalicylic per kilogram produced no apparent symptoms. Autopsies on animals which were fed 0.1 and 1 gm of Cu_2I_2/kg of food and 0.1 and 1 gm of 3-5-diiodosalicylic acid/kg of food for 5 months showed no liver, kidney or intestinal abnormalities. The animals gained weight normally and were in excellent health at the conclusion of the tests. In the amounts necessary to supply iodine needed to protect thyroid glands, Cu_2I_2 and 3-5-diiodosalicylic acid can be considered non-toxic.

SUMMARY

Iodine was found to be nutritionally available from insoluble cuprous iodide, diiododithymol, and 3-5-diiodosalicylic

acid. Acute and chronic toxicity tests indicated that Cu_2I_2 and 3-5-diiodosalicylic acid were non-toxic in amounts required to protect the thyroid gland.

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PHENYLALANINE AND TYROSINE UTILIZATION IN NORMAL AND PHENYLALANINE-DEFICIENT YOUNG MICE [1]

C. R. GRAU [2] AND ROBERT STEELE

Department of Biology, Brookhaven National Laboratory, Upton, New York

It has been known for a number of years that the conversion of phenylalanine to tyrosine proceeds even when high levels of tyrosine are fed along with phenylalanine (Moss and Schoenheimer, '40). Recently it has been shown that a potent liver enzyme system catalyzes the oxidation of phenylalanine to tyrosine (Udenfriend and Cooper, '52). The present study was undertaken to determine whether the oxidation of phenylalanine to tyrosine and thence to carbon dioxide proceeds to the same extent when an animal is fed a diet containing insufficient phenylalanine to allow normal growth as when an adequate level of phenylalanine is given.

Uniformly labeled C^{14} L-phenylalanine was fed in a test meal of the same composition as either the normal or the phenylalanine-deficient diet. After 30 minutes, the amount of the absorbed phenylalanine incorporated in liver protein as phenylalanine and as tyrosine, and the amount of labeled phenylalanine carbon expired as CO_2 were determined. Tyrosine was isolated directly from liver protein hydrolysate; phenylalanine in the hydrolysate was decarboxylated and isolated as phenylethylamine hydrochloride.

Uniformly labeled C^{14}-L-tyrosine was administered to a smaller number of mice for purposes of comparison.

METHODS

Young male mice of the C3H strain[3] were divided into groups and fed diets based on diet 39 of Maddy and Elvehjem ('49), in which the amino acids were supplied in crystalline form. Both diets contained 0.7% L-tyrosine. The phenylalanine-deficient diet contained 0.1% L-phenylalanine[4]; the normal diet contained 0.7% L-phenylalanine. All tracer experiments were begun at about 1:30 P.M. Although food and water were available at all times, a reduction in food intake was observed during the daytime, with a fluctuation in body weight of approximately 0.5 gm between 9 A.M. and 4:30 P.M. The animals were housed in a room maintained at 21°C.

A basal diet complete except for tyrosine and phenylalanine was used to prepare the tracer meal. A typical protocol was as follows (mouse 3): to 2.00 gm of basal diet were added 14 mg L-tyrosine, 6 mg L-phenylalanine and 1 ml water. The mixture was homogenized in a Potter-Elvehjem apparatus, and 75 mg of the aqueous mixture were weighed into a small glass cup. To this was added 16.2 µl of a radioactive phenylalanine solution containing 0.15 mg L-phenylalanine and 1.81 µc of C^{14}.[5] The tracer meal thus contained 50 mg dry diet, 0.7% L-tyrosine and 0.6% L-phenylalanine, as well as the normal content of all other nutrients.[6] To mix these ingredi-

[3] Obtained from Jackson Memorial Laboratory, Bar Harbor, Maine.

[4] Nutritional Biochemicals, Inc., lot 7372.

[5] The uniformly labeled C^{14} L-phenylalanine and C^{14} L-tyrosine were isolated from a hydrolysate of proteins of *Thiobacillus thiooxidans* after growth of this organism in $C^{14}O_2$, following the procedure of Frantz et al. ('52). Purities were estimated from radioautograph densities after 1-dimensional paper chromatography in phenol-water and aqueous butanol-propionic acid systems. The C^{14} L-phenylalanine contained about 2% of its radioactivity in an impurity which was shown not to be tyrosine. Comparison of the C^{14} L-phenylalanine and a sample of phenylethylamine obtained from it showed that the specific activity (mµc/mgC) was unaffected by decarboxylation.

[6] When C^{14} L-tyrosine was used as the tracer, the C^{12} L-tyrosine to be added to the diet was added in solution in N HCl, the mixture was homogenized, an aliquot was weighed into the cup, the C^{14} L-tyrosine (in N HCl) was added, and after thorough mixing the appropriate amount of N NaOH was added to neutralize the HCl.

ents, they were alternately sucked into and expelled from a special feeding syringe made from a piece of 3-mm glass tubing 10 cm long into which was inserted a closely fitted piece of glass rod covered with stopcock grease. In feeding the tracer meal, the mouse, while held on its back, was allowed to lick the mixture from the end of the syringe as the plunger was pushed in. The feeding process took one to two minutes. The animal was immediately placed in a metabolism cage (Roth et al., '48), and carbon dioxide samples collected for 4-minute periods at intervals during the run were precipitated as barium carbonate and counted in a methane flow counter (Van Slyke et al., '51). Specific activity was expressed as millimicrocuries per milligram of carbon, and from this was calculated the fraction of the absorbed C^{14} present per milligram respiratory carbon. At all other times respiratory CO_2 was collected in a 10% NaOH bubbler; thus total C^{14} excretion was determined over the course of the experiment.

The gastrointestinal tract from esophagus to rectum was removed from each carcass and slit open. The tissue was washed once with water and three times with a solution of carrier pheynlalanine or tyrosine in 5% trichloroacetic acid. The combined washings were filtered, extracted once with ether to remove part of the trichloroacetic acid, made to volume, and the total amount of C^{14} present was determined by combustion of an aliquot followed by gas-phase counting of the CO_2 (Van Slyke et al., '51).

Similarly the amount of C^{14} consumed by each animal was determined by difference by measuring the C^{14} content of an aliquot of a solution of the uneaten portion of the diet.

Except for mouse 19, which was kept for 90 minutes after feeding, each animal was kept for 30 minutes. At the end of the run, the animal was killed by a blow on the head, the liver was removed, weighed, homogenized at 0°C. with 5 ml of water, and 15 ml of 10% trichloroacetic acid were added. About 5 minutes elapsed between the death of the animal and the precipitation of the liver proteins.

The liver proteins were extracted in succession with trichloroacetic acid, with an ethanol-ether mixture, with hot trichloroacetic acid, with ethanol-ether, and were then dried (Schneider, '45). The dried protein was hydrolyzed by refluxing with 3 ml of 6.6 N HCl in an oil bath at 125°C. for 12 hours. To remove most of the HCl as NaCl, 3 ml of 5 N NaOH, 5 ml of absolute ethanol and 80 ml of acetone were added, the mixture was filtered, and the precipitate was washed with acetone. The filtrate was concentrated almost to dryness under vacuum, and the residue was transferred to a 12-ml centrifuge tube with the aid of several washings with live steam. NaOH (1 N) was added to pH 5 and the tube was heated in a boiling water bath for a few minutes, cooled, centrifuged to remove some humin, and the supernatant solution was decolorized with 15 mg of water-washed charcoal,[7] filtered with suction through Whatman No. 50 paper into a centrifuge tube, and concentrated to about 1 ml by heating the tube in a boiling water bath while directing a stream of nitrogen at the surface. After keeping the concentrate several hours in the refrigerator, crystals of crude tyrosine were centrifuged out and dissolved in about 1.5 ml of hot water. The resulting solution was centrifuged and the supernatant fluid was concentrated as before to about 1 ml, and cooled to crystalize out the tyrosine. The tyrosine was recrystalized once more in the same manner, dried, weighed and dissolved in 2 ml of N HCl.

The supernatant solutions from the first two tyrosine precipitations were treated with a phenylalanine-decarboxylase preparation as follows: To 3 ml of amino acid solution were added 3 ml of citrate buffer (0.2 M, pH 5.5) and 100 mg of an acetone powder of *Streptococcus fecalis.*[8] After incubating the mixture with stirring at 35°C. for 8 hours, 1 ml of 6 N

[7] Nuchar.

[8] This method of isolating phenylethylamine hydrochloride was suggested by Dr. S. Udenfriend, who also kindly supplied the culture of *S. fecalis.* The culture was grown and the acetone powder prepared after the manner of McGilvery and Cohen ('48).

HCl was added, the tube was heated gently, and the precipitated proteins were centrifuged. The supernatant solution was transferred to a separatory funnel, 1 ml of 10 N NaOH was added, and the liquid was extracted with 80 ml of ethyl ether. The water phase was transferred to another funnel and extracted again with 20 ml of ether. The ether extracts were combined and extracted three times with 100 ml portions of 0.1 N NaOH, followed by one extraction with water. Three successive extractions with 3 ml each of 0.1 N HCl converted the phenylethylamine formed by decarboxylation to the hydrochloride and extracted it from the ether layer. The combined acid extracts were evaporated to dryness on a steam bath, the residue was dissolved in 1 ml of absolute ethanol, and 40 ml of ethyl ether were added. The phenylethylamine hydrochloride precipitate was separated by centrifugation, dried, weighed and dissolved in 2 ml of water.

Aliquots of the isolated tyrosine or phenylethylamine hydrochloride were subjected to combustion and the C^{14} was counted as CO_2 in the gas phase. Specific activity of isolated phenylalanine or tyrosine was calculated from the total C^{14} of an aliquot and from the weight of amino acid contained in the aliquot. These specific activities were converted to total C^{14} content of liver protein phenylalanine or liver protein tyrosine by means of the weight of liver protein isolated plus the following figures: liver protein phenylalanine taken as 6% of liver protein, liver protein tyrosine as 4% of liver protein (amino acid figures based on figures for rats [Block and Bolling, '51]). In the case of mouse 19, where the isolated liver protein was not weighed, liver weight was assumed to be 5.7% of body weight and liver protein as 17.9% of liver weight, the average figures for our other animals.

RESULTS

The mice represented by the 6 lower growth curves of figure 1 were placed on the experimental diets at 4:30 P.M. when they weighed the amounts shown at zero days. On subsequent days all weights were taken at 9:00 A.M. Mice 2, 11 and 13

were from the same initial group but had been kept on the
pelleted stock diet for 12 days, only the last 6 days of which
are plotted here. All of these weights were taken at 9 : 00 a.m.
The last day plotted for each curve is the day on which the
C[14] feeding experiment was performed. Mouse 19, which was
from a different group of mice, was used for the 90-minute
experiment (see table 1).
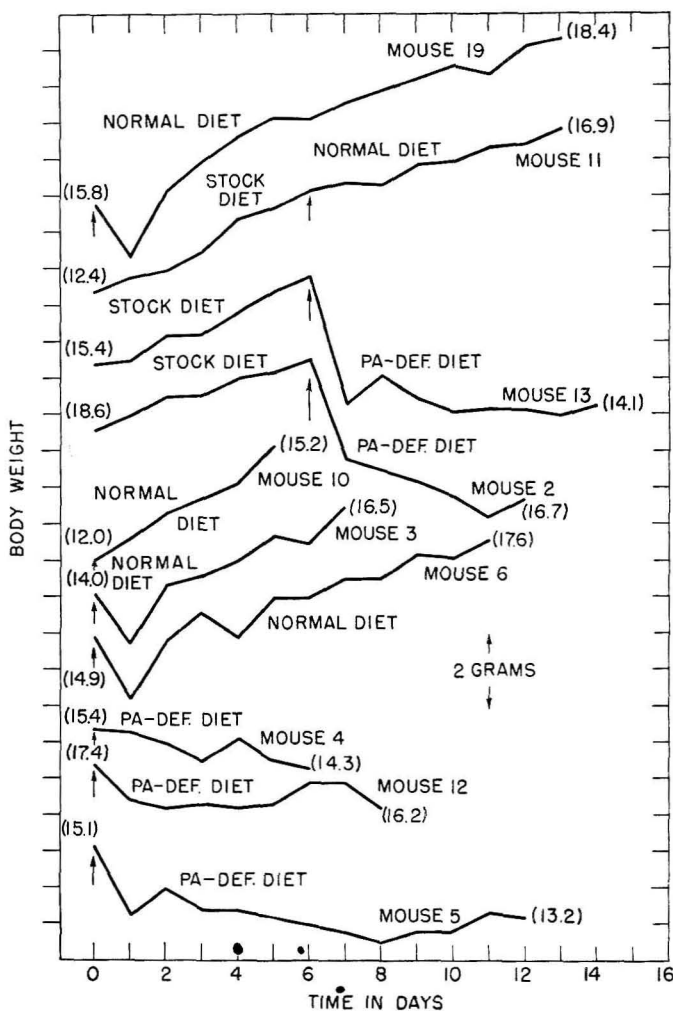
Fig. 1 Growth curves of mice on normal and phenylalanine-deficient diets.

PHENYLALANINE AND TYROSINE UTILIZATION

TABLE 1

The fate of uniformly labeled C^{14} L-phenylalanine and C^{14} L-tyrosine fed to normal and phenylalanine-deficient mice. Except for mouse 19, which was killed 90 minutes after feeding the tracer meal, all animals were kept 30 minutes

MOUSE NUMBER	DIET	TAGGED AMINO ACID INGESTED		TAGGED AMINO ACID ABSORBED		FRACTION OF ABSORBED C^{14} IN LIVER PROTEIN AMINO ACID			
		Identity	C^{14} Content	C^{14} Content	Weight	Phenyl-alanine	Tyrosine	Pa/Ty	In CO_2
			μc	μc	mg	%	%		%
19	Normal	Phenylalanine	2.63	2.54	0.48	10.5	2.1	5.0	[1]
10	Normal	Phenylalanine	1.22	0.90	0.15	10.2	1.7	6.0	2.0
3	Normal	Phenylalanine	1.43	1.19	0.20	10.5	1.9	5.5	1.5
4	Deficient	Phenylalanine	0.52	0.29	0.024	9.7	0.28	35	0.34
12	Deficient	Phenylalanine	0.71	0.39	0.032	18.9	0.91	21	0.75
6	Normal	Tyrosine	0.71	0.62	0.31	0	5.1	0	9.1
5	Deficient	Tyrosine	0.97	0.47	0.24	0	5.1	0	2.7

[1] Sample lost.

The effect on growth of feeding a diet containing only 0.1% L-phenylalanine is clearly shown in figure 1. The animals fed 0.7% L-phenylalanine grew at about the same rate as the mice on the stock diet.
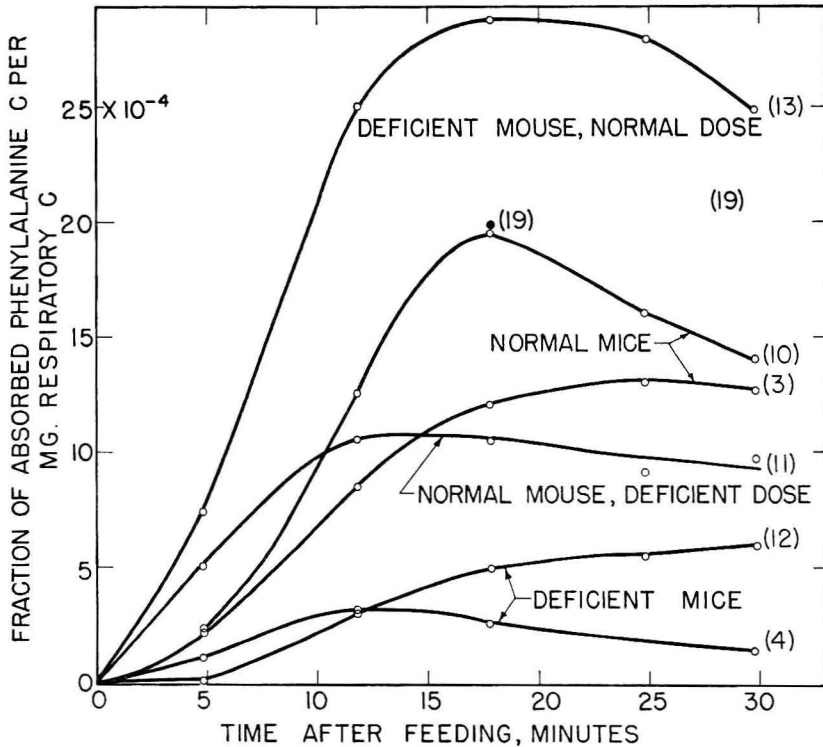
Fig. 2 The rate of excretion of absorbed phenylalanine carbon as respiratory CO_2.

The quantitative requirements of the growing mouse for phenylalanine and tyrosine (or additional phenylalanine) have not been closely established. The levels chosen for the present experiments were based upon data for the rat (Womack and Rose, '34) and the chick (Grau, '47). The lower level used here (0.1% L-phenylalanine — 0.7% L-tyrosine) is seen to have been sufficient to sustain nearly constant body weight after an initial weight loss lasting one or two days.

The higher level used was 0.7% L-phenylalanine — 0.7% L-tyrosine for the period covered by the growth curves, but the C^{14} L-phenylalanine doses were fed in meals containing 0.6% L-phenylalanine — 0.7% L-tyrosine (except for mouse 19 where the level was 0.7% L-phenylalanine — 0.7% L-tyrosine).

Figure 2 and the last column of table 1 show the extent to which the carbon of the absorbed, uniformly labeled C^{14} L-phenylalanine appeared in the respiratory CO_2. The mice fed the higher level of phenylalanine (3 and 10) excreted a much larger fraction of the absorbed dose than did the phenylalanine-deficient mice (4 and 12). Mouse 13, which was fed the C^{14} L-phenylalanine dose at the higher phenylalanine level after having been kept 8 days on the phenylalanine-deficient diet, suffered no impairment in ability to oxidize phenylalanine. In contrast, mouse 11, which was fed C^{14} L-phenylalanine in a meal deficient in phenylalanine after being kept on a diet having a high level of phenylalanine, converted phenylalanine carbon rapidly to CO_2 only during the first 10 minutes, after which time it responded in a manner similar to mice fed the phenylalanine-deficient tracer meal after having been kept on the phenylalanine-deficient diet.

Only two points in figure 2 were obtained for mouse 19, the animal fed the diet containing 0.7% L-phenylalanine and 0.7% L-tyrosine both prior to and during the experiment. Points subsequent to 30 minutes show a gradual decline in the curve up to the end of the experiment at 90 minutes.

Table 1 shows the fraction of the absorbed dose of C^{14} L-phenylalanine incorporated in the liver protein as phenylalanine and as tyrosine when high and low levels of phenylalanine were fed. Inasmuch as ingested C^{14} sucrose has been shown to contribute no C^{14} to either tyrosine or phenylalanine in the proteins of the young adult mouse (Steele, '52), it is clear that the C^{14} found in protein tyrosine and phenylalanine in the present experiments represents the incorporation of the intact phenylalanine-tyrosine carbon skeleton. The figures for incorporation into protein are consistent with

the figures for conversion of phenylalanine carbon to respiratory CO_2 in that less C^{14} tyrosine was incorporated (relative to the amount of C^{14} phenylalanine incorporated) when less C^{14} phenylalanine carbon was oxidized to CO_2. The major pathway of phenylalanine oxidation is through tyrosine (Udenfriend and Cooper, '52).

The incorporation of C^{14} L-tyrosine into protein in mouse 6 fed a diet adequate for normal growth might be expected to be higher than that for mouse 5 where no net synthesis of protein (no growth) was observed over a period of several days. However, a rough calculation [9] demonstrates that for the 30-minute period of the experiment at least 5 times as much tyrosine was incorporated in the liver protein as could be accounted for by the net increment in protein tyrosine, based on the observed growth rate of this mouse. Assuming that phenylalanine deficiency does not affect protein tyrosine turnover rate and ignoring the fact that more tyrosine is added to the body pool by conversion from phenylalanine in the normal animal (table 1) than in the phenylalanine-deficient animal, one would expect that C^{14} tyrosine incorporation in the phenylalanine-deficient animal would be less than 20% different from what it is in the normal animal. The data for the two animals (mice 5 and 6, table 1) show that there was, in fact, no difference in C^{14} tyrosine incorporation which could not be ascribed to the expected variation in response from animal to animal.

Table 2 shows the actual counting rates observed and the number of milligrams of amino acid (or derivative) carbon

[9] Mouse 6 was growing about 2% per day, or 0.04% per half-hour. Assuming that the total weight of liver protein tyrosine was increasing at the same rate, the 7 mg of total liver protein tyrosine of this mouse should have been increasing at the rate of 0.003 mg per half-hour. The amount of C^{14} tyrosine found incorporated per half-hour (see table 1) was 0.017 mg (5.5% of 0.31 mg). This is obviously a minimum figure for total tyrosine incorporation since it does not include (1) the tyrosine derived from protein degradation and reincorporated as C^{12} tyrosine or (2) the tyrosine derived from ingested C^{12} phenylalanine and incorporated into protein as C^{12} tyrosine. Thus at least 0.017/0.003 times or 5 times as much tyrosine was being incorporated into protein as could be accounted for on the basis of body growth.

for aliquots of the phenylethylamine hydrochloride and tyrosine isolated from the liver proteins of mice fed C^{14} L-tyrosine. The weight of carbon in the aliquot was calculated from the amount of isolated material weighed out for dilution to volume; carrier C^{12} compound was added prior to combustion to bring the total amount of carbon to 2 to 3 mg. In neither the normally-fed nor the phenylalanine-deficient animal was there any conversion of tyrosine to phenylalanine. This confirms the nutritional data on the irreversibility of the conversion of phenylalanine to tyrosine (Womack and Rose,

TABLE 2

Gas-phase counting rates actually observed for the carbon of isolated phenylethylamine hydrochloride and tyrosine from liver proteins of mice fed C^{14} L-tyrosine

MOUSE NUMBER	DIET	ACTIVITY AND CARBON IN AN ALIQUOT					
		Phenylethylamine · HCl			Tyrosine		
		cpm [1]	*mgC*	*mμc/mgC*	*cpm* [1]	*mgC*	*mμc/mgC*
6	Normal	− 2	0.067	0.00	2855	0.189	8.04
		1	0.067		2916	0.189	
5	Deficient	5	0.092	0.00	1576	0.107	7.77
		3	0.002		1585	0.107	

[1] Corrected for background counting rate.

'34; Bauer and Berg, '43; Grau, '47). The absence of C^{14} from the isolated phenylethylamine in these mice also demonstrated the purity of the phenylethylamine obtained, particularly with regard to freedom from contamination with labeled tyramine originating from the labeled tyrosine of the protein hydrolysate.

DISCUSSION

The data presented here show that when these young animals ingested a diet deficient in phenylalanine, less of the absorbed phenylalanine was converted to tyrosine and thence to carbon dioxide than when a normal diet was ingested. This effect can be explained without recourse to any special mechanism operating during periods of phenylalanine deficiency.

When adequate amounts of all amino acids except phenylalanine were present, the amount of phenylalanine available for conversion to tyrosine and thence to carbon dioxide was limited by the use of phenylalanine for protein synthesis. On the other hand when the phenylalanine content of the diet was increased to a level slightly higher than that needed for normal growth the rate of protein synthesis was not increased in proportion to the amount of phenylalanine present in the diet; under these conditions a larger fraction of the phenylalanine fed was left for degradation to tyrosine and thence to carbon dioxide.

It seems possible that the slope of the curve relating phenylalanine degradation to phenylalanine content of the diet may change more rapidly at the point at which the quantitative dietary requirement for phenylalanine is satisfied; if so this method may allow determination of the quantitative dietary requirements for phenylalanine or other essential amino acids in growing or adult animals.

An experiment was reported by Mackenzie et al. ('50), using methyl-labeled methionine, which is similar to the present work, except that both the diets used by these workers contained sufficient methionine to allow normal tissue synthesis.

SUMMARY

Young mice were maintained on normal or phenylalanine-deficient diets in which tyrosine and other amino acids were adequately supplied. Meals of similar composition but containing uniformly labeled C^{14} L-phenylalanine or C^{14} L-tyrosine were fed, expired $C^{14}O_2$ was collected and measured for 30 minutes, and the animals were then killed. Tyrosine and phenylalanine (as phenylethylamine hydrochloride) were isolated from the liver proteins and their C^{14} contents were determined.

Mice fed the phenylalanine-deficient diet converted less of the absorbed phenylalanine to liver protein tyrosine and less to CO_2 than did the animals fed the diet containing enough phenylalanine for normal growth.

Absorbed C^{14} L-tyrosine was not converted to liver protein phenylalanine by either the normal or phenylalanine-deficient mouse.

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STORAGE OF DIETARY MANGANESE AND THIAMINE IN THE RAT [1]

ROBERT M. HILL AND DORSEY E. HOLTKAMP [2]

Department of Biochemistry, University of Colorado Medical Center, Denver

The finding (Hill and Holtkamp, '48), that the manganous ion is a nutritional factor which if fed at different levels in the diet of lactating female rats may modify the age of development of body-temperature control in the young, led to further work on the effect of this ion on growth (Holtkamp and Hill, '50). In the course of this work it was found that after the first generation in a colony on dietary levels of manganese of not more than 30 µg per day, some rats developed ataxia (Hill, Holtkamp, Buchanan and Rutledge, '50). The investigation of body-temperature control (Hill and Holtkamp, '48) showed thiamine in low concentrations to be antagonistic to low concentrations of manganese. Similar antagonism, with respect to the toxicities of manganese and thiamine had been reported earlier by Perla and associates (Perla, '39; Perla and Sandberg, '39; Perla, Sandberg and Holly, '39; Sandberg, Perla and Holly, '39). Because of the apparent relationship between these two substances, it was considered advisable to determine the effect of various dietary levels of thiamine and manganese on the storage of these two substances in the liver. In our search of the literature we have not found analyses for manganese in the wall of the intestine of the rat except in a single one-dose experiment using only two rats (Greenberg and

Campbell, '40). Available data for other animals are few (Richards, '30). For these reasons and because Smith ('49) has shown the importance of manganese as an activator of intestinal peptidases, assay values of the manganese concentration in the wall of the small intestine are presented. We have also included a few assays for manganese in the wall of the cecum and the colon.

<div align="center">EXPERIMENTAL</div>

The rats used were of Wistar strain from our colony. Diet groups 9 and 10 (13 weeks old at time of sacrifice) received stock diet [3] and tap water ad libitum. The thiamine supplement (8 mg/rat/day) for group 10 was given in the drinking water. For the other groups (36 weeks old at time of sacrifice) the basal diet used was like that described previously (Holtkamp and Hill, '50) except that it did not contain the added thiamine. This basal diet provided about 30 μg manganese per adult rat per day. The average daily intake of an adult rat was about 100 ml of reconstituted milk, made up from 12.2 gm powdered whole milk supplemented with 1.0 ml corn oil, 15 U.S.P. units vitamin D_2, 5 mg iron, 0.1 mg copper, 0.1 mg niacin, 0.005 mg folic acid, 18 mg p-aminobenzoic acid, and sufficient distilled water to make 100 ml. Additional thiamine and manganese supplements were added to this basal diet to bring the daily intake levels to those given in table 1. The diet groups 2, 4, 6 and 8, received the manganese and thiamine in the weight ratio of 5 to 1, which Perla and Sandberg ('39) reported to be the optimum. The rats of diet groups 1 through 8 received the respective diets for more than 24 weeks whereas the rats of groups 9 and 10 received the diet for 4 weeks.

The most carefully prepared low manganese diets contain significant amounts of manganese as compared with the tissues of the experimental animals. For this reason most investigators, in order to avoid contamination from the residual intestinal contents, have removed the intestine immediately after sacrificing the animal and have not included the intestine in the

[3] Purina Fox Checkers.

analyses. In order to minimize contamination from this source, we put the rats of all diet groups (except groups 9 and 10) on the lowest manganese intake, 30 µg per day (table 1), 24 hours prior to the time of sacrifice, in order to flush unabsorbed dietary manganese from the lumen of the gastro-intestinal tract. The time on the low manganese diet, 24 hours, should be more than adequate as shown by studies with carmine marked food (Holtkamp, Whitehead and Hill, '51). Thus all analyses for manganese in the intestinal wall (except for groups 9 and 10) start from comparable base lines of possible contamination from intestinal contents.

The rats were killed by decapitation. The entire intestine was immediately removed and cut into segments 15 to 20 cm long. An open end of each segment was fitted over the nipple of a 20 ml syringe and the contents forced out with distilled water. After flushing with about 50 ml of water, each segment was opened longitudinally by a full length incision. Both surfaces (inside and out) were then washed thoroughly with a fine, steady stream of distilled water. After washing, the small intestine, cecum and colon were dried separately at 105°C. and ashed at 675°C. Weighed portions of the liver were dried and ashed in the same way.

Manganese was determined by the method of Ray ('40) with the modification that, as in earlier methods, periodate instead of persulfate was used to oxidize manganese to permanganate.

Thiamine was determined in homogenates of liver tissue by the manometric method of Atkin, Schultz and Frey ('39) except that the manganese sulfate concentration of the medium was doubled, and that thiamine standard solutions were used as described by Josephson and Harris ('42). In order to determine both free thiamine and cocarboxylase, the method of Kurachi ('48) (originally used for blood) was followed. In this method 1 ml of a solution containing 20 mg papain, 1 drop glycerine, and 20 mg takadiastase is added to a flask containing a measured amount of liver homogenate and 40 ml of a 0.5% acetate buffer at pH 4.5. The solution is covered with a layer of benzene, and incubated for 24 hours at 37°C. After

centrifugation the clear supernatant fluid is used for analysis. Inasmuch as our work failed to show an effect of dietary manganese on cocarboxylase content, the results have been expressed on the basis of total thiamine content.

RESULTS AND DISCUSSION

As shown in table 1, the average thiamine contents of the livers of groups 1, 2, 3, 5 and 7 (rats 36 weeks old, receiving 30 µg of dietary thiamine per day) ranged from 5.58 to 7.20 µg per gram. This value agrees well with the "maximum" values for liver thiamine of 7.6 µg reported by Williams ('43) and of 2.6 I.U. (7.8, 5.7–9.6 µg) reported by Leong ('37). Leong found maximum liver storage at daily intakes of 90 µg and reported that raising the intake to 1,500 µg did not further increase the storage of the vitamin. In Leong's experiments assays were made on "adult" rats by the "bradycardia" method. From our work it would appear that younger rats may attain considerably higher levels of liver thiamine even on low dietary thiamine levels. Thus our group 9 (table 1), 13 weeks of age, with a dietary level of only 50 µg per day showed liver storage values for thiamine of 9.66–13.60 µg per gram; and when a dietary supplement of 8,000 µg per day was given (group 10) the assay values at 13 weeks were 14.50–17.36 µg per gram. It is also of interest that when both managnese and thiamine supplements are given to older rats (groups 4, 6 and 8), liver thiamine values are reached that are considerably higher than the "maximum" values reported by Williams ('43) and by Leong ('37).

The metabolic interrelationship of manganese and thiamine as first reported by Perla, Sandberg and Holly ('39) is also apparent in the data of table 1. Supplementation of the basal diet to an intake of 0.17 mg of manganese per day (group 2) increased the average thiamine content of the liver from 5.58 to 6.78 µg per gram (P = 0.05 [Freeman, '42]), or 21%. Further increase of the manganese intake without additional thiamine did not affect the thiamine storage (groups 3 and 5) until the high level of 40 mg per day was reached (group 7). Here

TABLE 1

Manganese and thiamine content of rat liver as influenced by their level of dietary intake

GROUP NO.	NO. OF RATS	DAILY INTAKE		μG B₁/GM LIVER WET WEIGHT		μG Mn/GM LIVER WET WEIGHT [1]		No. of rats	μG Mn/GM LIVER DRY WEIGHT	
		Mn	B₁	Range	Average	Range	Average		Range	Average
		mg	mg							
1	10	0.03	0.03	3.9– 7.7	5.58	0.5–1.7	1.03	3	2.4– 5.8	4.17
2	11	0.17	0.03	4.6– 9.5	6.78 [2]	0.9–3.3	1.89 [3]	4	4.1– 9.8	6.36
3	11	1.0	0.03	5.0– 9.5	6.29	0.3–4.1	1.97 [2,6]	6	2.4– 9.1	6.66
4	9	1.0	0.2	7.0–16.8	10.23 [3]	0.6–4.5	2.72 [3]	4	4.1–16.7	10.0
5	11	10.0	0.03	4.4– 8.3	6.00	0.8–3.5	2.58 [3]	5	6.7–10.6	8.7 [8]
6	9	10.0	2.0	10.5–16.6	13.18 [3]	0.6–5.1	2.97 [3]	4	1.8–13.2	6.9
7	11	40.0	0.03	4.3– 8.9	7.20 [2]	1.7–5.0	3.28 [3]	6	8.5–16.9	11.9 [3]
8	9	40.0	8.0	11.1–16.8	14.10 [3]	1.0–5.8	3.52 [3]	6	8.2–13.4	10.2 [3]
9	7	1.1	0.05	9.7–13.6	12.00 [4]	1.3–2.7	2.31	4	4.1– 8.6	6.7
10	7	1.1	8.0	14.5–17.4	15.77 [5]	2.3–3.2	2.60 [7]	4	8.4–10.6	9.3 [8]

[1] Values per total liver show essentially the same relationships and statistical significance between diet groups.
[2] Comparison with group 1 gives P = < 0.05 by "t" test (Freeman, '42).
[3] Comparison with group 1 gives P = < 0.01.
[4] Comparison with group 3 gives P = < 0.01.
[5] Comparison with group 9 gives P = < 0.01.
[6] Comparison with group 4 gives 0.2 > P > 0.1.
[7] Comparison with group 9 gives 0.3 > P > 0.2.
[8] Comparison with group 9 gives P = 0.05.

the average liver storage, 7.2 µg per gram, is 29% higher
(0.05 > P > 0.02) than in group 1 where the manganese intake
is 0.03 mg per day.

Assay values for the manganese content of rat livers (table
1) when calculated on the basis of dry weight, are in agree-
ment with those reported by Skinner, Peterson and Steenbock
('31). Perla, Sandberg and Holly ('39) have shown by excre-
tion studies that, in the early phase of an experiment, the
dietary level of thiamine is a factor affecting the retention of
dietary manganese, although continued administration of thia-
mine does not cause further manganese retention. A compari-
son of groups 3 and 4 (table 1), when using the assay values
based on wet weight, shows that group 4, with the thiamine
supplemented to an intake of 0.2 mg, has an average concentra-
tion of 2.72 µg of manganese per gram of liver, and group 3,
without the supplement, an average concentration of 1.97 µg
per gram. This is an increased storage of 0.75 µg (38%) (0.2 >
P > 0.1) under the influence of thiamine administration. Assay
values based on dry weight confirm the significance of this
difference. A similar comparison of groups 9 and 10 (table 1),
using the dry weight values, shows a 30% increase (P = 0.05)
in the storage of manganese when a supplement of thiamine is
given. The difference based on wet weight values shows less
significance. These values suggest that, at low values of man-
ganese intake (1 mg per rat per day or less), thiamine ingestion
can increase the storage of dietary manganese. At higher
levels of manganese intake our data fail to give evidence of an
influence of thiamine on manganese storage.

There is no evidence of a maximum storage of manganese in
the liver, although the increases in storage are by no means
proportional to the increases in dietary intake. The data on
the effect of the level of manganese intake on the storage of
manganese in the wall of the small intestine are summarized
in table 2. There is no statistically significant evidence in
either age group or at any level of dietary manganese that the
level of thiamine intake has any influence on the storage of

TABLE 2

Effect of dietary manganese and thiamine on manganese content of rat small intestine

GROUP NO.	NO. OF RATS	DAILY INTAKE Mn	DAILY INTAKE B_1	µG Mn/GM SMALL INTESTINE WET WEIGHT Range	µG Mn/GM SMALL INTESTINE WET WEIGHT Average	µG Mn/GM SMALL INTESTINE DRY WEIGHT Range	µG Mn/GM SMALL INTESTINE DRY WEIGHT Average
		mg	*mg*				
1	7	0.03	0.03	< 0.2–1.0	0.34	< 1.0– 6.5	1.94
2	8	0.17	0.03	< 0.2–1.2	0.56	< 1.0– 8.4	3.46
3	7	1.0	0.03	0.5–1.2	0.74 [1]	2.4– 6.1	4.21 [1]
4	7	1.0	0.2	< 0.2–1.4	0.73	< 1.0– 9.9	4.78
5	7	10.0	0.03	0.6–2.0	1.24 [2]	4.3–13.3	7.92 [2]
6	6	10.0	2.0	0.9–1.9	1.40 [2]	5.3–12.8	8.66 [2]
7	7	40.0	0.03	1.1–3.8	2.42 [2]	6.9–30.3	16.05 [2]
8	6	40.0	8.0	1.2–3.8	2.32 [2]	7.6–18.7	13.65 [2]
9	7	1.1	0.05	0.8–1.4	1.07	5.2–11.4	7.33
10	7	1.1	8.0	0.7–1.4	0.97	5.4–11.4	6.84

[1] Comparison with group 1 gives P = < 0.05 by "*t*" test (Freeman, '42).
[2] Comparison with group 1 gives P = < 0.01.

TABLE 3

Manganese contents of rat ceca and colons at different levels of dietary manganese and thiamine

GROUP NO.	DAILY INTAKE Mn	DAILY INTAKE B_1	TOTAL WT. OF 2 CECA Wet	TOTAL WT. OF 2 CECA Dry	µG Mn/GM CECUM Wet	TOTAL WT. OF 2 COLONS Wet	TOTAL WT. OF 2 COLONS Dry	µG Mn/GM COLON Wet
	mg	*mg*	*gm*	*gm*		*gm*	*gm*	
1	0.03	0.03	5.85	1.06	0.69	3.52	0.85	< 0.2
2	0.17	0.03	5.67	1.14	0.57	4.12	1.10	
3	1.0	0.03	5.35	1.10	0.71	3.95	0.81	1.00
4	1.0	0.20	6.85	1.23	0.59	4.72	0.97	0.98
5	10.0	0.03	4.75	0.69	4.42	3.53	0.75	4.53
6	10.0	2.0	5.66	0.96	1.55	3.91	0.83	2.78
7	40.0	0.03	5.05	0.87	2.81	3.77	0.74	4.50
8	40.0	8.0	5.00	0.91	2.82	3.31	0.93	6.76

manganese in the wall of the small intestine.[4] However, the evidence is clear that the storage of manganese in the wall of the intestine is greatly influenced by the level of manganese in the diet. The rats of groups 3, 4, 9 and 10 were all on the same manganese intake, 1.0–1.1 mg per day. The rats of groups 3 and 4 were 36 weeks old and those of groups 9 and 10 were 13 weeks old at time of sacrifice. Although the rats of groups 3 and 4 received 0.03 mg and those of groups 9 and 10 1.1 mg of manganese during the 24 hours preceding sacrifice, a comparison of the manganese assays of the intestinal walls of these groups suggests that manganese is more readily stored in the intestine of the younger rats.

The manganese content of the wall of the cecum in rats on different levels of manganese and thiamine intake is shown in table 3. In these assays two ceca were pooled for each analysis and only one pair analyzed in each dietary group. Statistical analysis of the data, therefore, was not possible. It seems apparent, however, that the manganese content of the wall of the cecum increases with the increase in dietary manganese.

Similarly, the manganese content of the wall of the colon in rats on different levels of manganese and thiamine intake is also shown in table 3. As is true of the wall of the cecum the manganese content of the wall of the colon increases with increase in manganese intake. When the data for the 10 mg per day manganese intake are compared, they suggest that both the wall of the cecum and the wall of the colon may take up manganese more readily on a low thiamine intake. Perhaps this difference between thiamine effects on the manganese contents of liver and intestinal tissues is associated with differences in the function of manganese in the two organs.

SUMMARY

1. The livers of 95 rats on different levels of dietary thiamine and manganese have been assayed for thiamine and

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[4] Table 2 compares group 3 vs. 4, 5 vs. 6, 7 vs. 8 and 9 vs. 10. Lack of significance can be seen from inspection of data, and is not computed for table or text.

manganese. The small intestines of 69, and the ceca and colons of 8 pairs of these rats were assayed for manganese.

2. Storage of thiamine in the liver increases with supplementation of manganese and thiamine intakes until dietary levels of 10 mg and 2.0 mg respectively, per rat per day, are reached.

3. Maximum thiamine storage in the liver, 11–16 µg per gram wet weight, is higher than has been reported earlier.

4. There is some evidence that storage of thiamine in the liver occurs more readily in young adult rats (13 weeks) than in older rats (36 weeks).

5. With thiamine intake constant at 0.03 mg per rat per day, manganese supplementation of the diet from a level of 0.03 to 1.0 mg per rat per day increases the storage of thiamine in the liver.

6. Liver storage of manganese increases with increase in dietary intake up to 40 mg of dietary manganese per day. With manganese intake constant at 1 mg per rat per day, thiamine supplementation of the diet from a level of 0.03 to 0.2 mg per rat per day increases (but not statistically significant) the mean storage of manganese in the liver.

7. At low levels of dietary manganese, storage of manganese in the wall of the small intestine is from one-third to two-thirds as much per gram of tissue as in the liver. At dietary intakes above 10 mg per day storage per gram in the small intestine is somewhat greater than in the liver.

8. Storage of manganese in the wall of the cecum and large intestine is as great or greater per gram than it is in the liver.

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THE USE OF AA'-DIPYRIDYL FOR DETERMINING THE AMOUNT OF FERROUS IRON FORMED IN THE DIGESTIVE TRACT OF WOMEN BEFORE AND AFTER THE ADDITION OF BEEF TO THE DIET

FRANCES A. JOHNSTON, RUTH L. INGALLS
AND BETTY O. MUKA

*New York State College of Home Economics and the School of Nutrition,
Cornell University, Ithaca*

In 1933 Lintzel published a paper reporting evidence that iron is absorbed only in the ferrous form. He used $\alpha\alpha'$-dipyridyl which he believed formed an insoluble compound in the digestive tract with ferrous iron. He believed this because when a ferrous salt was mixed with $\alpha\alpha'$-dipyridyl and fed to rats, the iron appeared quantitatively in the feces. Also, rats fed food mixed with $\alpha\alpha'$-dipyridyl became anemic after 4 to 6 weeks although $\alpha\alpha'$-dipyridyl administered parenterally had no effect. Later, Lucas and Summerfeldt ('39) confirmed the production of anemia in rats by the oral administration of $\alpha\alpha'$-dipyridyl. Rechenberger and Pollack ('44) used it to study the amount of iron secreted into the gastro-intestinal tract in the bile. They fed a man a diet almost devoid of iron and measured the iron excreted in the feces before, during, and after the addition of $\alpha\alpha'$-dipyridyl. On a diet containing 0.37 mg of iron, the iron content of the feces of the subject rose during the administration of $\alpha\alpha'$-dipyridyl from an amount similar to that in the intake to 2.7 mg on one day and 3.2 mg on the other. Later, another subject, on a diet containing 3.2 mg of iron, excreted 4.1 mg on one day and 4.9 mg on another during which the compound was administered.

If $\alpha\alpha'$-dipyridyl combines with ferrous iron in the gastro-intestinal tract to form an insoluble compound, it should be useful for studying the amount of ferrous iron formed in the digestive tract during the digestion of food. The present study was undertaken to study the feasibility of its use for this purpose.

<center>EXPERIMENTAL</center>

The general plan was to measure the increase in fecal iron after the administration of $\alpha\alpha'$-dipyridyl on a diet nearly devoid of iron in order to determine the amount of ferrous iron entering the digestive tract in bile, then to add a test food to the diet and find the increase in iron in the feces when $\alpha\alpha'$-dipyridyl was given. The increase of iron when the test food was fed would be corrected for bile iron. In this way, the amount of ferrous iron formed from the food could be determined.

The subjects were two women who were in good health. One was 36 years of age, 150 cm tall and weighed 52 kg; the other was 50 years of age, 164 cm tall and weighed 50.5 kg.

The diet was planned to be adequate in energy and protein and as complete in other respects as was possible on an intake extremely low in iron. About one-third of the calories was supplied by fat. The diet included 1 kg of milk, enough pure fat, starch and sugar to supply an adequate caloric intake and 25 mg of ascorbic acid. Three grams of No. 44 Whatman filter paper were added for roughage.

Samples of milk from local dairies were analyzed to find the milk with the lowest iron content. Cornstarch was washed with hydrochloric acid. Sugar of the lowest iron content of several samples analyzed was employed. The iron-low food products were made into cookies, caramel pudding and a syrup for drinking. The $\alpha\alpha'$-dipyridyl powder was mixed with the syrup and sipped throughout the meal and was also taken in water between the meals. Ground beef which was approximately two-thirds lean and one-third fat was made into 100 gm cakes. Three of these were broiled and added to the diet of each subject every day during one period.

The low-iron diet was fed for 6 days to allow time for the iron content of the feces to fall to a level representative of the low-iron diet and remain at that level for two days as evidence that a plateau had been reached. On the 7th and 8th days 0.15 gm of $\alpha\alpha'$-dipyridyl was administered. The subjects were kept on the diet a few days longer to allow the fecal iron to return to the level to which it had fallen previous to the addition of $\alpha\alpha'$-dipyridyl. The same procedure was followed when beef replaced an isocaloric amount of the sugar.

Carmine was administered before breakfast on the first and 7th days and on the day following the end of the dietary period. The feces were collected in ribbon formation on strips of cellophane. The feces collected from one marker to the next were divided into daily portions by weight. The divisions were not accurate, because the water content of the feces varied and estimation of the amount to include in a portion was difficult. The daily samples were digested in HCl for several days on a steam bath.

Aliquot amounts of feces and of each food product were ashed in platinum dishes. The ash was heated with HCl on a steam bath to hydrolyze pyrophosphates to ortho-phosphates according to the method described in the Official Methods of Analysis of the A. O. A. C. ('50). Iron was determined by the o-phenanthroline method of Saywell and Cunningham ('37). The depth of color was determined in a colorimeter described by Ellis and Brandt ('49) which permitted reading transmission through a 50 mm light-path. Nitrogen determinations were made by a semi-micro Kjeldahl method on samples of food, feces and urine. The caloric intake was calculated from the weight of the foods and the caloric values listed in the U. S. D. A. Agriculture Handbook 8 ('50). The dry weight of the feces for each day was determined by drying 25 ml of slurry to a constant weight.

RESULTS AND DISCUSSION

After 4 days on the low-iron diet, the fecal iron fell to a plateau from which it did not rise as anticipated during the

7th and 8th days when $\alpha\alpha'$-dipyridyl was administered. In the first trial on subject A. J. (table 1) there appeared to be a rise of about a half-milligram on those two days. However, the dry weight of the feces was enough larger than the weight on other days to account for this rise. In the case of subject R. I. (table 1), due to the occurrence of diarrhea which began on the 7th day, it was impossible to divide the feces for the last 4 days into daily portions. The mean value for the 4 days was slightly higher than that for days 5 and 6, but here again the dry weight of the feces was higher. In all three trials on the low-iron diet (table 1), the mean iron content of the feces for the last 6 days was below that of the intake, indicating that either $\alpha\alpha'$-dipyridyl had not tied up the bile iron, or the amount of iron secreted into the digestive tract in bile was very small.

In order to decide which of those possibilities was correct, a trial was made in which beef was added to the diet. The iron in hematin is in the ferrous form. Even if it is changed into the ferric form before it reaches the intestinal tract, compounds probably are formed upon digestion which reduce some of the iron. Experimental work in this laboratory (Johnston, Frenchman and Boroughs, '48) has shown that the iron of beef is unusually well absorbed. If the theory that iron is absorbed in only the ferrous form is correct, this fact would be evidence that much of the iron of beef either remains in, or is converted into the ferrous form.

On the diet containing 300 gm of ground beef, there was no indication of a rise in the iron content of the feces on the 7th and 8th days when $\alpha\alpha'$-dipyridyl was administered (table 2). This finding proved that the compound did not combine with ferrous iron in the digestive tract.

It is difficult to understand why our findings did not agree with those of previous workers, although in the case of Lintzel ('33) the explanation may perhaps be found in the fact that he used rats, whereas our work was done with humans. Rechenberger and Pollack ('44) worked with humans, but they made only two trials and the second was not convincing be-

TABLE 1

Iron and nitrogen balances for subjects who were given aa'-dipyridyl orally on the
7th and 8th days of a period during which they were maintained
on a low iron diet

DAY	Fe IN FECES	DRY WT. OF FECES	N IN FECES	NITROGEN BALANCE	BODY WEIGHT [1]
	mg	*gm*	*gm*	*gm*	*kg*
	Subject A. J. Intake: 1.72 mg iron; 4.58 gm nitrogen; 2,225 C/day				
1	6.17	18.4	0.71	— 1.78	60.3
2	5.46	21.2	0.66	— 2.38	60.3
3	2.63	17.1	0.49	— 1.42	60.2
4	1.94	17.5	0.46 [2]	— 2.44	. . .
5	1.44	18.7	0.46	— 1.36	60.4
6	1.36	19.0	0.46	— 0.60	60.2
7	1.80	22.1	0.71	— 1.19	60.2
8	2.02	27.2	0.71	— 1.16	60.3
9	1.46	22.5	0.67	— 1.63	60.2
10	1.07	15.4	0.67	— 1.77	59.9
Mean for last 6 days	1.53	20.8	0.59 [3]	— 1.47 [3]	
	Subject A. J. Intake: 1.69 mg iron; 4.58 gm nitrogen; 2,450 C/day				
1	5.02	10.9	. . .	. . .	60.6
2	2.99	13.0	0.35	— 0.32	60.7
3	2.70	16.1	0.45	— 0.13	60.9
4	1.74	16.3	0.39	+ 0.29	61.0
5	1.21	10.7	0.39	— 0.59	61.4
6	1.21	10.7	0.39	— 0.18	60.8
7	1.76	16.4	0.42	— 0.63	60.4
8	1.25	14.5	0.42	— 0.14	60.6
9	1.11	13.9	0.42	+ 0.06	60.5
10	1.55	19.7	0.42	+ 0.13	60.8
Mean for last 6 days	1.35	14.3	0.41 [3]	— 0.15 [3]	
	Subject R. I. Intake: 1.48 gm iron; 4.58 gm nitrogen; 2,060 C/day				
1	3.09	8.5	. . .	. . .	51.9
2	2.68	15.5	. . .	. . .	51.6
3	3.08	28.5	. . .	. . .	51.6
4	1.07	18.0	. . .	. . .	51.8
5	0.96	15.0	0.56	— 1.99	51.7
6	0.84	14.6	0.56	— 1.99	. . .
7	1.25	20.6	0.74	— 0.63	51.2
8	1.25	20.6	0.74	— 0.63	51.1
9	1.25	20.6	0.74	— 1.06	51.0
10	1.25	20.6	0.74	— 1.06	. . .
Mean for last 6 days	1.14	18.3	0.68	— 1.23	

[1] All body weights were taken on the morning following the day of the diet.
[2] Brackets indicate samples were pooled.
[3] Mean for last 7 days.

cause very little iron was excreted on the day following αα'-dipyridyl administration. Therefore, the possibility exists that the apparent differences in the second trial were caused by errors in fecal division. They made no determinations of dry weight.

Subject A. J. absorbed 4% of the iron of the beef while subject R. I. absorbed 35%. The hemoglobin levels for both subjects lay between 14 and 15 gm per 100 ml of blood. In the case of subject A. J., the need for iron was low because no iron was required for the replacement of menstrual losses. The lower need may have been the reason for the absorption of less iron. These results are similar to those on two subjects studied by Moore and Dubach ('51): one subject absorbed 1.9% and another 33.1% of radioactive iron from chicken muscle. The large amount absorbed by subject R. I is comparable to the amount absorbed by 5 college women studied in this laboratory in 1948 (Johnston, Frenchman and Boroughs, '48).

There are some workers who believe that when a subject changes from a high iron intake to a low one, iron is excreted in progressively decreasing amounts through the intestinal wall during a so-called "adjustment period." This experiment supplied evidence contrary to this theory. Four days (tables 1 and 2) were usually required after the first appearance of the carmine marker before the subjects became "adjusted" to the low intake. That the extra iron in the feces during those days was largely, if not all, delayed food-residue was evident from the appearance of the feces. During those days, the feces were changing from a dark and brownish color to a light yellow. The consistency also changed from grainy to very smooth. Dubach et al. ('48) noted this same lag in excretion. After a single dose of radioactive iron, radioiron appeared in the feces for 6 or 7 days and in the case of two subjects for 12 days in quantities in excess of that found after the administration of radioiron intravenously.

Both subjects lost nitrogen in the first trial (table 1) on the low iron diet. This was probably because both had been

living on diets of higher nitrogen content and were not receiving an adequate energy intake. In the second trial, subject A. J. was almost in nitrogen balance. The improvement may have been due to the increased caloric intake. When the nitrogen content of the diet was increased by the addition of beef (table 2), subject R. I. retained a normal amount of

TABLE 2

Iron and nitrogen balances for two subjects who were given aa'-dipyridyl orally on the 7th and 8th days of a period during which they were maintained on a low iron basal diet plus beef

DAY	Fe IN FECES	DRY WT. OF FECES	N IN FECES	NITROGEN BALANCE	BODY WEIGHT [1]
	mg	gm	gm	gm	kg
Subject A. J. Intake: 5.68 mg iron; 11.86 gm nitrogen; 2,357 C/day					
1	8.80	15.9	. . .	. . .	61.7
2	7.59	15.1	. . .	. . .	61.8
3	8.01	17.4	.	.	61.8
4	6.44	18.1	0.36 [2]	+ 0.19	62.2
5	5.64	16.6	0.36	+ 0.19	61.6
6	5.53	17.3	0.33	— 0.34	61.4
7	5.18	16.6	0.33	— 0.34	61.1
8	5.29	16.6	0.35	— 0.13	61.0
9	4.90	15.2	0.35	— 0.13	61.1
10	5.43	17.2	0.37	— 1.00	61.1
11	4.55	15.5	0.37	— 1.00	61.1
Mean for last 7 days	5.22	16.4	0.35 [3]	— 0.32 [3]	
Subject R. I. Intake: 5.59 mg iron; 11.86 gm nitrogen; 2,200 C/day					
1	3.50	16.8	. . .	. . .	51.9
2	2.94	14.9	. . .	. . .	51.9
3	3.24	16.5	. . .	. . .	51.8
4	2.87	14.1	0.60	+ 0.46	51.6
5	3.18	15.6	0.60	+ 0.46	51.6
6	4.60	22.1	0.71	+ 0.56	51.3
7	2.88	13.5	0.71	+ 0.56	51.3
8	4.08	18.5	0.76	+ 0.35	51.4
9	3.94	18.5	0.76	+ 0.35	51.4
10	4.10	17.8	0.70	+ 0.91	51.2
11	3.38	16.4	0.70	+ 0.91	51.0
Mean for last 7 days	3.74	17.5	0.69 [3]	+ 0.57 [3]	

[1] All body weights were taken the morning following the day of the diet.
[2] Brackets indicate samples were pooled.
[3] Mean for last 8 days.

nitrogen, but subject A. J. lost a small amount. This may have been due to too low a caloric intake as evidenced by the small loss of weight. However, the other subject lost approximately the same amount of weight. With human subjects, there is always the possibility that emotional stress may play a part in nitrogen loss. According to the original plan, the caloric intake was to be increased in later experiments if the subjects were not in nitrogen balance on the first intake. An increase was made in the second trial on the low iron intake of subject A. J. Further experiments, in which the calories were increased, were not carried out because the results did not show an effect with αα'-dipyridyl.

SUMMARY

Two women consumed diets low in iron until the iron content of the feces reached a plateau. Then they were given 0.15 gm of αα'-dipyridyl orally on each of two days after which they continued on the low iron diet for two more days. The subjects repeated the same procedure with beef substituted for a part of the sugar in the diet. On neither diet did the iron content of the feces rise upon the administration of αα'-dipyridyl. This indicated that αα'-dipyridyl does not combine with ferrous iron in the digestive tract to form an insoluble compound and cannot be used to measure the amount of ferrous iron formed.

One subject absorbed very little and the other about one-third of the iron of beef.

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BASAL METABOLISM OF NINETEEN CHILDREN FROM TWO TO TEN YEARS OLD

MINA WOLF LAMB AND JONNIE McCRERY MICHIE

Department of Foods and Nutrition, Texas Technological College, Lubbock

INTRODUCTION

Since data on the basal metabolism of young children are limited, the authors decided to extend the study of this age group. As early as 1899 Magnus-Levy and Falk established the fact that metabolism was high in childhood; their 15 subjects indicated that there is a higher level of heat production between the ages of one and 10 years. The Benedict-Talbot figures published in 1921 on 244 subjects from birth to puberty are based on children who were usually asleep. Since present standards for basal metabolism determinations require that the subjects be awake, these data are not comparable to recent determinations.

Wang and her associates ('25) published a study on the basal metabolism of undernourished children, including the metabolic rates of a limited group of "vigorous normal children." Only one child was in the normal group between two and 10 years of age.

Robb ('34) published a report of the oxygen consumption of 29 normal children, 12 boys and 17 girls, three to 4 years of age. She called attention to the wide range of differences in the rates of the individual subjects and also noted that the standards proposed as indicators of normalcy are too low.

Lewis, Kinsman and Ilif ('37) published a study of 100 girls and boys equally divided as to sex. They noted the significant effect of body build on the comparative values obtained for the basal metabolic rate by several methods of

reference. They emphasized the need for carefully controlled conditions in determining the basal metabolism of children.

Lamb ('45) confirmed the previous findings that children vary greatly in basal heat production. Her data were on 8 nursery school children, three girls and 5 boys, two to 5 years of age. She emphasized that children must be compared on the basis of developmental progress as well as of age and that the procedure for the determination needs to be further standardized.

Macy ('46) reported the basal metabolism of 11 children, the determinations having been repeated on some over a period as long as 4 years. The age range at the beginning of the study was from 4 years and 8 months to 8 years and 9 months. From 19 to 26 basal metabolism determinations were made on 10 of the children. The calories are expressed on the basis of the total for 24 hours and no analysis has been published to date.

In this study basal metabolism determinations were made according to the following conditions: (1) complete muscular relaxation; (2) post-absorptive condition (12 to 14 hours after last food intake); (3) lying quietly awake in a comfortable environment; (4) minimum mental and nervous stimulation; and (5) freedom from physiological disturbances, as indicated by normal sublingual temperature, normally constant pulse and general healthy appearance.

SUBJECTS

The subjects were 19 children, 9 girls and 10 boys ranging in age from two to 10 years. They were selected at random from applicants for admission to the Nursery School of the Department of Child Development, Division of Home Economics, Texas Technological College. The children were healthy and of normal growth and development, as shown by pediatric examinations. None was excessively overweight or underweight. The variations in weight were caused by differences in body build ranging from a stocky to a slender structure, and by the stage of maturity ranging from chubby

babyhood to the slimness of early childhood. Each subject increased regularly in both weight and height throughout the study.

The subjects were trained for the determination of their basal metabolism by the procedure described by Ling ('46). They were cooperative, willing and capable in following the details of the procedure of the determination. At no time was difficulty encountered or any need found for modification of the procedure to accommodate the subjects.

PROCEDURE

A Collins-Benedict-Roth respiration apparatus was used for all determinations, with a rubber-cushioned nose clip and rubber mouth piece trimmed to accommodate the smaller face of a child.

The children were met at their homes as soon as they awakened and were brought to the laboratory. Upon arrival each child was put to bed and closely supervised by a trained assistant who read the child's favorite stories to him and kept him sleepy and relaxed. Sublingual temperature was determined and two one-half minute pulse counts were made during this initial rest period. The pulse was also counted twice during each determination of oxygen consumption.

At the end of the 20- to 30-minute rest period, the subject was attached to the apparatus and an 8-minute recording of the respirations and oxygen consumption was made on kymograph paper according to the accepted procedure for the apparatus. Two determinations of oxygen consumption were made, each of 8-minute duration, with a few minutes of rest between. The assistant was with the child at all times to see that he remained relaxed during the determinations.

At the end of the recordings of oxygen consumption the subject was weighed and measured while wearing only shorts. The height and weight were recorded to the nearest 10th of an inch and pound respectively.

Determinations of oxygen consumption were made on at least two mornings and continued when necessary until the

TABLE 1

Data on the basal metabolism of subjects

SUBJECTS		METABOLISM CALORIES PER 24 HOURS			
Sex	Age	Total	per kg	per cm	per m^2
	yr.-mo.-da.				
EB–f	2–4–12	710	55.5	8.3	1,316
	2–7–15	761	55.9	8.7	1,358
	2–10–27	713	49.5	7.9	1,229
	3–4–14	725	49.3	7.8	1,208
	3–7–2	713	46.3	7.6	1,150
	3–8–14	686	44.6	7.2	1,107
	4–3–8	773	49.6	7.8	1,189
	4–9–8	727	45.7	7.2	1,102
	5–2–27	775	46.6	7.5	1,140
	5–9–10	785	44.1	7.4	1,090
ED–f	3–6–23	811	54.1	8.1	1,267
	3–9–26	821	52.3	8.0	1,244
	4–0–16	874	55.3	8.4	1,285
	4–4–10	871	52.5	8.2	1,244
	4–6–25	883	51.1	8.3	1,244
	4–10–4	943	51.3	8.6	1,258
	4–11–21	871	47.1	7.8	1,162
	5–6–7	953	47.7	8.3	1,191
MH–f	3–3–29	799	53.3	8.5	1.310
	3–6–25	823	53.5	8.6	1,306
	3–9–26	852	52.6	8.6	1,311
	4–1–21	842	50.4	8.3	1,238
	4–4–13	895	51.2	8.7	1,229
	4–6–25	922	50.1	8.8	1,280
	4–8–0	893	48.0	8.4	1,257
	5–3–6	902	46.3	8.2	1,171
	5–9–17	905	43.3	7.9	1,117
	6–4–15	1,015	44.1	8.5	1,167
	6–10–1	1,025	39.4	8.4	1,114
JE–f	2–7–18	881	53.7	9.0	1,354
	3–2–10	792	43.5	7.9	1,131
	3–7–25	917	45.1	8.7	1,206
JM–f	3–11–12	912	52.4	8.8	1,305
	4–5–20	850	47.2	8.0	1,164
NC–f	4–2–6	737	47.8	7.3	1,133
LT–f	3–10–4	811	47.4	7.6	1,142
BH–f	6–11–5	1,003	44.8	8.2	1,153
MF–f	4–3–11	958	44.7	8.9	1,213
DW–m	3–2–12	833	52.4	8.4	1,281
	3–8–24	895	50.3	8.6	1,261
	4–1–0	926	49.3	8.8	1,268
	4–2–29	943	49.4	8.8	1,257
	4–9–16	967	47.6	8.6	1,224

TABLE 1 *(continued)*

SUBJECTS		METABOLISM CALORIES PER 24 HOURS			
Sex	Age	Total	per kg	per cm	per m²
	yr.-mo.-da.				
DW–m *(cont.)*	5–1–23	965	46.4	8.4	1,191
	5–2–22	938	43.2	8.2	1,131
	5–9–7	994	45.2	8.4	1,169
	6–2–16	967	42.8	8.0	1,111
	6–11–13	1,073	43.1	8.7	1,154
	7–3–8	1,027	40.6	8.0	1,070
	7–8–19	1,032	39.5	7.9	1,030
	10–3–10	1,188	34.6	8.2	1,006
SC–m	3–4–28	838	55.8	8.3	1,289
	3–8–16	934	59.1	9.0	1,393
	3–11–1	982	61.8	9.3	1,444
EP–m	3–6–0	840	55.6	8.4	1,313
	3–8–12	866	54.2	8.4	1,293
	4–0–0	874	53.6	8.3	1,266
	4–3–3	876	52.8	8.2	1,251
	4–5–22	905	52.6	8.4	1,257
	4–9–2	806	45.8	7.4	1,105
	4–10–6	917	51.2	8.3	1,238
	5–4–16	866	46.8	7.6	1,125
	5–10–9	919	47.6	7.9	1,163
BS–m	3–7–29	1,010	60.5	9.4	1,443
	3–11–2	958	55.0	8.8	1,312
	4–2–4	977	54.6	8.8	1,303
	4–5–7	1,001	54.1	8.9	1,317
	4–8–0	958	50.9	8.4	1,228
	4–10–3	972	49.8	8.4	1,215
	4–11–28	986	48.6	8.4	1,203
	5–6–25	943	45.3	7.7	1,109
	6–0–19	941	42.4	8.0	1,057
VB–m	4–2–16	905	56.2	8.9	1,359
	4–8–7	850	49.4	8.1	1,214
	5–0–8	922	51.8	8.7	1,281
	5–3–43	917	52.4	8.5	1,274
	5–8–22	953	50.4	8.5	1,238
	5–11–23	994	50.2	8.8	1,258
WD–m	3–8–8	948	53.3	9.5	1,374
	3–11–7	931	51.7	9.1	1,330
	4–2–10	962	51.7	9.3	1,336
OS–m	3–1–23	804	57.4	8.3	1,318
DW–m	3–5–0	912	50.7	9.1	1,303
	3–7–25	958	51.8	9.4	1,349
AM–m	3–11–16	835	52.9	8.1	1,246
BP–m	4–0–26	883	53.2	8.4	1,278

lowest figure obtained for calories per hour agreed within 5% with results obtained on a different morning. This procedure gave a minimum of 4 recordings for each subject and in some cases 6 recordings were made to obtain a "check." One cannot assume basal metabolism to be the single lowest figure but must consider that it may be any value within 5% of the lowest (Du Bois, '36). Therefore, all figures checking within 5% of the lowest were averaged with the lowest to represent the true basal metabolism of the individual.

True basal metabolism determinations of 10 of the subjects were made at three-month intervals the first year and at longer intervals thereafter. On each of these subjects three to 13 determinations were made over a period of one to 7 years. On 7 subjects only one basal metabolism determination was made, and on two subjects two determinations.

RESULTS

The data presented in table 1 give the individual results for all subjects. Calories are expressed as the total per 24 hours and as per kilogram of weight, per centimeter of height and per square meter of surface area per 24 hours. Deviations are calculated from 5 prediction standards — Benedict height, Dreyer, Talbot height, Talbot weight and Lewis.

Calories per 24 hours increased with increase in age and size. Calories per kilogram of weight decreased with increase in age, although not uniformly, either for any one subject, or for different subjects during the same age range. Calories expressed per centimeter of height decreased more consistently with increase in age than did calories expressed per kilogram of weight or per square meter of surface area. The girls showed a smaller percentage decrease from two and one-half to 7 years of age than the boys did from three to 10 years. This was true even when comparing only those children on whom repeated tests were done for the same age range. That is, consider girls EB, ED and MH, in comparison with boys DW, BS and VB, all of whom were studied from about three through 6 years. The decrease in calories for

the girls was as follows: 13.3% on the basis of weight; 1.3% on the basis of height and 9.4% on the basis of surface area, whereas, the decrease for boys was as follows: 18.3%, 6.7% and 14.7% respectively. Table 2 indicates that for each of these 7 subjects, calories per kilogram of weight showed the greatest, and those per centimeter of height, the least decrease.

For the group of children irrespective of age, calories per kilogram of weight showed the widest range from 61.8 to 34.6, a difference of 44%. Calories per centimeter showed the least range from 9.5 to 7.2, a difference of 24%, while the

TABLE 2

Percentage decrease in calories from three through 6 years

GIRLS	per kg	per cm	per m²
EB	10.9	6.3	11.3
ED	11.8	+ 2.5	6.6
MH	17.1	0.0	10.9
BOYS			
DW	8.3	4.8	13.2
EP	14.4	6.0	11.4
BS	29.6	14.9	26.7
VB	10.7	1.1	7.4
Av. for all subjects	16.1	4.4	12.4

calories per square meter ranged from 1,444 to 1,006, a difference of 30%.

When the data are grouped into age intervals of 6 months each, as shown in table 3, the results are more consistent. Girls still show a smaller range between maximum and minimum figures with calories per kilogram showing the widest variation and calories per centimeter the least.

The basal metabolism of children appears to parallel growth in height more than that in weight or surface area. An analysis of the data on children, especially when the calories are expressed per kilogram of weight, per square meter of surface area, indicates the prominence of heterogeneity among indi-

vidual children and among children of the same age group. Girls seem to show more uniformity and consistency than boys for these years from two to 10. The few cases after 6 years of age make results for the years of 6 to 10 inconclusive. Calories per kilogram of weight are inversely proportional

TABLE 3

Basal metabolism of age groups of children

AGE GROUP	SUBJECTS	BODY SIZE			CALORIES PER 24 HOURS			
		Ht.	Wt.	S. area	Total	per kg	per cm	per m²
yr.-mo.		*cm*	*kg*	*m²*				
Boys								
3–0 to 3–6	5	100	15.6	0.65	845	54.4	8.5	1,301
3–6 to 4–0	10	104	16.9	0.69	932	55.1	9.0	1,345
4–0 to 4–6	10	107	17.5	0.72	925	52.8	8.7	1,289
4–6 to 5–0	7	112	18.8	0.77	922	49.8	8.2	1,204
5–0 to 5–6	5	111	19.3	0.77	922	48.1	8.3	1,200
5–6 to 6–0	5	116	20.2	0.81	961	47.7	8.3	1,187
6–0 to 6–6	2	123	22.4	0.88	954	42.6	8.2	1,084
6–6 to 7–0	1	124	24.9	0.93	1,073	43.1	8.7	1,154
7–0 to 8–0	2	130	25.7	0.97	1,030	40.1	8.0	1,062
Girls								
2–0 to 2–6	1	86	12.8	0.54	710	55.5	8.3	1,316
2–6 to 3–0	3	92	14.8	0.60	785	53.0	8.5	1,314
3–0 to 3–6	3	96	16.0	0.64	772	48.7	8.1	1,216
3–6 to 4–0	9	100	16.4	0.67	816	49.8	8.1	1,226
4–0 to 4–6	8	104	17.1	0.70	850	49.8	8.2	1,218
4–6 to 5–0	6	106	17.9	0.72	873	48.9	8.2	1,217
5–0 to 5–6	2	107	18.1	0.73	839	46.5	7.9	1,156
5–6 to 6–0	3	112	19.6	0.78	881	45.0	7.9	1,133
6–0 to 6–6	1	119	23.0	0.87	1,015	44.1	8.5	1,167
6–6 to 7–0	2	122	24.2	0.90	1,014	42.1	8.3	1,134

to age whereas calories per centimeter of height are independent of age.

The standards available to predict the basal metabolism of any given child give calculated values which are very heterogenous in their predictions as can be seen in table 4. The observed results vary from more than 20% below to almost 20% above the calculated values from the 5 standards. The

most recently proposed standards by Lewis et al. ('43) give the best agreement with actual results. Of 86 results on all children only 4 determinations deviated from the Lewis standard by 10% or more. Of the total of 86 basal metabolism results, 48 were higher than those of the Lewis standards. Of the 19 children studied, 13 had an average deviation of from + 7.6 to + 0.3% higher than this standard. Basal metabolism values estimated from the Wetzel Grid were in close agreement with actual determinations.

TABLE 4

Deviation of observed basal metabolism based on age groups from prediction standards for children

AGE GROUPS	BASAL METAB. DETER- MINATIONS	PER CENT DEVIATION FROM STANDARDS				
		Benedict [1]	Dreyer	Talbot-ht.	Talbot-wt.	Lewis
yr.-mo.						
Boys						
3–0 to 3–6	5	+ 14.2	— 19.7	+ 8.2	+ 14.0	+ 0.5
3–6 to 4–0	10	+ 19.9	— 12.1	+ 16.2	+ 19.8	+ 5.7
4–0 to 4–6	10	+ 16.6	— 14.2	+ 13.4	+ 16.3	+ 2.9
4–6 to 5–0	7	+ 11.4	— 15.4	+ 8.0	+ 10.6	— 2.5
5–0 to 5–6	5	+ 10.1	— 16.3	+ 9.2	+ 9.5	— 1.1
5–6 to 6–0	5	+ 11.5	— 12.7	+ 7.8	+ 10.5	— 1.1
6–0 to 6–6	2	+ 3.5	— 17.9	— 1.3	+ 3.2	— 8.2
6–6 to 7–0	1	+ 8.6	— 10.5	+ 9.6	+ 5.1	— 1.0
7–0 to 8–0	2	+ 1.9	— 14.8	— 1.3	— 2.4	— 6.7
Average for boys		+ 10.9	— 14.8	+ 7.8	+ 9.6	— 1.3
Girls						
2–0 to 2–6	1	+ 16.9	— 16.6	+ 11.6	+ 9.6	+ 4.6
2–6 to 3–0	3	+ 24.8	— 12.9	+ 15.4	+ 10.4	+ 6.1
3–0 to 3–6	3	+ 19.7	— 17.3	+ 9.2	+ 3.9	— 0.4
3–6 to 4–0	9	+ 19.8	— 13.7	+ 10.5	+ 7.6	+ 1.8
4–0 to 4–6	8	+ 19.9	— 11.9	+ 11.6	+ 9.5	+ 3.8
4–6 to 5–0	6	+ 18.6	— 8.8	+ 11.9	+ 9.3	+ 4.1
5–0 to 5–6	2	+ 13.8	— 12.9	+ 6.9	+ 4.4	+ 0.3
5–6 to 6–0	3	+ 11.6	— 9.9	+ 6.8	+ 4.7	— 0.1
6–0 to 6–6	1	+ 18.9	— 4.2	+ 15.1	+ 10.3	+ 4.6
6–6 to 7–0	2	+ 15.1	— 4.7	+ 11.6	+ 7.4	+ 3.2
Average for girls		+ 17.9	— 11.3	+ 11.1	+ 7.7	+ 2.8
Average for all		+ 14.6	— 13.0	+ 9.2	+ 8.5	+ 0.3

[1] Benedict-Talbot was used for boys and Benedict-ht. for girls.

TABLE 5

Average basal metabolism of subjects classified into groups based on height, weight and surface area

GROUPS	NO. OF CASES	BASAL METABOLISM-CALORIES PER 24 HOURS		
		per kg	per cm	per m²
Ht. Groups *cm*	*Boys*			
97–103	11	53.7	8.8	1,322
103–109	15	53.6	8.6	1,296
109–115	11	49.2	8.4	1,214
115–121	5	46.8	8.2	1,172
121–127	3	43.6	8.1	1,107
127–133	2	40.1	8.0	1,050
	Girls			
85–91	3	53.6	8.3	1,301
91–97	5	49.4	7.9	1,216
97–103	10	49.6	8.0	1,211
103–109	12	49.1	8.1	1,142
109–115	4	47.0	8.1	1,177
115–121	3	45.5	8.3	1,170
121–127	1	39.4	8.4	1,114
Wt. Groups *kg*	*Boys*			
12–14	1	57.4	8.3	1,318
14–16	7	56.0	8.6	1,323
16–18	17	52.7	8.6	1,285
18–20	11	50.2	8.6	1,250
20–22	6	46.1	8.3	1,171
22–24	2	42.6	8.0	1,084
24–26	3	41.1	8.2	1,085
	Girls			
12–14	2	55.5	8.3	1,316
14–16	12	50.1	7.9	1,211
16–18	11	49.9	8.2	1,228
18–20	7	47.7	8.3	1,207
20–22	3	44.4	8.5	1,179
22–24	2	44.5	8.4	1,160
24–26	1	39.4	8.4	1,114
Surface area groups *sq. m*	*Boys*			
0.59–0.66	4	55.3	8.4	1,300
0.66–0.73	19	53.7	8.8	1,312
0.73–0.80	13	50.2	8.4	1,234
0.80–0.87	6	46.4	8.3	1,170
0.87–0.94	3	42.8	8.2	1,107
0.94–1.01	2	40.1	8.0	1,050
	Girls			
0.52–0.59	3	54.8	8.3	1,301
0.59–0.66	10	50.5	8.1	1,234
0.66–0.73	14	48.4	8.1	1,203
0.73–0.80	7	47.1	8.4	1,204
0.80–0.87	2	45.5	8.1	1,154
0.87–0.94	2	44.5	8.4	1,160

When the data are arranged according to age groups the results show good agreement with the Lewis standards. In 11 of the 19 groups there is a positive deviation of from + 6.1 to + 0.3%. All the data on girls average slightly below those of the Lewis standard, whereas those for boys average above. Data on these subjects are approximately 9% higher than Talbot's standards based on height and on weight. The data do not agree with the standards proposed by Benedict and Dreyer. This situation is understandable when one recalls that the conditions for determination of basal metabolism when Benedict was doing his work were not as clearly defined as they are today.

When the subjects were arranged in groups according to height, weight and surface area as shown in table 5, basal metabolism expressed as calories per kilogram of weight decreased consistently with increase in size irrespective of age within the range of two to 10 years. Calories per square meter of surface area also decreased consistently with increase in size. Since calories per centimeter of height do not decrease consistently with increase in size, this means of expressing basal metabolism has given the most constant values irrespective of age, size or sex.

On 7 subjects determinations of basal metabolism were made at from three- to 6-month intervals for from two to as many as 7 years on one subject. On the basis of weight and surface area the decrease in basal metabolism with increase in age was constant although not regular from year to year among the subjects of the same age span. For the period from three to 6 years calories per kilogram of weight decreased approximately 5 calories annually and calories per square meter of surface area decreased about 75 calories annually. The data are insufficient to permit definite conclusions.

SUMMARY AND CONCLUSIONS

From 86 basal metabolism determinations on 19 subjects, two to 10 years of age, the results indicate that age alone is

an inadequate criterion by which to classify basal metabolism of children. Basal metabolism changes more uniformly with change in size than in age. The results are more constant when expressed per unit of height than per unit of weight or surface area. The results obtained on these subjects do not conform with the prediction standards developed by Benedict and Dreyer but agree closely with those of Talbot and Lewis. This agreement can be attributed to the increased standardization of the procedures for determining basal metabolism and to the complete cooperation of these young subjects.

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OBSERVATIONS ON ODORATISM (SWEET PEA LATHYRISM) IN THE RAT

WALDEMAR DASLER

Department of Biochemistry, The Chicago Medical School, Chicago, Ill.

A perusal of the literature on lathyrism makes it appear doubtful that the term *lathyrism* has always been used to refer to the same syndrome. Denny-Brown ('47), for instance, questioned the identity of human lathyrism with the lathyrism produced in rats by the feeding of the seed of the flowering sweet pea *(Lathyrus odoratus)*. For this reason the present author favors the use of the term *odoratism,* proposed by Vivanco and Díaz ('51), when referring specifically to the lathyrism which results from the ingestion of *Lathyrus odoratus.* That other species of *Lathyrus* may give rise to a similar or identical syndrome in the rat is clear from the work of Lee ('50) and of Lewis et al. ('48).

The symptoms of odoratism in the rat have been described by Geiger, Steenbock and Parsons ('33), by Lewis et al. ('48) and by Vivanco and Díaz ('51). In this laboratory, hematuria and hemorrhage into the small intestine have been observed as occasional findings in the terminal phases of odoratism, but in rats which die or are killed in advanced stages of the disease, the gross appearance of the viscera frequently is normal (Vivanco and Díaz, '51). Numerous postmortem examinations in this laboratory, however, gave rise to the impression that the adrenal glands in these rats were larger than in normal rats of the same size. To obtain data bearing on this point, the adrenals of rats in advanced stages of odoratism were weighed and compared to those of control rats.

Hemoglobin and serum alkaline phosphatase values were also determined.

EXPERIMENTAL

The pea diets had the following composition in per cent: pea meal, 50, casein,[1] 10, corn starch, 25, sucrose, 5, brewer's yeast powder, 4, salts,[2] 4 and cod liver oil, 2. The dry ingredients were mixed separately and stored, the cod liver oil being added to portions of the mixture as needed. The pea meal was prepared by grinding the dry whole peas to a fineness which permitted all the material to pass through a 24-mesh sieve. The experimental groups received a diet in which the pea meal was made from the seed of *Lathyrus odoratus*.[3] A control diet was prepared from the meal of dried commercial edible peas.

Weanling albino rats, 22 days of age, of the Holtzman-Sprague-Dawley strain, were used. In the pair-fed groups, each of the experimental rats was paired with a control rat of the same weight ± 0.5 gm. The rats used to obtain the exploratory data in table 1 were 28 days of age.

The feedings were continued for three weeks. Since it was known in advance that, at the end of three weeks on 50% sweet pea diets, some rats are losing weight, moribund, or even dead, 20 rats were included in each of the pair-fed groups. Toward the end of the three-week period, the experimental rats were weighed daily. Animals which were losing weight were discarded together with their pair-fed controls. The tabulated data from the experimental groups therefore are uncomplicated by terminal changes and represent rats with advanced odoratism which were still active and gaining weight at the end of the experimental period.

Hemoglobin determinations were made with the aid of a Leitz clinical photoelectric colorimeter on blood obtained from the tail of each rat. The procedure was that which is described in the manual accompanying the instrument and was not re-standardized by comparison with a direct method.

[1] The casein was plain, untreated casein (not vitamin-free) purchased from General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] The salt mixture was that devised by Hubbell, Mendel and Wakeman ('37).

[3] The author gratefully acknowledges the generosity of Mr. Raymond H. Coulter of the Ferry-Morse Seed Company, Detroit, who supplied the sweet peas used in these and in related studies.

Blood for the determination of serum alkaline phosphatase was taken by heart puncture while the rats were under ether anesthesia. The determinations of serum phosphatase were made by the Bodansky method, modified to permit the use of the Fiske and SubbaRow method for liberated phosphate (Hawk, Oser and Summerson, '47). The readings were made on the Leitz photoelectric colorimeter.

Both adrenals of each rat were removed, carefully cleaned of all adhering tissue, and weighed together on a Roller-Smith torsion balance.

RESULTS

The results are presented in table 1. No terminal values are included in the tabulated data.

The hemoglobin levels of rats with advanced odoratism were somewhat higher than those of the control groups and, in the case of the males, the difference between the levels of the former and those of the pair-fed controls was significant at the 5% level of confidence. However, the two complete series of hemoglobin values given in the table indicate that the nature of the diet and the nutritional status of the growing animal exerted a greater effect on hemoglobin levels in this experiment than did the toxic factor.

The serum phosphatase values listed in table 1 indicate that here, too, the levels were influenced more by non-toxic variations in the diet and by the nutritional status of the growing animal than by the toxicity of the experimental ration.

Some terminal phase values for serum alkaline phosphatase in odoratism were determined in separate experiments. These values were invariably low, both in the experimental animals and in the pair-fed controls. In these rats the sharply decreased food consumptions accompanied by daily weight losses are adequate to explain the low values, since it has been shown that fasting gives rise to decreased serum phosphatase levels in the rat (Cantor, Wight and Tuba, '48; Tuba et al., '52; Jackson, '52; and others).

TABLE 1

Hemoglobin, serum alkaline phosphatase and adrenal weights in advanced odoratism

RATS	DIET	MEAN INITIAL WEIGHT	MEAN FINAL WEIGHT	MEAN HEMOGLOBIN ± S.E.[1]	MEAN PHOSPHATASE ± S.E.[1]	MEAN ADRENAL WEIGHT PER RAT ± S.E.[1]	MEAN ADRENAL WEIGHT PER 100 GM BODY WEIGHT ± S.E.[1]
		gm	*gm*	*gm/100 ml*	*Bodansky units*	*mg*	*mg*
10 ♂	50% sweet pea	48.7	119.3	13.6 ± 0.26 (8)[2]	87.8 ± 7.1 (9)[2]	32.6 ± 1.2	27.5 ± 1.3
10 ♂	50% edible pea, pair-fed	48.4	133.5	12.8 ± 0.25	128.0 ± 7.1	27.8 ± 0.8	21.0 ± 0.7
10 ♂	50% edible pea, ad libitum	47.6	146.1	12.1 ± 0.14	132.1 ± 10.4	26.9 ± 0.9	18.5 ± 0.8
10 ♂	Rockland rat diet ad libitum	52.0	178.7	10.3 ± 0.29	100.3 ± 5.4 (9)[2]	29.9 ± 0.6	16.8 ± 0.3
10 ♀	50% sweet pea	46.6	113.9	13.9 ± 0.43	97.0 ± 9.3	34.1 ± 1.3	30.1 ± 1.1
10 ♀	50% edible pea, pair-fed	46.5	125.1	13.2 ± 0.25	76.0 ± 8.7	31.8 ± 1.3	25.4 ± 0.8
10 ♀	50% edible pea, ad libitum	45.9	141.5	12.7 ± 0.42	91.8 ± 4.6	34.1 ± 1.2	24.2 ± 0.8
10 ♀	Purina fox checkers ad libitum	45.9	139.0	13.0 ± 0.42	71.2 ± 4.8 (9)[2]	34.8 ± 0.8	25.1 ± 0.7
7 ♀	50% sweet pea	53.3	128.2	...	98.0 ± 12.6 [3]		
8 ♀	50% edible pea, pair-fed	52.4	130.6	...	65.0 [3,4]		
8 ♀	50% edible pea, ad libitum	51.3	145.6	...	89.5 [3,4]		

[1] S.E. = Standard error.
[2] In instances where samples were inadequate, lost, or otherwise ruined, the numbers of samples analyzed are given in parentheses.
[3] Results from exploratory experiments.
[4] The average of 4 pooled samples from 8 rats in each case.

An effect of odoratism on the weights of the adrenal glands is clearly indicated by the results in table 1.

When the average adrenal weights are compared on the basis of body weight (table 1), the differences between the rats of the sweet pea groups and those of the pair-fed edible pea groups are highly significant statistically both for the males ($t = 4.371$; $P = < .001$) and for the females ($t = 3.487$; $P = < .005$). The differences between the sweet pea groups and the control groups fed ad libitum are even greater. If the average adrenal weights are compared on the basis of age of the animal, that is, if actual adrenal weights are compared (mean adrenal weight per rat, table 1), no statistically significant difference among the various groups is found in the case of the females. In the case of the males, however, the actual adrenal weights of the animals fed sweet peas are significantly higher than the adrenal weights of the larger control animals (for the pair-fed groups, $t = 3.511$; $P = < 0.005$). Even the adrenal weights of rats fed a stock diet ad libitum are somewhat smaller than those fed the sweet pea diet ($t = 2.083$; $P = < 0.06$; difference is probably significant), although the body weights of the former were 50% higher than those of the sweet pea group. It is seen that the effect of odoratism on the adrenals was greater in the males than in the females.

DISCUSSION

Hemoglobin values are of interest in connection with odoratism in rats because of the report by Geiger, Steenbock and Parsons ('33) that their rats showed a slight polycythemia and a tendency toward high hemoglobin. Lewis et al. ('48), on the other hand, reported that microscopic examinations of the bones of rats with odoratism revealed that there was much less hematopoiesis than in the control animals.

Serum alkaline phosphatase levels are of interest because of the spectacular skeletal changes which take place in rats consuming sweet pea diets.

The conclusion to be drawn from the present study is that an effect of the toxic factor of sweet peas on the levels of blood

hemoglobin and serum alkaline phosphatase, if any exists, cannot be determined by the use of edible pea control diets. The edible pea control diets used in this study and in those of other investigators provide very inadequate controls for this type of investigation because they introduce many variables by substituting the seed of a different genus of plant, *Pisum,* for that of *Lathyrus odoratus,* a major constituent of the experimental diet. Since, also, Tuba et al. ('52) have shown that serum alkaline phosphatase in the young rat is influenced by the level of fat intake and, indirectly, by the daily food consumption, it is not impossible to reconcile the findings of Chu et al. ('48), who found no significant differences in phosphatase values among adult rats in paired feeding experiments, and those of Vivanco and Díaz ('51) who found elevated values in young rats when compared to normals (diet of normals not specified).

With respect to the effect of odoratism on the adrenal glands, recent observations in this laboratory seem to indicate that odoratism in rats involves a disturbance of collagen metabolism. It appears, for instance, that the tissues which are high in collagen, namely, skin, bone and blood vessels, are the ones which are primarily affected in odoratism. The skin becomes extremely fragile in advanced odoratism and tears with ease in the living rat when a hypodermic needle is inserted subcutaneously. The total skin weight is reduced by 30% when compared on the basis of surface area to that of pair-fed control rats. The corium is usually hemorrhagic as are the bones and periosteal tissues. The bones are malformed. The incisors are much more easily extracted than in control rats, indicating a change in the periodontal membrane. Recently Ponseti and Baird ('52) have reported the occurrence of aneurysms of the thoracic aorta in rats with odoratism. The observation of Lewis et al. ('48) that new formation of bone around the shaft bears a resemblance microscopically to advanced scurvy is also consistent with this hypothesis. An involvement of the adrenal glands is not inconsistent with this hypothesis, since it is generally agreed that the adrenals are implicated in the so-called collagen diseases in humans.

It might also be postulated that the priapism and urinary distension of the bladder which are frequently seen in these rats might be referable to an hormonal imbalance, since it has been shown that priapism can be induced in rats by the administration of cortisone (Aterman and Greenberg, '52) and that squamous cell metaplasia in the urethra can be caused by the administration of estrogen (literature cited by Rezek et al., '51). It is probable, however, that these symptoms together with the frequently seen paralysis of the rear legs are caused by a spinal lesion resulting from the severe spinal curvature with consequent compression of the cord (Lewis et al., '48). Doctor Harold Koenig of our Anatomy Department has recently examined one of the author's rats suffering from paralysis and urinary distension and has reported that the spinal cord was completely severed at the point of angulation of the spine.

Table 1 shows that the effect of advanced odoratism on the adrenal glands is greater in the male than in the female. Although there is no proved relationship between human lathyrism and odoratism in the rat, it is of interest in this connection to note that the young human adult male is much more susceptible to lathyrism than are women, children or elderly men (Stockman, '29).[4] That the onset of human lathyrism may be occasioned in part by a reaction to stress under conditions of adrenal insufficiency might be inferred from the fact that "In man the symptoms usually begin suddenly and seem often to be precipitated by exposure to cold and wet and fatigue" (Stockman, '29).

SUMMARY AND CONCLUSIONS

1. Hemoglobin levels, serum alkaline phosphatase levels and adrenal weights have been determined separately in male and in female rats receiving diets containing 50% sweet peas *(Lathyrus odoratus)* for 21 days. These values have been

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[4] In rats consuming a diet containing 50% sweet peas, there is no distinguishable difference between males and females in the severity of the disease or in the rapidity of its development.

compared to those of rats pair-fed a 50% edible pea diet, to those of rats fed a 50% edible pea diet ad libitum, and to those of rats fed commercial stock rat diets.

2. The levels of hemoglobin and of serum alkaline phospha-tase appeared to be more dependent upon non-toxic variations in the diet and upon the nutritional status of the growing rat than upon the toxicity of the diet. It was concluded that an edible pea diet is a very inadequate control diet for a ration containing *Lathyrus odoratus* when investigations involving the determinations of the levels of certain constituents in the blood are to be made.

3. Adrenal weights in male rats suffering from advanced odoratism were significantly greater than the adrenal weights of larger control animals of the same age. This was true whether the control animals were pair-fed with the experi-mental group or whether they received the control diet ad libitum.

4. Adrenal weights in female rats suffering from advanced odoratism were not significantly different from adrenal weights of control animals of the same age.

5. Adrenal weights per unit of body weight were signifi-cantly higher both in female and in male rats suffering from odoratism than those in control animals pair-fed with the ex-perimental group or fed the control diets ad libitum.

6. It is suggested that the effect of odoratism on the adre-nals may be secondary to a disturbance in collagen metabolism.

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THE URINARY EXCRETION OF TRYPTOPHAN BY HUMAN SUBJECTS ON CONTROLLED DIETS VARYING IN LEVELS AND SOURCES OF PROTEIN [1]

ERNESTINE I. FRAZIER

WITH THE ASSISTANCE OF ALLENE L. STUTTS

Department of Home Economics, Agricultural Experiment Station,
Alabama Polytechnic Institute, Auburn

Since the development of microbiological procedures for the quantitative estimation of tryptophan in urine (Schweigert, Säuberlich and Elvehjem, '45), a number of studies have been reported on 24-hour excretion levels of "free" tryptophan (Steele, Säuberlich, Reynolds and Baumann, '47; Frankl and Dunn, '47; Woodson, Hier, Soloman and Bergeim, '48; Eckhardt and Davidson, '49; Harvey and Horwitt, '49; and Nasset and Tully, '51). Berg and Rohse ('47), using a chemical method studied the excretion of "free" tryptophan by 7 normal men. Two of the studies cited above, Woodson et al. ('48) and Eckhardt and Davidson ('49), reported values for excretion of the "free" and the "bound" forms while Steele and her associates ('50) studied the total amount of tryptophan available to the test organism in the urine of human subjects after alkaline hydrolysis. The values reported in the literature have, for the most part, been on uncontrolled dietary intakes or on calculated dietary nitrogen and tryptophan.

Steele et al. ('47) were unable to find any correlation between the source of dietary protein and the excretion of "free" tryptophan by human subjects maintained for 6 days on diets with the protein derived mainly from eggs and soybeans. These findings were confirmed by Nasset and Tully ('51), who studied the relation of the biological value of the protein ingested and the level of excretion of 10 essential amino acids, and found no correlation between the "free" tryptophan excreted and the source of protein when a nitrogen-free diet was supplemented with casein, eggs, wheat gluten or dried beef. Steele and her associates ('50) further reported no significant differences in the amounts of total tryptophan in the urine of 4 men subjects on three widely varying levels of dietary protein, namely, 25, 100 and 200 gm per day.

In the course of a series of studies on the metabolism of nicotinic acid by human subjects maintained on diets varying in levels and sources of protein, data have been obtained on 24-hour excretion levels of "free" and total tryptophan on constant dietary intakes of nitrogen over periods of from 14 to 21 days. The results of these studies are reported here.

EXPERIMENTAL SUBJECTS [2]

Twelve college women ranging in age from 19 to 24 years served as experimental subjects during three metabolism studies. The subjects were all in good general health as confirmed by the results of physical examinations. The basal energy requirement reported for each subject is the average of duplicate determinations made with a Benedict-Roth respiration calorimeter. The physical characteristics of the three groups of subjects are listed in table 1.

PLAN OF STUDY AND EXPERIMENTAL DIETS

The total length of time of the studies varied from 5 to 9 weeks and was divided into a control and a basal period. All

subjects ate weighed diets of constant composition throughout the entire study. After an adjustment period of from three to 7 days on each of the two dietary regimes (control and basal), weekly 4-day collection periods were instituted.

The control diet provided, from a variety of ordinary food sources, the National Research Council's (NRC) recommended allowances ('48) of the known dietary essentials for

TABLE 1

The experimental subjects

STUDY	SUBJECT	AGE	WEIGHT NET	HEIGHT	SURFACE AREA	BASAL MET.[1] CAL./M²/ HR.
		yrs.	*kg*	*cm*	*m²*	
I	M.H.	20	65.1	165.6	1.73	35.3
	L.W.	20	53.4	163.9	1.57	29.7
	E.W.	19	65.4	173.4	1.78	. . .
II	D.K.	23	51.4	165.1	1.56	. . .
	B.S.	22	50.1	165.1	1.54	30.0
	L.W.	21	53.4	165.1	1.58	30.0
	M.T.	20	55.2	172.1	1.65	. . .
	G.D.	20	64.5	165.1	1.71	. . .
III	M.H.	22	65.0	165.1	1.73	35.2
	F.P.	24	71.4	167.6	1.81	32.4
	E.P.	23	71.0	160.0	1.74	33.5
	B.W.	19	68.4	172.0	1.81	32.2
Average		21	61.2	166.7	1.68	32.3

[1] Dubois and Dubois formula.

the age and sex group studied. This diet supplied approximately 1 gm of protein per kilogram of body weight. The protein of the basal diet, which was planned to furnish a minimum level of nicotinic acid from dietary sources with all other nutrients approaching the NRC allowances, was derived mainly from washed cottage cheese, skim milk and corn products.

Variations in the composition of the basal diets between studies were as follows: in study I, 100 gm of cottage cheese were included in the diet with all other foods remaining

constant (basal I); in study II, periods (a) and (b), the amount of cottage cheese was increased to 110 gm (basal II a) and 150 gm (basal II b), respectively; and in study III, 50 gm of the cottage cheese in basal I were replaced with 75 gm of raw peanuts (basal III a) and 50 gm of peanut butter (basal III b).

The number of collection periods and the order of control and basal dietary regimes varied between studies. For example, in study I the control diet preceded the basal diet with three collection periods during each dietary regime, while in study II the basal diet was fed for a total of 5 weeks followed by a two-week control period. Similarly, two of the subjects in study III were on the basal supplemented diet for three weeks (two collection periods) before receiving the control diet. The other two subjects were studied during the control regime period before being placed on the basal diet.

During the control periods the average total nitrogen intake was 9.80 gm per day with a range of 9.50 to 10.11 gm. The average tryptophan content of the control diet was 885 mg per day. The total nitrogen intakes during the basal periods were 7.10, 7.34, 8.42, 9.51 and 8.85 gm per day for basal diets I, II(a), II(b), III(a) and III(b), respectively. The corresponding tryptophan intakes in milligrams per day were 521, 540, 616, 594 and 552.

The estimates of the tryptophan content of the diets obtained by assay of diet composites were corrected for losses during hydrolysis. An over-all correction factor of 1.47 was derived from data obtained in a study of internal recoveries of DL-tryptophan added to individual foods prepared for serving (Frazier and Stutts, '52). Since this factor is identical to the one found by Futrell et al. ('52) to correct for losses incurred in the hydrolysis of samples of diet composites, the values reported here probably approach the actual amounts ingested.

The calorie intakes during both control and basal regimes were adjusted to body weight maintenance levels. The range

was from 31 to 40 calories per kilogram. The average total calorie intake found to be adequate for maintaining body weight for this group of subjects was approximately 2,000 with a range of from 1,950 to 2,073 calories.

COLLECTION OF SAMPLES AND ANALYTICAL PROCEDURES

A one-day diet composite containing an extra weighed portion of all foods eaten except sugar and margarine was made during each 4-day collection period. The foods were homogenized in a Waring Blendor. The homogenate was either sampled for immediate analysis, or portions stored at 0°C. Four 24-hour urine collections were made during each weekly study period in bottles containing 5 ml of toluene and refrigerated at 5°C. Composites containing one-tenth of each of the 4 daily urine volumes were frozen and stored at 0°C. until analyzed for nitrogen and tryptophan. Fecal collections were made over the same 4-day period with carmine as a marker. Five per cent by weight of concentrated H_2SO_4 was added to each collection which was immediately frozen. At the time of analysis the samples were thawed, homogenized in a Waring Blendor and sampled for nitrogen determination. Analysis of all stored samples was completed within one month after the end of the study.

Total nitrogen in the diet, urine and fecal composites was determined by the Macro-Kjeldahl procedure according to the modifications of Hiller, Plazin and Van Slyke ('48). Tryptophan assays were carried out according to the microbiological method of Säuberlich et al. ('46) with *Lactobacillus arabinosus* as the test organism. "Free" tryptophan represents that amount available to the organism in diluted unhydrolyzed urine samples adjusted to pH 6.8. For total tryptophan in urine composites, 10 ml aliquots were autoclaved with 5 ml of $5 N$ NaOH for 8 hours at 15 pounds' pressure. Two-gram samples of the diet composites were hydrolyzed with 8 ml of $5 N$ NaOH for a similar period of time. The urine and diet hydrolysates were adjusted to pH 6.8, filtered and diluted to appropriate volumes. The values

TABLE 2

Nitrogen balance and tryptophan excretion per 24 hours [1]

SUBJECT	NITROGEN BALANCE	TRYPTOPHAN EXCRETED			INTAKE EXCRETED AS "FREE" TRYPTO-PHAN	RATIO "BOUND" "FREE"
		Total	"Free"	"Bound"		
	gm	*mg*	*mg*	*mg*	*%*	
Control diet						
M.N.	0.47 (2)	36.0 (3)	13.5 (3)	22.4	1.5	1.66
L.W.	1.62 (2)	26.2 (3)	7.9 (3)	18.3	0.9	2.31
E.W.	0.44 (2)	53.6 (3)	17.6 (3)	36.0	2.0	2.05
D.K.	1.46 (1)	26.6 (1)	9.9 (1)	16.7	1.1	1.68
B.S.	1.22 (1)	25.0 (1)	9.5 (1)	15.5	1.1	1.63
M.T.	0.77 (1)	29.6 (1)	11.1 (1)	18.5	1.3	1.67
D.G.	0.89 (1)	29.1 (1)	11.5 (1)	17.6	1.3	1.53
M.H.	— 0.23 (1)	34.5 (1)	11.6 (1)	22.9	1.3	1.97
F.P.	0.16 (1)	30.9 (1)	13.0 (1)	17.9	1.5	1.38
E.P.	0.84 (1)	23.1 (1)	7.0 (1)	16.1	0.8	2.30
B.W.	1.02 (1)	22.7 (1)	6.8 (1)	15.9	0.8	2.34
Mean		30.6	10.8	19.8	1.2	1.83
S.D.		± 1.91	± 0.73	± 1.38		
Basal no. I and II (a)						
M.N.	0.20 (3)	28.5 (2)	9.6 (2)	18.8	1.8	1.96
L.W.	0.89 (2)	24.1 (2)	7.1 (2)	17.0	1.4	2.39
E.W.	0.14 (2)	32.5 (3)	10.0 (3)	22.5	1.9	2.25
D.K.	— 0.63 (1)	22.7 (3)	11.2 (3)	11.5	2.1	1.02
B.S.	0.08 (1)	23.4 (3)	10.4 (3)	12.9	1.9	1.24
L.W.	0.02 (1)	22.7 (3)	9.5 (3)	13.1	1.8	1.38
M.T.	. . .	26.1 (1)	11.8 (1)	14.3	2.2	1.21
G.D.	. . .	27.7 (1)	12.9 (1)	14.8	2.4	1.15
Mean		25.9	10.3	15.6	1.9	1.51
S.D.		± 2.24	± 0.86	± 1.62		
Basal no. II (b)						
D.K.	0.82 (1)	27.9 (2)	10.5 (2)	17.3	1.7	1.65
B.S.	0.98 (1)	23.1 (2)	11.2 (2)	11.5	1.8	1.03
L.W.	— 0.22 (1)	24.3 (2)	9.4 (2)	14.9	1.5	1.59
M.T.	0.50 (1)	26.0 (2)	11.8 (2)	14.7	1.9	1.25
G.D.	. . .	32.0 (1)	13.5 (1)	18.5	2.1	1.37
Mean		26.6	11.3	15.3	1.8	1.35
S.D.		± 2.83	± 1.08	± 2.05		
Basal no. III (a)						
M.H.	0.98 (2)	37.6 (2)	13.6 (2)	23.7	2.3	1.74
F.P.	0.38 (2)	34.6 (2)	15.2 (2)	19.8	2.6	1.30
Mean		36.1	14.4	21.7	2.4	1.51
Basal no. III (b)						
E.P.	0.70 (2)	31.1 (2)	9.7 (2)	21.4	1.8	2.21
B.W.	— 0.23 (2)	24.4 (2)	7.3 (2)	17.4	1.3	2.38
Mean		27.7	8.5	19.4	1.5	2.28

[1] The numbers in ( ) indicate number of study periods included in average values.

reported represent two replicates at three dilutions on duplicate samples in an assay volume of 2 ml. "Bound" tryptophan represents the difference in the values obtained from hydrolyzed and unhydrolyzed samples of the urine composites.

RESULTS AND DISCUSSION

The average daily excretion of total, "free" and "bound" tryptophan for each of the 12 subjects on the control and basal diets as well as nitrogen balance data for corresponding collection periods are shown in table 2.

All subjects except one in each group were either storing nitrogen or approaching equilibrium. The amount of total nitrogen stored or lost in the urine did not seem to affect the amount of tryptophan excreted.

The results confirm the observations of Eckhardt and Davidson ('49), Steele et al. ('50) and Nasset and Tully ('51) that the level of total nitrogen intake and the source of dietary protein have little effect on the absolute amount of tryptophan excreted by humans. The average amounts of "free" tryptophan excreted by this group of subjects were approximately the same, 10.8 ± 0.73, 10.3 ± 0.86 and 11.3 ± 1.08 mg per day, on the control diet and on basal diets I and II, respectively. The average excretions of the "bound" form of tryptophan were slightly higher on the control diet. The average of 5 mg per day increase in the amount excreted during this period over the basal level of excretion is not proportional, however, to the differences in the nitrogen and tryptophan content of the diets during the two dietary regimes: namely, 2.58 gm of nitrogen and 355 mg of tryptophan. Similarly, an increase of approximately 1 gm of dietary nitrogen with a resulting increase of 70 mg of tryptophan had no effect on the average amount of tryptophan excreted by 5 subjects (basal periods I and II).

In general there was a fairly characteristic pattern of excretion of the "free" form of tryptophan by the individual subjects between collection periods as well as between study periods. The per cent of intake excreted in the "free" form

ranged from 0.7 to 2.4 with over-all averages of 1.2, 1.9, 1.8, 2.4 and 1.5 for the control, basal I and II(a), basal II(b) and basal III(a) and (b), respectively. Any increase in the total amount excreted was due to an increase in the "bound" form, or peptide. That the increased amount of individual amino acids available to the test organism after hydrolysis of human urine represents conjugated forms is supported by the recent work of Stein ('53) who studied the amounts of individual amino acids in untreated and hydrolyzed samples of urine

TABLE 3

Variation in nitrogen balance and tryptophan excretion

STUDY PERIODS	CONTROL						BASAL			
Collection period	I	II	III	I	II	III	IV	V	VI	VII
Subject, E. W.										
N Balance, gm		0.91	0.03	0.08	— 0.33	0.84				
Tryptophan, mg										
Total		49.9	57.4	31.7	25.6	40.3				
"Free"		16.6	18.4	10.9	6.6	12.5				
"Bound"		33.3	39.0	20.8	19.0	27.8				
Subject, L. W.										
N Balance, gm		1.80	1.44	1.60	0.20			— 0.22		
Tryptophan, mg										
Total	24.6	24.3	29.7	23.2	25.0	22.4	23.9	21.7	22.1	26.5
"Free"	7.6	7.5	8.7	6.7	7.6	9.2	10.7	8.8	9.3	9.5
"Bound"	17.0	16.8	21.0	16.5	17.4	13.2	13.2	12.9	12.8	17.0

by means of chromatographic techniques. The author found "about 2 gm of amino acids (are) excreted per day in the conjugated forms." The average amount of tryptophan in the urine of the subjects in the present study increased from two and one-half to three times after hydrolysis. The ratio of the "free" to the "bound" form excreted per 24 hours ranged from 1.02 to 2.38 (last column, table 2).

Although it may be assumed that subjects on a weighed diet of constant composition for periods of from 14 to 21 days were in a fairly stable state of equilibrium with respect to

total nitrogen, some variations in the pattern of excretion of individual subjects were observed. Variability in nitrogen balance and the amount of tryptophan excreted by two subjects is shown in table 3. The data for E. W. showed greater variation between collection periods than any of the other subjects, and for the most part, a higher level of excretion. On the other hand, the data for L. W. showed a more uniform pattern of excretion which was also typical of the other subjects. This subject was studied during the basal periods of the first and second study. The lack of conformity of the metabolic pattern of E. W. as compared to that of the other subjects was also observed in the parallel study of nicotinic acid metabolism (Frazier, '52).

The amount of nicotinic acid ingested during this series of studies ranged from 6.46 to 22.1 mg per day. There was no apparent relationship between the nicotinic acid intake and the excretion of tryptophan on intakes of 500 mg or more of the amino acid (Frazier, '52).

Thompson and Kirby ('49) reported gross correlations between the amounts of amino acids excreted and body weight. On the other hand, Frankl and Dunn ('47), Schweigert et al. ('46), Woodson et al. ('48) and Steele and her associates ('50) were unable to find any correlation between levels of excretion and body weight. A study of the data in the present investigation failed to show any apparent relationship between body weight in kilograms, surface area, creatinine excretion, urine volume or basal calories.

The relatively constant level of excretion of "free" tryptophan by humans and the constant ratio of the "free" to the conjugated form suggest a selective mechanism of reabsorption by the tubules from the glomerular filtrate. Eckhardt and Davidson ('49) and Steele and associates ('50) have pointed out the phenomenal power of reabsorption of amino acids by the tubules. This observation is confirmed in the present study for the essential amino acid tryptophan. Beyer et al. ('46) were unable to exceed the reabsorptive capacity of the tubules at feasible levels of infusion of L-tryptophan

TABLE 4

Summary of data from the literature on tryptophan excretion by human subjects on controlled diets

INVESTIGATOR	SUBJECTS NO.	INTAKE		TRYPTOPHAN EXCRETION					
		Nitrogen	Trypto-phan	Total		"Free"		"Bound"	
				Av.	Range	Av.	Range	Av.	Range
		gm	mg	mg	mg	mg	mg	mg	mg
Present study	11	9.80	885	30.6	22.7–53.6	10.8	6.8–17.6	19.8	15.9–36.0
	8	7.22	530	25.9	22.7–32.5	10.3	7.1–12.9	15.6	11.5–22.5
	5	8.42	616	26.6	23.1–32.0	11.3	9.4–13.5	15.3	11.5–18.5
	2	9.51	594	36.1	34.6–37.6	14.4	13.6–15.2	21.7	19.8–23.7
	2	8.85	552	27.7	24.4–31.1	8.5	7.3–9.7	19.4	17.4–21.4
Harvey and Horwitt, '49	20	8.88[1]	520			11.6	6.0–22.0		
Steele et al, '47	4	5.15	480			8.0	5.0–11.0		
Eckhardt and Davidson, '49	1	0		19.4		15.8		3.6	
		12.0[1]		30.3		23.4		6.9	
		16.0[1]		36.2		27.4		8.8	
Steele et al, '50	4	4.0[1]	310	31.0	22.0–46.0				
		16.0[1]	1160	41.0	29.0–70.0				
		32.0[1]	2370	33.0	22.0–42.0				
Nasset and Tully, '51	10	0.29	290			6.1			
		4.88	430			7.4			
		4.54	304			7.8			
		5.33	312			7.8			
		4.96				7.6			

[1] Calculated from author's data.

in dogs. These investigators found that at increased plasma concentrations of 10 times above fasting levels reabsorption occurred at the rate of 7.5 mg per minute.

The physiological economy in the retention of this amino acid by the body is of particular interest in view of its role as a precursor of nicotinic acid. Rose ('49) found that an intake of 250 mg of tryptophan per day was sufficient to maintain nitrogen equilibrium in adult man. The "recommended intake" of 500 mg per day from dietary sources seems to be adequate or in excess of the body's requirement under the conditions of this study.

The amounts of total, "free" and "bound" forms of tryptophan excreted by the 12 women studied are within the range of the values reported by other investigators for human subjects maintained on controlled diets using microbiological techniques. This is shown in the data from the literature in table 4.

The low values for "free" tryptophan reported by Steele et al. ('47) and Nasset and Tully ('51) were probably due to the preservation of the urine samples with acid. It may be observed that the average amount of "free" tryptophan excreted by the 20 men subjects in the study reported by Harvey and Horwitt ('49) is practically the same as the average values found for the women subjects in the present study. The conditions of the two studies were comparable with respect to intake and length of time.

SUMMARY

The amounts of "free" and total tryptophan excreted per 24 hours by 12 young adult women maintained on diets varying in levels and sources of protein have been studied. The amounts of the conjugated forms in the urine were derived by difference.

On constant intakes of a control diet providing 9.8 gm of protein and 885 mg of tryptophan over periods of from 14 to 21 days, the mean levels of excretion were, 30.6 ± 1.91, 10.8 ± 0.73 and 19.8 ± 1.38 mg per day for total, "free" and "bound" tryptophan, respectively.

The same subjects were fed a basal diet with total nitrogen intakes of from 7.10 to 8.42 gm and tryptophan intakes of from 521 to 616 mg per day for 14 to 35 days. On this regime there was a slight decrease in the average amount of the "bound" form excreted (from 19.8 to 15.5 mg per day) while the average amount of "free" tryptophan excreted was the same: namely, 10.8 mg per day on both control and basal dietary regimes.

There was no apparent relation between the amounts of total, "free" and "bound" tryptophan excreted and the source or amount of dietary nitrogen. Further, the amount of tryptophan excreted was not related to body weight, surface area, basal calories or urine volume. The data presented indicate a selective reabsorption of this amino acid by the tubules from the glomerular filtrate.

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THE EFFECTS OF VITAMIN B_{12}, ORGAN EXTRACTS, YEAST AND ANTIBIOTICS ON EMETINE TOXICITY IN RATS

K. GUGGENHEIM AND S. HALEVY

Laboratory of Nutrition, Department of Biochemistry, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

The toxicity of various drugs may be counteracted by vitamin B_{12}, liver extract or yeast. Thus rats and mice were protected by vitamin B_{12} from the toxic action of carbon tetrachloride (Hove and Hardin, '51; Mushett, '50; Popper, Koch-Weser and Szanto, '49; Koch-Weser, Szanto, Farber and Popper, '50), pyridine (Dinning, Keith, Parsons and Day, '50), atabrine (Ershoff, '50a), and DL-thyroxine (Sure and Easterling, '50). Whole liver or its fractions have been shown to minimize the deleterious effects of massive doses of strychnine (Batelli, '40), sulfanilamide (Chamelin and Funk, '43), promin (Higgins, '44; Ershoff and Williams, '49), atabrine (Ershoff, '48), dinitrophenol (Ershoff, '48) and pyridine (Dinning, Keith, Parsons and Day, '50). Yeast has been demonstrated to increase the tolerance of rats to atabrine (Ershoff, '48). It was found in this laboratory that rats maintained on a low protein diet exhibit a diminished tolerance to emetine (Guggenheim and Buechler, '48). This paper reports the results of a study on the effect of vitamin B_{12}, organ extracts, yeast and antibiotics on the tolerance to emetine of both protein deficient and well nourished rats.

METHODS

Young male rats, weighing 40 to 50 gm were fed diets of normal or restricted protein content (table 1). After three weeks on the diet each rat was given daily 0.3 mg emetine

hydrochloride per 100 gm body weight by subcutaneous injection. The diet was maintained during the period of treatment.

The survival time from the beginning of treatment to death from emetine poisoning was used as an index of drug tolerance.

The organ extracts were prepared by the manufacturer [1] as follows: dried liver powder, fresh beef muscle, heart of kidney, respectively, were digested with papain, extracted with alcohol and filtered. The filtrate was concentrated by vacuum distillation until it contained 280 mg of dry substance per milliliter. A tenth or 0.25 ml of this extract was injected subcutaneously each day.

TABLE 1

Composition of diets

INGREDIENT	C 3	C 9	C 18	Y 6	C 15 Y 6
	%	%	%	%	%
Casein	3	9	18		15
Dried yeast				6	6
Corn starch	88	82	73	85	70
Olive oil	5	5	5	5	5
Salt mixture	4	4	4	4	4
Protein content, %	2.4	7.2	14.4	2.4	14.4

The diets were supplemented with the following vitamins (mg per 100 gm ration): Thiamine 0.2, riboflavin 0.3, pyridoxine 0.1, calcium pantothenate 1.6, niacin 5.0, pteroylglutamic acid 0.25, and choline chloride, 100. Each rat received 100 I.U. vitamin A and 4 I.U. vitamin D twice weekly.

Aureomycin and streptomycin were each incorporated into the rations at a level of 50 mg per kilogram of diet.

Liver nitrogen was determined by the Kjeldahl method, amino nitrogen by the formol titration procedure described by Melnick and Oser ('49), and vitamin B_{12} according to Burkholder ('51).

RESULTS

The effect of various levels of dietary protein on emetine tolerance is shown in table 2. As can be seen from the table a

[1] Organ extracts and vitamin B_{12} were kindly supplied by Teva, Middle East Pharmaceutical and Chemical Works, Jerusalem, Israel, aureomycin by Lederle Laboratories Division, and streptomycin by E. R. Squibb and Sons.

TABLE 2

Tolerance of rats to emetine

EXP.	NO. OF RATS	DIET	TREATMENT	DURATION OF TREATMENT [1]	AVERAGE FOOD CON- SUMPTION BEFORE EMETINE TREAT- MENT	AVERAGE WEIGHT CHANGE DURING FIRST 3 WEEKS	SURVIVAL TIME MEANS AND STANDARD ERRORS
					gm/day	*gm*	*days*
1	38	C 3	..	..	4.8	— 9	9.6 ± 0.68
2	19	C 3	Vitamin B_{12}	Experiment	4.6	— 5	11.5 [2]± 0.66
3	15	C 3	Vitamin B_{12}	Emetine	3.8	—11	9.1 ± 0.66
4	16	C 3	Liver, 0.1 ml	Experiment	3.6	—10	10.6 ± 0.62
5	14	C 3	Liver, 0.25 ml	Experiment	4.4	— 9	14.9 [3]± 1.85
6	17	C 3	Muscle, 0.25 ml	Experiment	4.9	—10	14.6 [4]± 0.95
7	15	C 3	Heart, 0.25 ml	Experiment	5.0	—10	14.9 [4]± 0.27
8	18	C 3	Kidney, 0.25 ml	Experiment	4.3	—11	12.9 [3]± 1.00
9	18	C 3	Liver, 0.25 ml	Emetine	4.8	—11	16.7 [4]± 1.44
10	17	C 3	Amino acids	Experiment	4.2	— 9	12.9 [4]± 0.54
11	17	Y 6	Yeast	Experiment	5.7	— 2	11.4 ± 0.80
12	17	C 3	Aureomycin	Experiment	5.0	— 4	13.2 [4]± 0.40
13	17	C 3	Aureomycin	Emetine	4.5	— 6	12.5 [4]± 0.49
14	17	C 3	Streptomycin	Experiment	5.9	+ 2	13.8 [4]± 0.41
15	16	C 9	..		7.6	+ 23	12.5 ± 0.48
16	16	C 9	Vitamin B_{12}	Experiment	7.1	+ 20	12.6 ± 0.53
17	38	C 18	..		8.6	+ 53	11.9 ± 0.47
18	22	C 18	Vitamin B_{12}	Experiment	9.2	+ 53	12.0 ± 0.36
19	18	C 18	Liver, 0.25 ml	Experiment	9.6	+ 54	15.6 [4]± 1.00
20	17	C 18	Muscle, 0.25 ml	Experiment	8.7	+ 53	15.0 [3]± 0.95
21	16	C 18	Heart, 0.25 ml	Experiment	9.6	+ 60	13.6 [2]± 0.72
22	18	C 18	Kidney, 0.25 ml	Experiment	9.2	+ 58	14.7 [4]± 0.74
23	18	C15 Y6	Yeast	Experiment	7.2	+ 42	12.8 ± 0.55
24	16	C 18	Aureomycin	Experiment	8.4	+ 60	12.4 ± 0.52
25	17	C 18	Streptomycin	Experiment	8.3	+ 59	12.9 ± 0.38

[1] Indicates whether the supplement was given from start of experiment or start of emetine treatment only.

[2] Significant at 5% level.

[3] Significant at 1% level.

[4] Significant at 0.1% level.

low protein diet (experiment 1) decreases the resistance to emetine in comparison with diets of moderate or normal protein content (experiments 15 and 17), thus confirming our previous observations (Guggenheim and Buechler, '48).

In experiments 2, 3, 16 and 18, the effect of daily subcutaneous injections of 0.5 µg vitamin B_{12} was studied. It was found that vitamin B_{12} significantly increased the emetine tolerance of rats maintained on a low protein diet, only if administered from the beginning of the diet. If, however, vitamin B_{12} was given immediately before emetine treatment was started and after the rats had been on the low protein diet for three weeks, no protection was obtained. In rats on normal or only slightly reduced protein content, vitamin B_{12} was without effect on tolerance to emetine. It is noteworthy that the protective effect of vitamin B_{12} was not accompanied by stimulation of food consumption or diminished weight loss. This is at variance with the findings of Black and Bratzler ('52), who noted improved food utilization by rats under the influence of vitamin B_{12}.

The results of the subcutaneous injection of liver extract (experiments 4, 5, 9 and 19) are also shown in table 2. Small doses of the liver extract, such as 0.1 ml per day, were without effect on emetine tolerance. A dose of 0.25 ml per day, however, prolonged significantly the survival time of rats on a low protein diet, whether given from the start of the diet, or only when emetine was started. Moreover, liver extract increased to a highly significant degree the emetine tolerance even of rats fed a full diet. This effect of liver extract was not accompanied by stimulation of appetite or improved food utilization. Similarly, extracts of muscle, heart and kidney administered from the start of the diet, increased significantly the survival time of rats on both protein-deficient and full diet (experiments 6, 7, 8, 20, 21, 22). These extracts, too, were without any influence on food intake or weight increase.

The question arose as to whether the protective effect of organ extracts in protein-depleted rats might be due at least partly to their content of essential amino acids. Since liver

extract proved to be the most effective among the organ extracts, an amino acid mixture was prepared so as to contain the same amounts of essential amino acids present in the liver extract. The concentration of amino acids in liver extract according to Block and Bolling ('51) was adjusted on the basis of 280 mg dry substance per milliliter. Daily injections of the amino acid mixture also protected the protein depleted rats against emetine poisoning (experiment 10).

On the other hand, as demonstrated by the following experiment, the protective effect of liver extract in protein depleted rats is not caused by the essential amino acids it supplies.

TABLE 3

Nitrogen content of livers of rats

DIET	NO. OF RATS	TREATMENT	NITROGEN CONTENT MEANS AND STANDARD ERRORS
			mg/gm
C 18 [1]	25	....	32.7 ± 0.88
C 3	24	....	22.7 ± 0.53
C 3	12	Amino acids	26.3 [2] ± 0.65
C 3	11	Liver extract, non-hydrolyzed	22.5 ± 0.54
C 3	11	Liver extract, hydrolyzed	25.0 [2] ± 0.48
C 3	18	Vitamin B_{12}	22.6 ± 0.49
C 3	12	Aureomycin	23.4 ± 0.36

[1] Guggenheim and Buechler-Czaczkes ('50).
[2] Significant at 0.1% level.

Harrison and Long ('45); Guggenheim and Buechler-Czaczkes ('50); and Henry, Kosterlitz and Quenouille ('53) showed that the regeneration of liver protein in fasted or in protein depleted rats serves as a useful tool in assessing the nutritional value of food proteins. Groups of rats were therefore put on a low protein diet and injected daily with 0.25 ml liver extract or the amino acid mixture. After 12 days the rats were killed and the concentration of liver nitrogen determined.

As can be seen in table 3, this diet considerably reduced the level of liver nitrogen. Daily injections of amino acids partially prevented this fall in liver nitrogen. Liver extract, however, had no effect on the level of liver nitrogen. This apparent

discrepancy was resolved by injecting liver extract which was completely hydrolyzed by autoclaving with hydrochloric acid for 10 hours and supplemented with tryptophan. This treatment increased the amount of amino nitrogen of the preparation from 10.2 to 18.3 mg per milliliter. Daily injections of this completely hydrolyzed liver extract into rats on a low protein diet (C 3) significantly raised the level of liver nitrogen over that of untreated controls or of rats treated with the non-hydrolyzed extract. On the other hand, the difference in the level of liver nitrogen between the rats which received the hydrolyzed extract and those given the amino acid mixture was not significant. It follows, therefore, that the action of liver extract in increasing the tolerance to emetine in protein depleted rats is not due to its amino acid content, since these amino acids after subcutaneous injection were utilized metabolically only after complete hydrolysis of the liver extract. Neither can the effect of the liver extract be explained by its content of vitamin B_{12}, for 0.25 ml of the extract was found to contain only 0.07 μg vitamin B_{12}, whereas doses of 0.5 μg vitamin B_{12} were without influence on the toxicity of emetine, if the injections were started with the beginning of the emetine treatment.

In contrast to organ extracts, dried brewers' yeast was without any influence on the survival time, either in protein depleted or in normally fed animals (experiments 11 and 23).

In a last series of experiments the effects of aureomycin and streptomycin were studied (table 2, experiments 12, 13, 14, 24 and 25). Both these antibiotics significantly increased the survival time in protein-depleted rats, and in the case of aureomycin even when started at the beginning of emetine treatment. These antibiotics proved, however, to be without any influence on the emetine tolerance of rats kept on a full diet. It should be noted that feeding streptomycin along with the low protein diet C 3 stimulated food consumption and prevented loss of weight.

Neither vitamin B_{12} nor aureomycin increased the level of liver nitrogen in protein-depleted rats (table 3). Their effect

on emetine tolerance cannot, therefore, be explained by an improvement in the nitrogen economy of the body.

DISCUSSION

Our experiments show that vitamin B_{12}, organ extracts, aureomycin and streptomycin are able to increase tolerance to emetine in protein depleted rats, while yeast proved to be without any effect. Moreover, organ extracts even prolong the survival time of emetine-treated rats on full diets.

It may be argued that the effect of vitamin B_{12} may be ascribed to its action in promoting protein utilization. Vitamin B_{12} has been reported to improve protein efficiency (Goyco, '51; Rosenberg and Rohdenburg, '52) and utilization of protein (Henry and Kon, '51) and circulating amino acids for the formation of fixed tissues in normal (Charkey, Wilgus, Patten and Gassner, '50) and hyperthyroid rats (Rupp, Paschkis and Cantarow, '51), and to increase liver protein (Sahasrabudhe and Rao, '51; Dumm, Ralli, Gershberg and Laken, '52). However, other authors did not find any effect of vitamin B_{12} on protein utilization (Chow and Barrows, '50; Black and Bratzler, '52; Knoebel and Black, '52) and believe rather that it may play an important role in carbohydrate and fat metabolism (Chow and Barrows, '50; Ling and Chow, '52; Arnrich, Lewis and Morgan, '52). In our experiments vitamin B_{12} failed to increase growth or liver protein in protein-depleted rats, suggesting that its effect on emetine tolerance was due to some mechanism other than improved nitrogen utilization.

The beneficial effect of whole liver feeding against various noxious agents such as thyroxine, cortisone, dinitrophenol, atabrine and cold has been pointed out by Ershoff ('47; '48; '50b; '50c; '50d; '51a; '51b). Moreover, mammalian muscle (Tappan, Boldt and Elvehjem, '53) as well as desiccated and defatted kidney (Ershoff, 50d) have been found to counteract the toxicity of iodinated protein and of desiccated thyroid. It is possible that our injectable partially hydrolyzed organ extracts contained the same protective factor or factors as the preparations used by the above authors. But in contrast to

Ershoff's experiments ('48), we did not find any protective action of yeast.

Aureomycin has been reported to exert a sparing action on vitamin B_{12} (Cravioto-Munoz, Poncher and Waisman, '51), either by stimulating the intestinal synthesis of the vitamin or by inhibiting its destruction or utilization by intestinal micro-organisms (Davis and Chow, '51). It is difficult, however, to explain the action of aureomycin on emetine poisoning as an indirect vitamin B_{12} effect, since aureomycin was found to be active when administered after the rats had already been kept for three weeks on a low protein diet. Vitamin B_{12} proved to be ineffective under these conditions.

SUMMARY

1. Vitamin B_{12}, partially hydrolyzed extracts of liver, muscle, heart and kidney, aureomycin and streptomycin were found to increase the tolerance to emetine in protein-depleted rats, whereas yeast was without any effect.

2. The organ extracts exerted their protective action even in rats fed a full diet.

3. The protective effect of liver extract in protein depleted rats cannot be ascribed to its content of essential amino acids.

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THE AMINO ACID CONTENT OF ROE AT DIFFERENT STAGES OF MATURITY FROM THE FIVE SPECIES OF PACIFIC SALMON

HARRY L. SEAGRAN, DAVID E. MOREY
AND JOHN A. DASSOW

Fishery Products Laboratory, U. S. Fish and Wildlife Service and the Fisheries Experiment Commission of Alaska, Ketchikan

Many fishery products not used for human consumption have long been considered as valuable protein supplements for animal feeding. A typical example is the use of fish waste as feed for hatchery fish. According to a survey made by Tunison et al. ('49), over 29 million pounds of feed, including fish waste, were used in one year by hatcheries of the federal government and those of 38 states. The tremendous growth of animal feeding industries, coupled with increased feed costs and shortage of commonly used feed materials (Wigutoff, '52), such as flukey beef livers and horsemeat, has stimulated research into the possibilities of utilizing the huge potential of Alaska salmon cannery waste for animal feeds.

In an attempt to determine a component of salmon viscera that would, by reason of its superior food value for hatchery fish, allow at least a partial economical utilization of Alaska salmon cannery waste, experimental feeding tests with fingerling salmon have been carried out on the visceral components under actual hatchery conditions (Robinson et al., '49; Burrows et al., '51). The results of these investigations demonstrated that in all respects (total growth, conversion rate and mortality), salmon roe was definitely superior to any of the other visceral components.

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The purpose of the present study was to investigate the amino acid content of salmon roe, to make possible a further comparison between the quality of salmon roe protein and other protein supplements commonly used for hatchery fish and other animal feeds. The factors of species, maturity and individual variation have been considered in determining the amino acids arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. The microbiological method was used. The possible relation of the amino acid distribution in roe to the sexual maturity of the fish is important in view of the physical and chemical changes that occur in salmon during the spawning migration (Davidson and Shostrom, '36).

<div align="center">EXPERIMENTAL</div>

Source and preparation of samples

The roe from king *(Oncorhynchus tschawytscha)*, chum *(O. keta)*, sockeye *(O. nerka)*, coho *(O. kisutch)* and pink *(O. gorbuscha)* salmon were obtained locally. The skeins (and free eggs) of each species were sorted with respect to relative maturity, sealed in cans and stored at 0°F. until analyzed.

The maturity of the roe was estimated by means of the scale of Davidson and Shostrom ('36), which consists of giving arbitrary values of from 1 to 4 to the size and condition of the unfertilized eggs as they are found, either in ovaries or loose in the body cavity of the salmon. Eggs that were found to be small and compact in the ovaries were given a grade of 1. Those that had been shed from the ovaries into the body cavity were given a grade of 4. Intermediate stages of development were graded 2 or 3.

Samples were taken from the roe according to the two methods. In the first, which was used for a study of individual variation, 8 individual whole skeins of eggs of maturity grade 3 were selected from 8 pink salmon and blended separately with equal volumes of acetone in an electric blender.

In the second method, which was used for a study of group variation due to species and maturity, representative portions of roe were obtained for each sample from 6 or more fish. For grade-1, -2 and -3 roe, each of 6 or more individual skeins was cut at right angles to the skein length into three equal sections. The respective midsections for each sample were then combined and homogenized with an equal volume of acetone. In the case of the grade-4 eggs, a representative sample was withdrawn from the mixed eggs of 6 or more fish and treated as described above.

Lipid- and water-free materials for analysis were prepared from the resulting homogenates as follows: A portion of each homogenate was covered with an equal volume of acetone and permitted to stand overnight. Each sample was then transferred to an extraction thimble and subjected to continuous Soxhlet extraction with acetone for 16 hours, and with ethyl ether for 6 hours. (The ethyl ether extraction greatly facilitated the reduction of the resulting material to a fine powder by removing waxy components, such as lecithin, from the protein.) The preparations were air-dried at room temperature, finely ground, dried at 40°C. under 29 inches of mercury vacuum for one hour, and stored in airtight containers at room temperature.

Acid hydrolysates were prepared by autoclaving approximately 0.5 gm (accurately weighed) of the dried lipid-free preparations in sealed pyrex tubes with 20 ml of 3 N hydrochloric acid for 5 hours at 15 pounds pressure. Nitrogen determinations were carried out on triplicate aliquots of the filtered acid hydrolysates by the micro Kjeldahl method of Ma and Zuazaga ('42). Samples were digested for one hour after the first fuming. The slight residue remaining after acid hydrolysis contained no detectable nitrogen by the above method. Alkaline hydrolysates for tryptophan assays were prepared by autoclaving approximately 0.5 gm (accurately weighed) of the dried lipid-free preparations with 10 ml of 5 N sodium hydroxide for 10 hours at 15 pounds pressure in covered, stainless steel beakers. Nitrogen values

for tryptophan calculations were assumed from the nitrogen determinations on the acid hydrolysates. Aliquots of the hydrolysates were adjusted to pH 6.9 immediately before use.

TABLE 1

Organisms used in microbioassay for amino acids

AMINO ACID [1]	MICRO-ORGANISM	MAXIMUM STANDARD DOSAGE (L-isomer)
		µg per tube
Arginine	*S. faecalis* R	11.7
Histidine	*L. mesenteroides* P-60	3.2
Isoleucine	*L. mesenteroides* P-60	8.5
Leucine	*S. faecalis* R	10.4
Lysine	*L. mesenteroides* P-60	16.5
Methionine	*S. faecalis* R	3.1
Phenylalanine	*L. mesenteroides* P-60	5.9
Threonine	*S. faecalis* R	9.8
Tryptophan	*L. arabinosus* 17-5	1.4
Valine	*S. faecalis* R	9.8

[1] With the exception of L-arginine, all of the standards were of the DL-forms. All amino acid standards were dried *in vacuo* at 30°C. and kept over anhydrous calcium chloride *in vacuo*.

Assay procedure

The microbiological procedure and media [1] of Henderson and Snell ('48), with minor modifications, were used in this investigation. The different cultures used were as indicated in table 1.

Cultures were maintained by weekly transfers as recommended by the American Type Culture Collection. Inoculum was prepared and handled as described by Henderson and Snell ('48) with the exception that 10% of filtered, neutralized tomato juice was included in the inoculum growth medium.

[1] It was found necessary to use the DL-form of glutamic acid in the media for arginine, leucine and methionine assays, since objectionably high blanks resulted when the L-form (Nutritional Biochemicals Corp.) was used. The use of L-leucine (same source) in the media for methionine assays also resulted in high blanks, which were corrected by use of the DL-form. L-leucine was used in the media for isoleucine assays, because of the common occurrence of isoleucine in DL-leucine.

The addition of the tomato juice produced better growth with *L. mesenteroides* P-60 and yielded cells that were more easily centrifuged.

The lactic acid produced by the assay organisms was electrometrically titrated directly in the assay tubes with 0.04 N sodium hydroxide as described by Henderson, Brickson and Snell ('48). Each assay was made with 6 tubes at each of 6 levels of the amino acid standard and with three tubes at each of 5 levels of the samples.

RESULTS AND DISCUSSION

All amino acid values reported and discussed in this paper are expressed as per cent of protein, calculated to 16% nitrogen (grams amino acid per 16 gm nitrogen).

The results of the test for individual variation, which have been treated statistically (Youden, '51), showed no variation (at the 5% level) in amino acid distribution in the 8 pink salmon roe beyond that normally due to experimental error (table 2). The data in table 3 reveal a marked similarity in the "essential" amino acid content of salmon roe regardless of species and, within limits, of maturity.

In order to obtain more specific information concerning possible differences in the amino acid content due to species, the agreement among the means of the different species at maturity grade 3 was compared statistically (Youden, '51). With the exception of arginine, the difference between these means was not significant (at the 5% level) as measured by the agreement among determinations within species (table 4). The agreement among these means was quite variable, ranging from 1 (tryptophan) to 136 (arginine) % of the limiting value at the 5% level. Thus, additional determinations might detect greater differences between the species.

The data in table 3 suggest greater differences in amino acid distribution in salmon roe due to maturity than to any other factor considered. These results indicate an increasing content of most of the amino acids (on the basis of nitrogen content) with increasing maturity. The arginine and tryp-

tophan levels proved quite constant, however, while there was an indication that the threonine content decreased with maturity. These trends were much more noticeable during the most immature stages of the roe.

TABLE 2

Individual variation in amino acid content of roe from eight pink salmon of similar maturity [1]

(Values given as per cent of protein, calculated to 16% N)

AMINO ACID	MEAN	RANGE	STANDARD DEVIATION	F RATIO [2] F CRITICAL (5% LEVEL)
Arginine	7.23	6.78–7.59	0.29	0.39
Histidine	2.85	2.81–2.90	0.03	0.00
Isoleucine	6.94	6.74–7.12	0.13	0.01
Leucine	9.44	8.98–9.75	0.30	0.03
Lysine	8.86	8.74–9.08	0.13	0.02
Methionine	3.04	2.70–3.32	0.25	0.03
Phenylalanine	4.87	4.66–5.20	0.20	0.18
Threonine	5.14	5.01–5.41	0.13	0.03
Tryptophan	1.10	1.04–1.17	0.04	0.50
Valine	8.12	7.61–8.78	0.43	0.41

[1] All roe were moderately mature (maturity grade 3) with egg diameters 6.0 ± 0.5 mm.

[2] This term expresses to what degree individual variation of the roe has influenced amino acid content beyond the variation normally expected from experimental error. Only for numbers greater than unity is there a statistical indication (at the 5% level) that this variation exceeds experimental error (Youden, '51). The term ''F ratio/F critical (5% level)'' is derived as follows:

$$\frac{\text{F ratio}}{\text{F critical}} = \frac{\dfrac{\text{variance}_x}{\text{variance}_{STD}}}{\text{critical value F (5\% level)}},$$

where ''variance$_x$'' is the square of the standard deviation calculated for the 8 roe; ''variance$_{STD}$'' is the square of the standard deviation calculated from three to 5 separate assays for each amino acid on one homogeneous sample of pink salmon egg protein. ''Variance$_{STD}$'' is thus an estimation of the precision of the method (experimental error).

The means of the 4 maturity grades for king salmon roe were also compared statistically (Youden, '51). These statistical comparisons confirmed the apparent relation between amino acid content and maturity of the roe (table 4). At least for king salmon roe, the differences for arginine and

TABLE 3

Amino acid content¹ of salmon roe

(Values given as per cent of protein, calculated to 16% N)

	Species																
	CHUM			COHO			KING				PINK			SOCKEYE			
MATURITY GRADE²	2	3	4	1	2	3	1	2	3	4	2	3	4	1	2	3	4
EGG DIAMETER min./max. (in mm)	4.0/5.0	7/7.5	8/9	2/2.5	3.5/4.5	5.5/7	2/2.5	3.5/4.5	6/7	7.5/8	4.5/5.5	5.5/6.5	7/7.5	1/3	4/4.5	5/6.5	7/8
NO. OF SAMPLES	1³	4	3	2³	3	3	5	2	3	3	3	3	3	2³	3	4	3
Arginine	6.3	6.8	6.7	6.8	7.0	7.0	7.5	7.3	7.7	7.5	6.7	7.3	6.8	6.9	7.2	7.2	7.3
Histidine	2.7	2.8	3.0	2.4	2.7	2.8	2.3	2.5	2.6	2.5	3.0	2.8	2.9	2.5	2.7	2.7	2.9
Isoleucine	7.2	7.3	7.4	5.8	6.7	7.5	5.6	6.7	6.8	7.2	7.1	7.1	7.6	5.5	7.0	7.5	7.3
Leucine	9.0	9.8	10.0	8.8	10.7	10.0	8.2	9.3	9.4	9.8	10.4	9.9	10.6	9.1	10.1	10.2	10.7
Lysine	8.7	8.8	8.6	7.5	8.5	8.8	7.1	8.4	8.8	8.8	8.7	8.9	8.7	7.2	8.4	8.5	8.7
Methionine	2.6	2.9	2.9	2.5	2.7	2.7	2.6	2.8	3.0	3.0	2.8	2.8	2.7	2.2	2.8	2.8	2.8
Phenylalanine	5.1	4.8	5.0	4.4	5.0	4.9	4.4	4.9	4.8	4.8	4.9	5.0	4.8	4.2	4.8	4.8	5.0
Threonine	6.3	6.1	6.4	6.4	6.1	5.9	6.0	5.7	5.8	5.8	6.3	5.7	6.4	6.2	5.8	5.8	5.9
Tryptophan	0.9	0.9	1.0	0.9	1.0	0.9	0.9	0.8	0.9	0.8	1.0	1.0	1.1	0.8	1.0	0.9	1.1
Valine	8.3	7.1	7.2	6.8	7.1	7.1	6.1	7.0	7.0	7.1	7.0	7.7	7.3	6.3	7.6	7.3	7.7

¹ All values represent the mean of the individual determinations.

² An arbitrary scale indicating the degree of sexual maturity. Grade-1 roe are very immature; grade-4 roe are very mature.

³ Each sample thus noted is representative of one fish. All other samples are representative of 6 or more fish.

tryptophan were minor, whereas those for isoleucine, leucine, lysine, methionine and threonine were significant (at the 5% level). Valine and histidine differences approached the significance level. In addition, it is felt that these calculated

TABLE 4

Variation in amino acid content of salmon roe due to species and maturity differences

AMINO ACID	SPECIES VARIATION [1] F ratio [3] F critical (5%)	MATURITY VARIATION [2] F ratio [3] F critical (5%)
Arginine	1.36	0.11
Histidine	0.26	0.75
Isoleucine	0.58	1.72
Leucine	0.73	2.02
Lysine	0.53	3.88
Methionine	0.15	2.46
Phenylalanine	0.31	0.46
Threonine	0.61	1.05
Tryptophan	0.01	0.14
Valine	0.37	0.95

[1] Agreement among the means of the different species at maturity grade 3.

[2] Agreement among the means of the individual maturity grades (1, 2, 3 and 4) for king salmon roe.

[3] This term expresses to what degree differences in amino acid distribution exist due to species and maturity differences. Only for numbers greater than unity is there an implication (at the 5% level) that differences exist among the materials (Youden, '51). The term ''F ratio/F critical (5%)'' is derived as follows:

$$\frac{F \text{ ratio}}{F \text{ critical}} = \frac{\dfrac{\text{variance}_b}{\text{variance}_w}}{\text{critical value F (5\% level)}},$$

where ''variance$_w$'' is a measure of the agreement between determinations within a species or a maturity grade; ''variance$_b$'' is an estimate of the variance of the species or maturity grade means.

values imply a minimum difference, since the nitrogen content for the lipid- and water-free materials prepared from the roe of maturity grade 1 averaged only about 86% of that prepared from the mature roe. The lipid- and water-free samples prepared from the mature roe averaged about 16% nitrogen on an ash- and moisture-free basis. This variation in nitrogen

content may well be explained by a greater relative amount of lower nitrogen-containing amino acids in the egg-casing, which increases at a lower relative rate with maturity than does the sac contents (Young and Inman, '38).

From the standpoint of protein quality, as determined by "essential" amino acid content, salmon roe must be considered to contain protein of the finest nutritive quality, equalling

TABLE 5

Comparison of amino acid content of salmon roe with protein supplements commonly used for fish-hatchery feed

(Values given as per cent of protein, calculated to 16% N)

AMINO ACID	SALMON ROE [1]	PINK SALMON [2] VISCERA	BEEF LIVER [3]
Arginine	7.2	7.3	6.6
Histidine	2.7	3.1	2.5
Isoleucine	7.2	8.4	4.8
Leucine	9.9	10.7	8.4
Lysine	8.8	7.9	7.0
Methionine	2.9	3.3	3.2
Phenylalanine	4.8	5.1	6.1
Threonine	5.9	5.2	5.3
Tryptophan	0.9	1.1	1.5
Valine	7.2	6.6	6.0

[1] Average values of the mature roe (maturity grade 3) from the 5 species of salmon determined in the present work.

[2] Ney et al. ('50).

[3] Block and Bolling ('51).

that of meat and fish (Neilands et al., '49). As a feed supplement there would be very little difference in the "essential" amino acid content of salmon roe from different lots, since the very immature roe are not present in any significant quantity in salmon cannery waste. It is difficult to explain the results of Robinson et al. ('49) and of Burrows et al. ('51) on experimental fish-hatchery feeds solely on the basis of amino acid content. Although salmon roe was more effective as a hatchery feed than was beef liver (the control diet) or salmon viscera (one of the more effective components of salmon waste), the "essential" amino acid content of salmon

roe is very similar to that for beef liver and pink salmon viscera (table 5). The findings of Cooke et al. ('49) that salmon viscera contain a growth factor or factors for young trout and salmon, concentrated particularly in the roe, suggest a possible explanation for the excellent conversion rate when salmon roe is used as a hatchery feed.

SUMMARY

The "essential" amino acid content of roe (eggs) at different stages of maturity from the 5 species of Pacific salmon has been determined by microbiological methods. The relative distribution of amino acids in the roe was generally uniform, being significantly altered only by maturity. Most of the amino acids were present in increasing quantities with increasing maturity. The arginine and tryptophan levels proved quite constant, however, whereas the threonine content decreased with maturity. These trends were much more noticeable during the most immature stages of the roe.

The average amino acid content (expressed as per cent of protein, calculated to 16% nitrogen) of mature roe from the 5 species of salmon was arginine 7.2, histidine 2.7, isoleucine 7.2, leucine 9.9, lysine 8.8, methionine 2.9, phenylalanine 4.8, threonine 5.9, tryptophan 0.9 and valine 7.2.

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THE EFFECT OF CARBOHYDRATE-FREE AND CARBOHYDRATE-LOW DIETS ON THE INCIDENCE OF DENTAL CARIES IN WHITE RATS [1]

JAMES H. SHAW

Harvard School of Dental Medicine, Boston, Massachusetts

In a variety of experiments in the field of dental research, there arises the need for a diet or a series of diets which are completely noncariogenic for experimental animals, even during the penalty of such severe caries-producing situations as prolonged desalivation. Such diets, to be most useful, should be nutritionally adequate in all regards in order to permit normal growth and well-being of the experimental subjects throughout the entire period of their ingestion. One experimental condition which permitted a complete prevention of tooth decay even in the absence of the salivary glands was the tube feeding of the usual cariogenic diet 100 used in this laboratory (Kite, Shaw, and Sognnaes, '50). A modification of this procedure, in which only the sucrose of cariogenic diet 100 was introduced into the stomach and the remainder of the diet was consumed in normal fashion, permitted major reductions in the dental caries incidence (Haldi, Wynn, Shaw and Sognnaes, '53). In addition, the feeding of cariogenic diets to germ-free rats resulted in the complete prevention of dental caries (Orland, '50). However, each of these three procedures, although effective in the experiment for which it was designed, was sufficiently laborious and time-consuming as to be completely unfeasible for extensive experiments in the ordinary

laboratory. Hence, the studies reported herein were conducted in the interest of developing a caries-preventing dietary regimen for strains of rats which, by reason of their genetic, dietary, and environmental background, were highly caries-susceptible under the usual experimental conditions.

EXPERIMENTAL

The rats used in these experiments were offspring of the caries-susceptible strains maintained in this laboratory. In all cases, the parents of these rats had been maintained through-

TABLE 1

Composition of diets

INGREDIENTS	RATION 100	RATION 101	RATION 161	RATION 170	RATION 180
	gm	gm	gm	gm	gm
Sucrose	67	. .	5	. .	. .
Starch	. .	67	. .	. .	. .
Lard	. .	. .	12	12	24
Casein (with added B vitamins)[1]	24	24	24	24	24
Casein (as purchased)	. .	. .	35	40	13
Corn oil (with added fat-soluble vitamins)[1]	5	5	5	5	5
Salt mixture [1]	4	4	4	4	4
Whole liver substance	2	2	2	2	2
Liver concentrate powder (1: 20)	2	2	2	2	2

[1] Shaw, J. H.: J. Dent. Res., *26*: 47, 1947.

out their entire lives on ration 100, the composition of which is given in table 1. The rats were weaned at 21 days of age and distributed according to litter-mates and sex among the several groups of each experiment. Throughout the entire experimental period, the rats were maintained in single, screen-bottomed cages with ad libitum access to tap water and the ration under investigation.

The rations which were to be tested in this investigation for their caries-producing properties were designated by the code numbers 101, 161, 170 and 180. Their composition is given in table 1. Rations 170 and 180 were completely free of carbo-

hydrate except for the minute traces contributed by the liver concentrates. The composition of these rations was determined by a complete isocaloric replacement of the sucrose in ration 100 by varying amounts of casein and lard. In ration 170, most of the sucrose had been replaced by protein; in ration 180, most of the sucrose was replaced by lard. Ration 101 was a modification of ration 100 in which all the sucrose was replaced by finely ground corn starch. Ration 161 contained approximately 6% of sucrose and also is an isocaloric modification of ration 100.

In experiment 1, 60 rats were distributed into 4 equal groups. The first group was maintained on ration 100 for 20 weeks and then sacrificed. The second group was fed carbohydrate-free diet 170 for 20 weeks and then sacrificed. The third group was maintained on ration 170 for 104 weeks before being sacrificed. The rats in the 4th group were allowed to eat ration 100 for 20 weeks and then transferred for an additional 20 weeks to ration 170, at the end of which time they were killed. The number of carious molars, the number of carious lesions and the extent of carious lesions were evaluated according to previously established criteria (Shaw, Schweigert, McIntire, Elvehjem and Phillips, '44).

In experiment 2, 40 rats were distributed into 4 equal groups. For three days they were allowed to acclimatize themselves to the diets. On the 4th day, the principal salivary glands (parotid, submaxillary, major sublingual and extraorbital lacrymal) were removed under ether anesthesia through a single midline ventral incision. The first group was maintained on ration 100 for 12 weeks. The second group was maintained on ration 170 for the same period. Group 3 was fed ration 170 for 104 weeks. Group 4 was allowed to eat ration 100 for 12 weeks and then was transferred to ration 170 for an additional 92 weeks. At the end of the above periods, all rats were sacrificed and the incidence of dental caries determined.

In experiment 3, 24 rats were distributed into 4 equal groups. The details were identical to those in experiment 2 with the

single exception that ration 180 was compared with cariogenic diet 100 instead of ration 170.

In experiment 4, 40 weanling rats were divided into 8 groups. As in experiment 2, the rats were desalivated on the 4th day. The first and second groups were fed ration 100 for periods of 12 and 52 weeks, respectively. The rats in the third group were maintained on ration 100 for 12 weeks and then on ration 160 for 40 additional weeks. The animals in groups 4 and 5 were fed ration 161 for periods of 12 and 52 weeks, respectively. Group 6 was maintained on ration 161 for 20 weeks and then on ration 100 for another 12 weeks. The rats in group 7 were maintained on ration 101 for 12 weeks. The rats in the 8th group were allowed to eat laboratory chow for 12 weeks. The laboratory chow used in this experiment was a commercial product whose detailed composition has been described by Sognnaes ('48). At the end of these periods, the rats were killed and the dental caries experience evaluated.

RESULTS

In the first experiment, the rats in the 4 groups grew at normal and practically identical rates and attained comparable adult body weights. The average number of carious molars, the average number of carious lesions and the average extent of carious lesions for the rats in this experiment are presented in table 2. The rats in the first group demonstrated an incidence of dental caries that was typical of intact representatives of this strain of caries-susceptible rats, when maintained on cariogenic ration 100 for 20 weeks. The average number of carious teeth, the average number of carious lesions, and the average extent of carious lesions were 5.8, 8.7, and 25 +, respectively. The carious lesions were almost entirely at the bases of the sulci of the molars and were soft and fairly heavily pigmented, indicating that they were actively, but not rampantly, progressing. At the end of 20 and 104 weeks, respectively, none of the 30 rats in groups 2 and 3 which were fed carbohydrate-free diet 170 had any evidence of any carious lesions. The rats in group 4 which were maintained for 20

TABLE 2

The effect of a carbohydrate-free diet on the dental caries incidence in white rats

GROUP	RATION	DURATION WEEKS	NO. OF RATS	NO. OF CARIOUS TEETH AV.	SEM [1]	CR [2]	NO. OF CARIOUS LESIONS AV.	SEM [1]	CR [2]	EXTENT OF CARIOUS LESIONS AV.	SEM [1]	CR [2]
Experiment 1: Intact rats												
1	100	20	15	5.8	0.6		8.7	0.8		25 +	3 +	
						9.7			10.9			8.3
2	170	20	15	0	0		0	0		0	0	
						0.3			0.4			0.9
3	170	104	15	0	0		0	0		0	0	
						10.1			11.9			7.0
4	100 / 170	20 / 20	15	5.6	0.5		8.3 [3]	0.7		21 +	3 +	
Experiment 2: Desalivated rats												
1	100	12	10	10.8	0.9		28.4	2.4		110 +	9 +	
						12.0			11.8			12.2
2	170	12	10	0	0		0	0		0	0	
						0.3			0.1			0.2
3	170	104	10	0	0		0	0		0	0	
						11.2			10.6			11.3
4	100 / 170	12 / 92	10	11.2	1.0		28.7 [4]	2.7		113 +	10 +	
Experiment 3: Desalivated rats												
1	100	12	6	11.3	0.8		29.7	3.0		119 +	10 +	
						14.1			9.9			11.9
2	180	12	6	0	0		0	0		0	0	
						0.5			0.2			0.8
3	180	104	6	0	0		0	0		0	0	
						11.9			9.0			12.0
4	100	12	6	10.7	0.9		29.1 [4]	3.2		108 +	9 +	

[1] SEM: Standard error of mean.
[2] CR — Critical ratio. The critical ratio is the ratio of the difference between two means to the standard error of the difference between the means. Wherever the critical ratio is less than 2.0, the difference between the means is considered to be statistically insignificant; when from 2.0 to 2.9, moderately significant; when 3.0 or higher, highly significant.
[3] Arrested carious lesions — extremely hard and shiny with moderate amount of dark brown or black pigmentation.
[4] Arrested carious lesions — broad, smooth, highly polished, saucer-like regions.

weeks on ration 100, followed by 20 weeks on ration 170, had no
active carious lesions. However, they had an incidence of ar-
rested carious lesions that was almost identical in number and
size with the incidence of active lesions in group 1. These ar-
rested lesions were extremely hard and shiny, with a moderate
amount of dark brown or black pigmentation. The soft carious
enamel and dentin which must have been present at the time
of the change in rations seemed to have been abraded away in
the course of mastication. Where the lesions existed at suffi-
cient depth in the sulci to escape abrasion during mastication,
little change in the general contours of the lesions had occurred,
but the same hardening and increased pigmentation of the
lesions were noted.

The rats in the 4 groups of experiment 2 grew at somewhat
slower rates than those in experiment 1. Typical of desalivated
rats, their hair coats were in general much more disorderly and
dirty than the intact rats in the previous experiment. Like-
wise, their oral cavities were continually dirty, with large
masses of ration material becoming caked around the bases of
the incisor and molar teeth and in all oral folds and crevices.
The incidence of dental caries in these rats is presented in table
2. The incidence of carious lesions among the rats in group 1
was much greater than for the comparable intact group in ex-
periment 1. Averages of 10.8 carious molars, 28.4 carious le-
sions, and a caries extent score of 110 + were observed. As
would be expected from these values, the lesions were ram-
pantly progressing, extremely soft, and with practically no pig-
mentation. Almost all lesions had progressed to the point
where appreciable fracturing of the cusps had occurred by
reason of the extent to which they were undermined. Typical
of desalivated rats, the lesions gave evidence of beginning on
wide areas of the sulci instead of the small localized areas
which are typical of intact rats. Despite desalivation and the
extensive accumulation of food materials around the teeth of
the rats in groups 2 and 3, no carious lesions whatever were
observed, regardless of whether the rats were maintained for
the normal experimental period of 12 weeks or for the un-

usually long period of 104 weeks. Again, the rats in the 4th group of experiment 2 had no active carious lesions. Instead, there were many broad, smooth, highly-polished, saucer-like regions in the approximate position of the sulci. These undoubtedly represented the areas where actively fulminating lesions had been at the time the diet change from 100 to 170 was made. Evidently the forces of mastication had abraded away the soft carious material. Because of the large extent of these lesions at that time, none had been inaccessible to the abrasion. Rarely was there any evidence of pigmentation in the areas where carious lesions had been.

The results of experiment 3 are also presented in table 2. In every detail, the substitution of ration 180 for ration 170 produced the same results.

In each of these three experiments, the differences in caries incidence between the control rats fed caries-producing diet 100 and the rats fed either diet 170 or 180 were tested by statistical methods and found to be highly significant.

The rats in the first 7 groups of experiment 4 grew at approximately the same rates for the first 12 weeks of the experimental period. Those in group 8 which were fed laboratory chow grew somewhat less rapidly than those in the rest of the groups. During the last 40 weeks of the experiment, the rats in groups 4 and 5 fed ration 161 grew appreciably more rapidly than those in group 2 fed ration 100. The incidence of dental caries for the rats in this experiment is presented in table 3. The rats in group 1, fed ration 100 for 12 weeks, had a moderately high incidence of carious lesions, which was comparable to the incidence of lesions in experiments 2 and 3. The rats in group 2, fed ration 100 for 52 weeks, had carious lesions in each one of their 12 molar teeth. This did not represent a statistically significant increase in carious molars as an average of 11.0 molar teeth were carious in the rats in group 1. However, the increase in the number of carious lesions observed in the rats in group 2 was statistically significant and the increase in extent of carious lesions was highly significant. In the rats in group 3 where the ration was shifted from 100 to

161 after 12 weeks, there were no active carious lesions, but the molar teeth bore evidence of previous caries activity by reason of the presence of arrested carious lesions. Their frequency indicated a caries incidence at the time of the diet change that

TABLE 3

The effect of a carbohydrate-low diet on the dental caries incidence of desalivated white rats

GROUP	RATION	NO. OF WEEKS	NO. OF RATS	NO. OF CARIOUS TEETH		NO. OF CARIOUS LESIONS		EXTENT OF CARIOUS LESIONS	
				Av.	SEM	Av.	SEM	Av.	SEM
1	100	12	3	11.0	0.7	22.5	2.1	77 +	6.9 +
2	100	52	3	12.0	0.0	29.7	2.6	141 +	10.3 +
3	100	12	7	10.8	0.6	21.7 [1]	2.1	65 +	6.1 +
	161	40							
4	161	12	5	0	0	0	0	0	0
5	161	52	7	0	0	0	0	0	0
6	161	20	5	7.0	0.6	11.4	1.3	40 +	4.7 +
	100	12							
7	101	12	5	10.1	0.9	14.0	1.5	49 +	4.3 +
8	Laboratory chow	12	7	9.8	0.8	15.3	1.3	41 +	3.9 +

Comparison of critical ratios
    between groups

1 and 2	1.6	2.2	5.2
1 and 3	0.2	0.3	1.3
1 and 4	15.7	10.7	11.2
2 and 5	Infinity	11.4	13.7
1 and 6	4.4	4.4	4.4
1 and 1	0.8	3.3	3.5
1 and 8	1.1	3.0	4.6
6 and 7	2.8	1.3	1.4
4 and 8	12.3	11.8	10.5
7 and 8	0.3	0.7	1.4
6 and 8	2.8	0.4	1.3

[1] Arrested carious lesions — broad, smooth, highly-polished, saucer-like regions.

was highly similar to that of the animals in group 1. All rats in groups 4 and 5 were completely free of any evidence of carious lesions after 12 and 52 weeks, respectively, on ration 161. The differences between groups 1 and 4 and between groups 2 and 5 were highly significant for the number of cari-

ous molars, the number of carious lesions and the extent of carious lesions. In group 6, where the ration was shifted from low carbohydrate diet 161 to cariogenic diet 100 after 20 weeks, there was an appreciably lower incidence of carious lesions than in the rats in group 1. In the comparison of groups 1 and 6, the differences in the three criteria for expressing prevalence of dental caries were highly significant. This would suggest that the animals in group 6 which were 12 weeks older when first exposed to a caries-producing regimen had a markedly lower susceptibility than the animals in group 1. The rats in the 7th group fed ration 101 had a caries incidence which was somewhat lower than for the rats in group 1 fed the sucrose-containing cariogenic diet 100 from the beginning of the experiment; however, the caries incidence in group 1 was slightly higher than for the rats in group 6 fed diet 100 after 20 weeks on ration 161. The difference in number of carious molars between groups 1 and 7 was insignificant; however, the differences in number of carious molars and extent of carious lesions were highly significant. The slightly higher values seen in the rats in group 7 compared with those of group 6 differed by a statistically significant amount only in the case of the number of carious molars. The rats in group 8 fed laboratory chow had a lower incidence of carious lesions than the rats in group 1 fed ration 100, but a great deal higher incidence of lesions than the rats fed ration 161. The differences between the caries incidences in groups 1 and 8 were highly significant for the number of carious lesions and the extent of carious lesions. The differences between the caries experience in groups 4 and 8 were highly significant.

DISCUSSION

The two carbohydrate-free diets and the low carbohydrate ration which were used in these 4 experiments were found to be completely incapable under the conditions in this laboratory of producing carious lesions in our caries-susceptible strain of rats. This was true not only in intact rats, but also in desalivated rats which were maintained on these diets for as long as

one and two years. In addition, when intact or desalivated rats were maintained on caries-producing diets for sufficiently long periods to produce clinically demonstrable lesions, the transfer of these rats to one of the carbohydrate-free or carbohydrate-low diets resulted in an arrest of the caries process, a wearing away of the soft areas of any carious lesions which were exposed to attritional forces, and a hardening of the underlying regions of tooth substance.

These observations suggest that a source of carbohydrate is necessary in the oral cavity of experimental animals for the initiation of carious lesions and also for the continued development of lesions. These findings are in harmony with earlier studies. The introduction of all components of the cariogenic purified ration 100 into the stomach by means of tube feeding resulted in a complete prevention of carious lesions in intact and desalivated rats (Kite, Shaw and Sognnaes, '50). Similarly, desalivated rats which were tube-fed only the sucrose of the purified ration, and ate the remaining components developed a very low incidence of carious lesions (Haldi, Wynn, Shaw and Sognnaes, '53).

It is obvious from the results of experiment 4 that the carbohydrate-free and the carbohydrate-low rations had a much greater caries inhibiting ability than either laboratory chow or ration 101 in which starch is the only carbohydrate, at levels of 48.5 and 67%, respectively. Though the two latter rations have a significantly lower caries-producing potentiality than ration 100 in the intact rat (Sognnaes, '48), they are both capable of permitting the initiation and development of carious lesions at a reasonably rapid rate in desalivated rats. This fact makes their utilization impossible in any experiment where absolute prevention of dental caries must be guaranteed for any appreciable period.

The complete prevention of dental caries in the rats receiving rations 161, 170 and 180 may have been partially due to the physical consistency of the diet. Obviously the inclusion of increased amounts of lard in these diets resulted in rations of less granular nature. This was especially true in the case of

ration 180, where the diet was quite pasty. Earlier studies with a variety of similar diets with different textures suggested that the physical consistency of the diets within the range tested was not an important factor in producing alterations in the caries incidence (Shaw, '49). Hence, in the present studies it is expected that the physical consistency was of little or no importance in the caries-prevention observed.

There is evidence in experiment 4 that the rats in group 6 which did not receive the cariogenic diet until 161 days of age after a period of 20 weeks on a caries-preventing low carbohydrate diet had an appreciably lower incidence of carious lesions than did the litter-mates in group 1 who were fed the caries-producing ration immediately after weaning. This observation suggests a lower susceptibility to tooth decay with increased tooth age; this is a problem concerning the maturation and aging of teeth which needs further investigation.

From the standpoint of dental research, there is another more important aspect to the results of these experiments. The demonstration that these rations were capable of preventing the initiation of carious lesions, and the further development of existing lesions, in a susceptible strain of rats for prolonged periods despite the absence of the major salivary glands makes it possible to design, conduct and evaluate several types of experiments which were heretofore not feasible. The following examples will indicate briefly some of the potential experimental uses of a caries-preventing regimen for investigations concerning the teeth:

1. Histochemical and radiochemical studies of the dental structures during the maturation of the teeth (the caries-preventing diet is needed here to guarantee that any changes noted in the dental structures were due to the maturation of the teeth rather than to the superimposition of effects attributable to caries initiation).

2. Contributions of the saliva to the enamel and dentin during the maturation period (the caries-inhibiting ration in this case is a requirement for a period of maintenance after the earlier age groups of animals are desalivated in order to

guarantee a common non-cariogenic period for all groups [Fanning, Shaw and Sognnaes, '53]).

3. The relationship of tooth age to caries-susceptibility (the caries-preventing regimen would be used here for the period of maintenance of the animals up until the age when the caries-producing regimen would be begun; this is mandatory in order to be able to guarantee that no lesions had developed prior to the specific experimental period under investigation).

Studies of various phases of each of these problems are underway in this laboratory. The majority of these are with caries-susceptible rats but some are with rhesus monkeys. On the basis of present findings, there appears to be ample justification for the belief that these diets are capable of preventing carious lesions in the rhesus monkey also.

SUMMARY

Two carbohydrate-free rations and a low carbohydrate ration were found under intensive tests to be incapable of producing tooth decay in a highly-caries susceptible strain of rats. Even in trials as long as one and two years after the rats were desalivated to increase the susceptibility to tooth decay, no carious lesions developed.

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