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(January 3, 1883 – October 22, 1952)

The lean dark-eyed man who sat often at nutrition conference tables spoke seldom and then briefly. But others always listened attentively to his precise and concise comments, for Dr. Jeans had that rare ability to extract the essence of a problem immediately and discard the nonessentials. A completely logical man, yet one so practical he never let the ideal supersede the achievable, his comments were always valuable. A medical man who received his training before medicine acknowledged nutrition as its handmaiden, he became an authority on nutrition of the infant and child.

Philip Charles Jeans grew up in the typical midwestern small town of Hillsboro, Ohio. He went to the University of Kansas because his maternal grandmother lived in Lawrence. He majored in Chemistry; the ruling that the laboratory was not open for student work evenings seemed to him unwarranted, so he ignored it. The fire escape was a simple means of entry for a long-legged youngster. That calm refusal to recognize dicta which seemed to him illogical was characteristic throughout life. He thought out carefully what he wanted to do and quietly did it, regardless of obstacles.

Philip Jeans completed his B.A. in 1904, worked as chemist in a sugar mill for a year, then went to Johns Hopkins Medical School. He earned his way by running a boardinghouse for students and by doing odd jobs. He completed his studies in the year 1909. In his senior year, two courses were given in pediatrics at Hopkins, both elective. Philip Jeans signed up for both, went to one session of each, and skipped the remainder. He not only got his degree, but was offered the post of physician-in-residence at a small country hospital maintained for sick infants and young children of Baltimore slums. So

this student with two lectures in pediatrics as training suddenly found himself caring for a group of infants and children, most of whom were seriously ill of dysentery. This experience determined Dr. Jeans' career. Poor nutrition and lack of sanitation were the great killers of infants. He must protect them. He determined to be a pediatrician.

The 16-month rotating internship he had accepted at Hartford, Connecticut, beginning October 1909, was carried through faithfully, but the subsequent invitation to join the practice of the chief surgeon did not attract him. In Boston, 6 months as House Officer of Children's Hospital and a summer's internship at Boston Floating Hospital showed the same problems he had found in Baltimore. As intern, he had little opportunity to work on his own, but he seized every chance to learn more about the effect of dysentery on the body and to devise formulas both digestible and safe for babies. Five months as Senior Interne in Pathology completed his Boston studies. His gaiety and humor made many friends.

He went to the Department of Pediatrics of the Medical School of Washington University, St. Louis, in 1913, as assistant. At Christmastime, 1914, he returned to Boston to marry Miss Grace Cushing. Their son, Robert Philip, was born in 1923.

Dr. Jeans found St. Louis, like Baltimore and Boston, a city of large and unsanitary slums, with its infant death rate appallingly high. In charge of the free out-clinic, his youthful appearance, enchanced by his engaging grin, caused concern among mothers accustomed to formality in physicians. Some even asked to have their infants treated by a "white coat doctor" (student) rather than "that boy."

The high incidence of congenital syphilis among infants and children of the large and poorly educated negro population of St. Louis offered an unparalleled opportunity to find out how syphilis was transmitted from mother to infant. Again the nutrition problem had to take second place, while a more pressing medical problem was investigated. Yet the importance of nutrition in pediatric training seemed to him so great

that his lectures to students started with a section on nutrition. By 1920 half a semester was devoted to nutrition and questions on the nutritional status of the patient were an accepted and routine part of staff-student discussions.

Research was interrupted by the great epidemic of poliomyelitis in New York in the summer of 1916. Dr. Jeans answered the city's call for help, returning to St. Louis only in time to take up his classes in the fall.

About this time, he wrote his first paper for publication. No one had given him any advice about such writing. The paper was accepted, but returned so rewritten by the editor as to be scarcely recognizable to its author. Characteristically, Dr. Jeans went over the paper, word by word, satisfying himself that the editorial changes were factually correct, and learning how these changes had improved on his original. He then defined the ideal paper as one in which no word could be substituted or omitted without altering the meaning. Constant striving for this ideal undoubtedly helped to develop the brevity of his speech as well as of his writing.

W. McK. Marriott became head of Pediatrics at Washington University. Interested in nutrition as a chemical problem, Marriott assumed enthusiastically the problem of devising and testing a milk formula safe against dysentery. When Marriott published his formulas for acid milk, the infant feeding that needed no refrigeration and had as small a curd, therefore was as digestible, as human milk, Dr. Jeans accepted it as the fulfillment of a long quest for an artificial feeding suitable for infants in hot climates and in homes without good sanitation.

Study of the syphilis problem had been completed when the Red Cross in 1921 asked Dr. Jeans to go to southeastern Europe to help establish child health clinics. So Dr. and Mrs. Jeans sailed for Europe in the fall of 1921. He spent most of his time on shipboard writing his book on Prepubescent Syphilis.

In Europe his work took them through Austria, Hungary, Czechoslovakia, Albania and Montenegro. They travelled hard through southeastern Europe, seldom staying more than a day

or so in a place, constantly seeing people deprived of needed food for long periods because of the war. He set up clinics for the care of infants and children of these countries. Again he was impressed by the degree of malnutrition and its importance as a factor in the high death rate of infants and children. Here he saw for the first time the nutritional disease then called Mehlnährschaden, or disturbance of nutrition caused by too much cereal—a disturbance now known as malignant malnutrition, or kwashiorkor. He met with pediatricians to devise means for improving the care of infants and children of these war-ravished countries. His accomplishments in Hungary are best told by the following quotation:

> ". . . Dr. Philip C. Jeans . . . brought to the work not only great professional skill and knowledge . . . but his fairness, common sense and diplomacy were of inestimable value. It is not usual for us in the American Red Cross to give individual praise, but we hold Dr. Jeans and his work in such high esteem that we thought it our duty to report to you especially in the matter."
>
> —Jas. G. Pedlow, Director, Hungarian Unit, American Red Cross, to Col. Ernest P. Bicknell, American Red Cross Commander for Europe, Paris, France.

In 1924, Dr. Jeans came to the State University of Iowa as Head of the Department of Pediatrics. The department at Iowa was already committed to studies of the nutrition of the infant and child. Under the direction of Dr. Amy Daniels of its Child Welfare Research Station, working in the Department of Pediatrics, a metabolic ward for the study of infant nutrition had been started in the fall of 1920 with one assistant, Genevieve Stearns, to carry the analytical work.

The Child Welfare Station, started in 1916, had grown large by 1926 and wanted to unify its staff. So Dr. Daniels moved across the river to pursue her nutrition studies independently.

The first nutritional study started by Dr. Jeans was the quantitative study, with Martha Nelson, of retention of nitrogen and minerals by babies fed the acid milk.

Early studies concerned questions of immediate practical importance to pediatricians, such as the effect of the substitution of evaporated for fresh milk, and the studies designed to prove whether the ease of digestibility of the acid milk feedings was due primarily to the lowering of the pH (human milk has a lower pH than cow's milk), or merely to the fineness of the curd. The curd won.

A long series of studies concerned the vitamin D requirement of the infant. The variable potency of cod liver oil and the use of other sources of vitamin D previous to standardization of unitage had resulted in considerable confusion as to the efficacy of the vitamin in relation to the source. Since from 60 to 90% of urban infants were reported to be rachitic unless vitamin D was given, the question of dosage was important. The amount required, whether the two chief types of the vitamin were equivalent for the infant, and the effect of the dispersion of the vitamin on its utilization, all needed clarification. The difficulties were heightened by the over-refinement of techniques for the diagnosis of rickets. Prevention of rickets was the common criterion for requirement of the vitamin, but Dr. Jeans insisted that the requirement should be determined by the dosage permitting best growth and development of the normal baby, not merely that which just prevented disease. The Iowa studies showed that the moderate intake of 300 to 400 units was ample for the well baby.

The Council on Foods, organized in 1929 by the American Medical Association as an authoritative body to inform physicians as to the truth of claims in food advertising, made Dr. Jeans a member in 1930, an association which continued until his death. In 1935, the Council assigned him the task of analyzing the publications on rickets and writing an authoritative review on the relative merits and desirable dosages of the various types of vitamin D milk then on the market. Dosages of the vitamin advocated for normal infants varied from 135 to 3000 international units daily. Similar confusion existed in diagnosis of rickets. Unity of thought among leading experts in the field was certainly desirable. Data and roent-

genograms were exchanged; after several weeks of discussion, at times rather warm, all agreed with the Iowa findings that 300 to 400 units daily were ample for the well baby. The exchange of data and roentgenograms among pediatricians considered authorities on rickets resulted in the unexpected finding that a healthy well-fed baby during his rapid early growth may show a slight fuzziness in the roentgenograms of his epiphyses, indistinguishable from that which heralded the beginning of rickets in the child receiving no vitamin D. Also, the baby given 135 units of vitamin D milk, while fully protected from rickets, did not grow fast enough to show any fuzziness of epiphyseal margins. The confusion on diagnosis was resolved. As often happens, calm and complete study of all available data not only cleared up the points at issue, but added to human knowledge. The number of publications on vitamin D requirement dropped precipitously.

Continued general use of dosages of vitamin D greater than 2000 international units daily prompted a study of the effects of such dosage. It was discovered that babies so fed grow very well for a time but at about 6 months of age they lose appetite and linear growth slows significantly. These results were greeted by considerable disbelief at the time, but are generally accepted now.

In addition to the above studies, Dr. Jeans directed clinical research by residents, and devised a means of determining vitamin A deficiency in children by means of a test of dark adaptation. This test also came under severe criticism. A personal meeting with the chief critic and check of the test as a measure of vitamin A deficiency showed that the cones of the retina, as well as the rods, were affected by bright light and that vitamin A is associated with visual violet (iodopsin) formation as well as that of visual purple (rhodopsin). The accuracy of the test as a survey measure, also criticized, was found to be dependent on the freedom of the subject from recent illness, especially upper respiratory infection. By the time this evidence was clear cut, however, better methods for determination of serum vitamin A had been devised, so the

latter study was never published, as the original test was no longer needed.

After the vitamin D and A studies were completed, attention was turned to other nutritional requirements of infants and to those of older children. The studies of children had been begun almost as early as those of infants, but progressed far more slowly, as they were necessarily subordinate to the studies of infants.

Dr. Jeans carried a crowded schedule as administrator, teacher, clinician, clinical advisor to the physicians of the state, research director and "finance minister" for research funds. Prior to the availability of foundation and government funds, research money came largely from commercial organizations. Its contributors understood well that no study would be financed entirely from one source, to prevent unconscious bias, and that publication of results was not to be restricted. It is a tribute to the far-sightedness of these groups and to their trust in Dr. Jeans' ability and integrity that money was always available for the studies.

The second World War brought more responsibilities. The Food and Nutrition Board was reorganized as a section of the National Research Council. Dr. Jeans was an obvious choice for this group and so to his active work with the AMA Council on Foods, were added the frequent and arduous wartime trips to Washington. Committee work was heavy and despite efforts of his staff to relieve him of departmental responsibilities, he showed the strain. After the war he assumed still other tasks. He became a member of the steering committee of the Food and Nutrition Board, of the Advisory Committees of the Nutrition Foundation and of the U. S. Bureau of Human Nutrition, and a consultant on Nutrition for the Public Health Service. The Food and Drug Administration knew him as a perfect witness. The Public Health Service sent him to Hawaii in 1950 to give a series of lectures on nutrition. He was president of the American Pediatric Society in 1950–51, having previously served on its governing council from 1938 to 1946. He became chairman

of the Council on Foods and vice-chairman of the Food and Nutrition Board in 1951.

With all these added cares, his teaching remained as logical, up-to-date and thorough as ever. As one former resident said: "All the pediatricians in my city have been well-trained clinically. When I have succeeded where others failed, it has been because I watched the child's nutrition as carefully as his illness. As a resident, I wasn't aware I was learning nutrition. I thought it was all Pediatrics." And so it always was to his chief—an essential part of Pediatrics.

Dr. Jeans' calm logic, unhampered by any feeling of professional pride, made him an excellent mediator whenever the furtherance of knowledge was impeded by difference of opinions among investigators. His most difficult task of this type was assumed at the instigation of the Food and Nutrition Board of the National Research Council; chairmanship of a committee to mediate the question of cause of dental caries, that serious nutritional disease which had been widely studied only to give rise to two utterly opposed schools of thought. Dr. Jeans set out to assemble all the known facts in a series of articles written by leading investigators in each field. Characteristically, he chose not only sound investigators but those whose names were not involved in controversy to be authors of the reviews.

Assembling the mass of proved data, subjecting each article to a jury of experts in the given field, revision and re-revision, was a heart-breaking task. Authors defected under the strain; others took their places. The task of summarizing was given to a foreign investigator, Dr. Guttorm Toverud, who was completely outside any of the controversies which racked investigators here. Finally, nearly ten years after its inception, the book was published. It reflected its editor in its calm unimpassioned logic. As with the controversy on diagnosis of infantile rickets, so the great controversy on the cause of dental caries died, quietly and immediately, once the entire body of evidence was assembled and analyzed logically and unemotionally. Unfortunately, Dr. Jeans did not live to enjoy any satis-

faction from this long labor of love, for the book was not published until a few weeks after his death.

Dr. Jeans was a Fellow of the American Medical Association, and a member of the American Pediatric Society, American Institute of Nutrition, Society for Experimental Biology and Medicine, Society for Research in Child Development, Society for Pediatric Research, Nu Sigma Nu, Alpha Omega Alpha and Sigma Xi. He received (with Stearns) the Borden Award from the American Institute of Nutrition in 1946 for work on infant and child nutrition.

In addition to his book on Prepubescent Syphilis, and the editing of the review on Dental Caries, Dr. Jeans was the author of two other books. His Textbook of Pediatrics for Nurses revolutionized nurses' texts, for it made obsolete all textbooks of Pediatric (or other specialty) Nursing. After Marriott's death, Dr. Jeans took over his book on "Infant Nutrition" which ran through several revisions, but the complete re-writing, postponed until after retirement, was never started.

In July 1952, he retired from his headship of the Department of Pediatrics, according to University regulations. Much was planned for the ensuing years; writing that had been long postponed for other, more urgent, matters.

In October 1952, he went to Guatemala as a consultant for the INCAP group there. Once again he saw the malignant malnutrition he had seen after World War I in Europe. He worked as hard as the group would let him. His Central American trip was to culminate in a series of lectures in Costa Rica and Panama. His sudden death in Panama, only a few hours after a gala welcome from a large group of Central and South American physicians, ended his career, October 22, 1952.

Throughout Dr. Jeans' career, his ideas, his actions, were solely the products of his logic and his practicality. Very few men are willing, as he was, to go completely counter to accepted thought and practice of their time. If customary practice or theory appeared illogical to Dr. Jeans, he discarded

it forthwith. If further data showed his own accepted conclusions to be in error, they too were discarded without ado. Yet, no matter how logical a certain course might be, if it proved impractical, the course was altered to that which could be accomplished. He never tilted at windmills.

To his students and associates his outstanding characteristics were his wisdom, his kindness, his humor and his modesty. Wisdom rather than brilliance, warmth of friendship, a quick humor that was never barbed, and above all, simplicity and utter lack of any striving for personal aggrandizement were the traits that made Dr. Jeans a good friend, an inspiring teacher, and a leader in his field. A bronze plaque in the Pediatrics Library at the State University of Iowa reads: "This library is dedicated in appreciation of Philip Charles Jeans, M. D., Professor and Head, Department of Pediatrics, 1924—1952. A wise clinician, thorough investigator, humble teacher and understanding friend."

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NUTRITIONAL STUDIES WITH GLYCINE, AMINOETHANOL AND RELATED COMPOUNDS IN THE CHICK [1,2]

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(Received for publication July 18, 1957)

INTRODUCTION

The chick has a nutritional requirement of 11 amino acids, i.e., the 10 essential amino acids of the weanling rat plus glycine. The earlier demonstrated need for dietary glycine by the young chick (Almquist and Mecchi, '40) and its interrelationship with dietary arginine for creatine formation has been amply confirmed by recent studies (Wietlake et al., '54, Fisher et al., '55, '56; Hogan et al., '57). However, in view of the present knowledge of the metabolism of glycine-related compounds, it is highly desirable to inquire further into the specificity of the glycine requirement (Almquist and Mecchi, '40). In this respect, our previous experiments have established that serine could substitute for glycine as measured by the criterion of chick growth (Wixom et al., '55). This report describes further experiments to clarify the specificity of the glycine requirement of the chick.

[1] Part of the experimental data in this paper was taken from a thesis submitted by George E. Pipkin to the Graduate School of the University of Arkansas in partial fulfillment of the requirements for the Master of Science degree. Present address, Department of Biochemistry, School of Medicine, Tulane University, New Orleans, Louisiana.

[2] This investigation was supported by research grant no. 227, Nutrition Foundation, Inc., and grant no. G-2173, National Science Foundation. Preliminary reports were presented at the annual meetings of the American Institute of Nutrition at Atlantic City, April 16–20, 1956 and at Chicago, April 15–19, 1957.

The method of growth may also be used to examine the metabolism of a test substance. If the incorporation of a test substance in a glycine-deficient basal diet increases the growth rate of chicks, then this substance may, tentatively, be considered a metabolic precursor of glycine. This explanation is similar to that given for the precursor-product relationship between phenylalanine and tyrosine, or between methionine sulfur and cystine sulfur; in nutritional literature this relationship is referred to as a sparing of the latter for the former. Such an *in vivo* nutritional approach is a desirable confirmation of other methods of study of metabolism, such as isotopic tracer studies which, while invaluable in detecting biosynthetic pathways, cannot always clarify the magnitude of such reactions.

In recent years numerous investigations have been made to identify the pathway(s) of biosynthesis of glycine and serine (Arnstein, '54). That there must be some pathway for their formation, other than the interconversion of glycine and serine, is evident from the normal growth of rats on diets devoid of glycine and serine (Rose et al., '52). In view of the slow growth of chicks on glycine-deficient diets, the same conclusion is applicable to the avian species. Investigations, primarily with isotopically-labeled intermediates, have established the following biosynthetic pathways in the rat: betaine to dimethylglycine to sarcosine to glycine, aminoethanol to glycolaldehyde to glycolic acid to glyoxylic acid to glycine, and triose to hydroxypyruvate to serine. Several of these pathways have been examined in the present nutritional or growth studies with the young chick, and the results are discussed with respect to the comparison of these metabolic reactions in the two species.

EXPERIMENTAL

One-day-old Single Comb White Leghorn cockerels were given the earlier described casein-gelatin starting diet (Wixom et al., '55) for one week. Their handling and division into experimental groups of 12 chicks each at one week of age was carried out in the same manner previously outlined in

order to minimize variability. By this selection process, the overall mean initial weight for experiments XV through XIX was 78.3, 80.1, 83.9, 77.0 and 74.5 gm, and the range of chick weights in each experiment was not more than 23 gm. Three groups of 12 chicks each were placed on each experimental diet for two weeks. Since the direction of change of the dependent variable was the same for each of the three groups of 12 chicks in all experiments, only the overall averages for 36 chicks are presented in the tables. The data are reported as feed efficiency, average gain during the two week experimental period, and as the average percentage gain per day (Wixom et al., '55). There were no fatalities during the test period in any of the experiments reported.

The basal, glycine-deficient, choline-containing ration during the experimental period was similar to the ration of Wiatlake et al. ('54). It contained the following constituents in per cent: casein 35.0, L-arginine·HCl 1.24, L-cystine 0.3, cellulose 3.0, hydrogenated vegetable oil 3.0, corn oil 0.9, salt mixture 5.7, vitamin mixture 0.4, and glucose hydrate 50.46 (Wixom et al., '55). The test substances, which were generally used in an amount equimolar to 1.50% glycine, were added to the basal diet by substitution for an equal amount of glucose hydrate. Most of the test substances were purchased from commercial sources.[3] All of the amino acids and other nitrogenous test supplements were examined for purity by a macro Kjeldahl nitrogen analysis. The data for betaine·HCl were: found 9.05% N, calculated 9.12% N. Sarcosine·HCl was purified by recrystallization from glacial acetic acid (found 10.98% N, calculated 11.16% N). The purity of glycolic acid was checked by paper chromatography in three different solvent systems, and by determining its neutralization equivalent after drying (found 75.8, calculated 76.0). The three amines were purified by fractional distillation, and then checked by Kjeldahl analysis (aminoethanol, B.P. 170–170.1°C; found 22.96% N, calcu-

[3] Betaine·HCl and sarcosine·HCl were obtained from Nutritional Biochemicals Corp.; glycolic acid from Matheson, Coleman and Bell Corp.; aminoethanol and dimethylaminoethanol from Eastman Organic Chemicals Co., and monomethylaminoethanol by gift from Carbide and Carbon Chemicals Company.

lated 22.92% N; N-methylaminoethanol, B.P. 158.5–160°C; found 18.50% N, calculated 18.65% N; N,N'-dimethylaminoethanol, B.P. 134°C; found 15.05% N, calculated 15.72% N).

It was necessary to synthesize two of the test supplements: sodium dimethylglycine (Michealis and Schubert, '36) (found 11.12% N, calculated 11.19% N) and sodium glyoxylate (Radin and Metzler, '55). The latter substance reduced ammoniacal silver nitrate; it was homogeneous and free of both oxalic and glycolic acid by paper chromatography in two different solvent systems. Its derivatives had the following properties: 2,4-dinitrophenylhydrazone, M.P. obs. 189–190°, lit. 191° (Weissbach and Sprinson, '53a); *p*-nitrophenylhydrazone, M.P. obs. 198–199° dec., lit. 200° dec. (Heilbron, '46); semicarbazone, M.P. obs. 215° dec., lit. 211° dec. (Campbell, '55).

TABLE 1

The growth response in chicks resulting from the addition of various nitrogenous compounds

EXPERIMENT NO.	SUPPLEMENT TO BASAL DIET	FEED EFFICIENCY	AVERAGE GAIN ± STANDARD ERROR	AVERAGE GROWTH RATE
	%	*gain/feed*	*gm*	%
XV	None	0.56	145 ± 3	6.9
	Glycine, 1.50	0.63	165 ± 3[1]	7.3
	DL-Alanine, 1.78	0.58	147 ± 4	6.9
	L-Glutamic acid, 2.94	0.57	146 ± 3	6.9

[1] By comparison with the basal diet the difference is statistically significant at the 1% level by the "t" test.

RESULTS AND DISCUSSION

The growth response due to the addition of various nitrogenous supplements. Before planning experiments with compounds possibly related to glycine, it was necessary to study the specificity of the glycine nitrogen requirement. An earlier experiment pertinent to this point (Wixom et al., '55) demonstrated that the addition of an amount of diammonium citrate that was isonitrogenous with 1.50% glycine to a glycine-deficient basal ratio did not alter the growth rate. In experiment XV (table 1), the addition of either 1.78% DL-alanine or 2.94%

L-glutamic acid did not improve the chick growth rate in the same manner as the 1.50% glycine supplement. The addition of 2.28% diammonium citrate in experiment XIX (table 3) also failed to give growth comparable to that with the glycine supplement.

In the above two experiments and in the remaining ones to be described, the feed efficiency was highest for the glycine-supplemented group, and lowest for the basal group. With the other test substances, the feed efficiency was directly related to the growth rate. Thus the evidence from the above three supplements lends further support to the earlier conclusion that the attainment of maximum rate of growth is not restricted by insufficient total nitrogen in the diet. The utilization of the extra dietary nitrogen in the test substances is probably related to the carbon skeleton attached to the nitrogen. Apparently then, the rate of synthesis of the carbon chain of either glycine or serine is limiting the growth rate in these chicks.

The growth response due to the addition of both glycine and serine. In three previous experiments, it was found that the addition of 2.10% DL-serine improved the growth rate of chicks on a glycine-deficient ration to almost the same extent as 1.50% glycine (Wixom et al., '55). These observations might be explained by the presence of a reversible metabolic transformation of serine and glycine, or on the basis of a multiple essential amino acid deficiency, namely glycine and serine. The latter hypothesis, if valid, would require a growth increase due to glycine plus serine supplementation greater than the growth response due to glycine or serine alone.

Experiment XVII (table 2) was designed to differentiate between these opposing hypotheses. It was found that the simultaneous addition of 1.50% glycine and 2.10% DL-serine to the basal diet did not improve, but actually decreased, the growth rate of chicks as compared with those which received only a glycine supplement. This decrease might be due to the presence of relatively high levels of potentially available glycine and therefore expained on the basis of glycine toxicity (*cf.*

Arnstein, '54). Confirmation of this latter interpretation is found in experiment XVIIIA (table 2) where half of the above levels, i.e. 0.75% glycine plus 1.05% DL-serine gave essentially the same growth rate as 1.50% glycine. Thus the evidence in both experiments eliminates the hypothesis of a multiple amino acid deficiency; this sparing effect of serine for glycine is in accord with the presence of a metabolic interconversion of glycine and serine (fig. 1).

The growth response due to the addition of methylated glycines. Earlier experiments by other investigators with the rat and rabbit suggested the need to test the possible metabolic

TABLE 2

The growth response in chicks resulting from the addition of glycine and serine

EXPERIMENT NO.	SUPPLEMENT TO BASAL DIET	FEED EFFICIENCY	AVERAGE GAIN ± STANDARD ERROR	AVERAGE GROWTH RATE
	%	*gain/feed*	*gm*	%
XVII	None	0.55	14[illegible] ± 3	6.5
	Glycine, 1.50	0.63	165 ± 2[1]	7.1
	Glycine, 1.50 + DL-serine, 2.10	0.57	[illegible]4 ± 4	6.5
XVIIIA	None	0.52	138 ± 3	6.7
	Glycine, 1.50	0.57	157 ± 3[1]	7.2
	Glycine, 0.75 + DL-serine, 1.05	0.55	156 ± 3[1]	7.2

[1] By comparison with the basal diet the difference is statistically significant at the 1% level by the "t" test.

conversion of tri-, di-, and mono-methylated glycines to glycine in the chick. In experiments XVI, XVIII, and XIX the calculated equivalent amount of sodium chloride, or sodium bicarbonate or hydrochloric acid was added along with the test substance so that all of the individual experimental diets had a total sodium chloride content of 1.17%; this amount was higher than the usual 0.65% but no adverse changes were observed. The data for experiments XVI and XIX (table 3) indicate that the growth rate and feed efficiencies of diets containing betaine, dimethylglycine, sarcosine and of the basal group were essentially the same. The finding with betaine was not unexpected

in view of the data of Almquist and Mecchi ('40). Our experiments, which employed a larger number of chicks and an improved basal diet, confirmed their brief report. While the earlier results excluded the overall conversion of betaine to glycine, the response of metabolic intermediates between betaine and glycine in the chick was unknown. The present

TABLE 3

The growth response resulting from the addition of glycine or aminoethanol derivatives

EXPERIMENT NO.	SUPPLEMENT TO BASAL DIET	FEED EFFICIENCY	AVERAGE GAIN ± STANDARD ERROR	AVERAGE GROWTH RATE
	%	*gain/feed*	*gm*	%
XVI	None	0.57	153 ± 3	6.9
	Glycine, 1.50	0.59	160 ± 4	7.1
	Betaine, 2.34	0.54	146 ± 4	6.8
	Dimethylglycine, 2.06	0.57	154 ± 4	7.0
	Sarcosine, 1.78	0.57	153 ± 3	7.0
XVIIIB	None	0.52	138 ± 3	6.7
	Glycine, 1.50	0.57	157 ± 3[1]	7.2
	Glyoxylic acid[2], 1.48	0.49	130 ± 3	6.5
	Glycolic acid[2], 1.52	0.44	100 ± 4[1]	5.6
	Aminoethanol, 1.22	0.57	154 ± 3[1]	7.1
XIX	Diammonium citrate, 2.28	0.55	135 ± 3	6.8
	Glycine, 1.50	0.66	159 ± 3[1]	7.4
	Aminoethanol, 1.22	0.65	152 ± 3[1]	7.2
	Methylaminoethanol, 1.50	0.51	84 ± 3[1]	5.1
	Dimethylaminoethanol, 1.78	0.58	138 ± 3	6.9
	Betaine, 2.34	0.57	131 ± 4	6.7
	Sarcosine, 1.78	0.56	128 ± 3	6.6

[1] By comparison with the basal diet the difference is statistically significant at the 1% level by the "t" test.

[2] Diammonium citrate 2.28% included in these diets to make them isonitrogenous with the glycine-containing diet.

nutritional observations clearly establish the inability of the chick to transform sarcosine to glycine.

The above results are in sharp contrast to the known presence of these metabolic reactions in the rat. Isotope and enzyme studies from several laboratories have demonstrated that in the rat, choline must first undergo oxidation to betaine,

which, after the transfer of a methyl group, yields dimethylglycine (Stetten, '41; du Vigneaud et al., '46b; Borsook and Dubnoff, '47; Dubnoff, '49; Muntz, '50). Both the oxidation and transmethylation steps to form methionine proceed as readily in liver homogenates from the chick as those from the rat (Dubnoff, '49). Furthermore, nutritional studies with the chick showed that the addition of betaine to a choline-deficient, dimethylaminoethanol-containing ration improved growth and prevented perosis (Jukes and Stokstad, '52; Young et al., '55). Since the transfer of a methyl group away from betaine can occur readily, a later step is rate-limiting for possible glycine formation in the chick.

The subsequent steps between dimethylglycine and glycine have also been studied in the rat. On the basis of nutritional and isotopic experiments, dimethylglycine (Moyer and du Vigneaud, '42; du Vigneaud et al., '46b) and sarcosine (du Vigneaud et al., '39, '46c) were demonstrated to be ineffective methyl donors for choline formation, showing that only one methyl group of betaine can be transmethylated. Both isotopic and enzymatic studies indicate that dimethylglycine is oxidized to formaldehyde and sarcosine, which upon further oxidation, yields glycine and formaldehyde (Bloch and Schoenheimer, '40; Handler et al., '41; Mitoma and Greenberg, '52; Mackenzie et al., '53; Mackenzie, '55). As sarcosine has a growth-stimulating effect with rats on a sodium benzoate-containing diet (White, '41), demethylation of sarcosine must proceed fairly readily in the rat. Another indication of the overall rapid rate of conversion of betaine to glycine in the rat is the highly pertinent observation that N^{15} choline or N^{15} betaine is as effective as N^{15} serine in being a precursor of N^{15} glycine in hippuric acid (Soloway and Stetten, '53).

In the rabbit, administration of sarcosine with sodium benzoate was found to increase urinary hippuric acid excretion (Abbott and Lewis, '39). By the same criteria, the rabbit was shown to be unable to carry out the conversion of betaine or dimethylglycine to glycine.

Recent enzymatic studies also demonstrate a species difference in the *in vitro* oxidation of the methylated glycines. Mackenzie ('55) found active enzymes for the oxidation of dimethylglycine and sarcosine in preparations of rat, rabbit, hamster and guinea pig liver, but similar preparations of chicken liver were devoid of dimethylglycine or sarcosine oxidase activity. In summary, the above evidence from isotopic

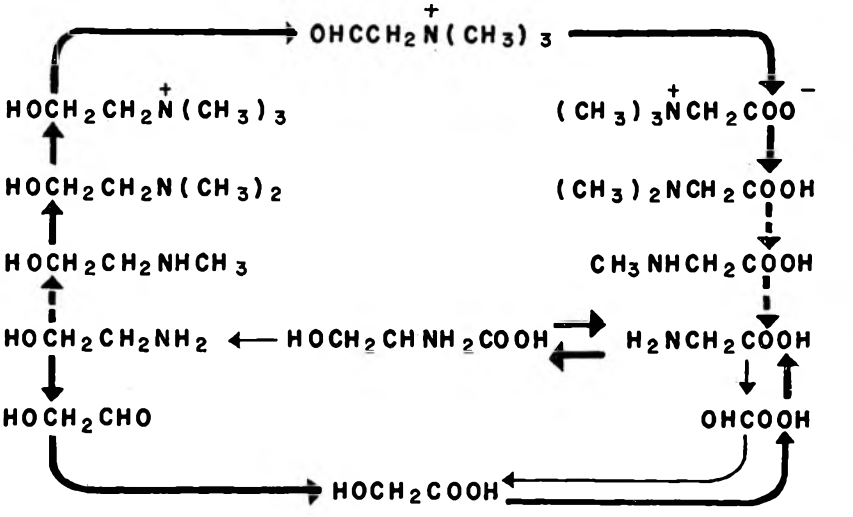

Fig. 1 Metabolic relationships of glycine, serine, aminoethanol and related compounds. All of the above reactions have been shown to be present in the rat. The arrows with heavy lines indicate reactions that occur readily in the chick. The arrows with broken lines represent reactions that have such a slow rate that they cannot be detected by nutritional studies in the chick. The reactions shown by arrows with the thin lines have not been evaluated in the chick.

tracer, hippuric acid formation, enzymatic and the present nutritional studies are in remarkable agreement regarding species differences for the rate of metabolism of the methylated glycines. The rat can readily carry out all steps of the conversion of betaine to glycine; the rabbit can complete only the last step of oxidation of sarcosine to glycine; by contrast, while the chick can readily transfer one methyl group of betaine, this species cannot perform the last two reactions at a measurable rate (*cf.* fig. 1).

The growth response due to the addition of glycolic acid and glyoxylic acid. Evidence for the reversible reactions between glycolic acid, glyoxylic acid and glycine in the rat has been described in recent publications. For this reason, experiment XVIIIB (table 3) was performed to test the presence of these reactions in the chick. Equimolar amounts of glycine, sodium glyoxylate and glycolic acid, respectively, were added to the basal diet of groups of 36 chicks each. The ionic content was adjusted, as described earlier for experiment XVI, to give a sodium chloride content of 1.17%; diammonium citrate was added to the appropriate groups to make the experimental diets isonitrogenous. Glycine gave the usual statistically valid increase in growth rate, from 6.7 to 7.2%. By contrast, glyoxylic acid and glycolic acid led to a depression of the growth rate. The significance of these results will be considered in the next section on aminoethanol.

Several experimental approaches have demonstrated that glycolic acid may be oxidized to glyoxylic acid, which in turn may undergo transamination to form glycine in the rat. Unequivocal evidence for these reactions is found in recent radioactive carbon studies (Weinhouse and Friedmann, '51; Weissbach and Sprinson, '53a; Chao et al., '53) and *in vitro* enzymatic studies (Nakada and Weinhouse, '53a, b; Kun et al., '54; Nakada et al., '55). Earlier growth studies with incorporation of benzoic acid into the diet of the rat have shown that glycolic acid minimized the toxicity of benzoic acid (Griffith, '30) and improved the growth rate (White, '41). In the rabbit administration of glycolic acid with sodium benzoate led to the formation of extra hippuric acid (Abbott and Lewis, '41).

By contrast, the growth of chicks on a glycine-deficient ration was depressed by the dietary glycolic acid in both the present and earlier studies (Almquist and Mecchi, '40). In a report that appeared after the completion of the present experiment (Sullivan and Bird, '57), the simultaneous addition of sodium glycolate and urea produced a growth increase comparable to that of a glycine supplement. This positive

observation may be due to their use of markedly different dietary conditions—i.e., a low-protein basal diet (12%), and a low level of sodium glycolate (0.147%). In view of the positive findings with aminoethanol to be described in subsequent paragraphs, it is essential to consider the nature of the growth-depressing action of glycolic and glyoxylic acid. One indication is found in *in vitro* experiments wherein glyoxylate yielded oxalate when present at high concentrations, and not when formed transiently in low physiological concentrations (Nakada and Weinhouse, '53a). A single intraperitoneal injection of glyoxylic acid produced tetany and death in rats (Weissbach and Sprinson, '53a). Another clue is that the toxicity of a large single oral dose of glyoxylic acid for chicks was found to be similar to that of oxalic acid, as folic acid failed to prevent the toxic symptoms; by contrast, folic acid helped the chick to withstand an otherwise lethal dose of glycine (Naber et al., '56). Evidently the toxicity of glyoxylic acid and glycolic acid in the present experiment may depend on the rate of release of these acids in the tissues, high levels leading to increased oxalic acid formation, and lower physiological levels being more efficiently used in metabolic reactions.

The growth response due to the addition of aminoethanol. Recent studies with radioactive aminoethanol have indicated that this substance is utilized for glycine synthesis in the rat. The possible presence of this metabolic transformation may also be examined in the chick by growth studies. In experiment XVIIIB (table 3) the addition of aminoethanol to a glycine-deficient basal ration led to an increase in the growth rate from 6.7 to 7.1%, an increase of the same magnitude as that resulting from glycine supplementation. Application of the "t" test showed that the probability that the observed difference in the mean gain of the basal and aminoethanol-supplemented group was due to chance was less than 1%. In view of the uniqueness of this observation, the experiment was repeated. Again aminoethanol (experiment XIX, table 3) increased the growth rate to almost the same extent as glycine, from 6.8 to 7.2 and from 6.8 to 7.4% respec-

tively. Both the increase due to glycine and that due to aminoethanol were statistically significant at the 1% level.

It has been previously demonstrated that in the rat the formation of aminoethanol proceeds by the intermediary conversion of glycine to serine and then decarboxylation of serine to aminoethanol (Stetten, '41, '42b; Levine and Tarver, '50; Arnstein, '51; Elwyn et al., '55). The possibility of direct reduction of glycine to aminoethanol has been eliminated (Greenberg and Harris, '50; Arnstein, '51).

The carbon skeleton of aminoethanol may form the two-carbon chain of glycine by two pathways—via choline or via glycolaldehyde. Investigations of the metabolic distribution of radioactive aminoethanol, glycolaldehyde and glyoxylic acid in the rat established that one metabolic pathway of aminoethanol involves the sequence of steps from aminoethanol to glycolaldehyde to glycolic acid to glyoxylic acid to glycine (Weissbach and Sprinson, '53a, b; Pilgeram et al., '53; Friedmann et al., '56). After administration of radioactive aminoethanol or glyoxylic acid, the uric acid excreted by a pigeon was found to have the same carbon-14 distribution as that from radioactive glycine. Evidently the oxidative metabolic pathway of aminoethanol is the same in the avian species as in the rat. The above interrelationships are summarized in figure 1.

The growth response due to addition of methylated aminoethanols. Owing to the positive findings with aminoethanol, and since there are two known metabolic pathways for this substance in the rat (fig. 1), experiment XIX (table 3) was performed. In marked contrast to aminoethanol, the addition of either N-monomethylaminoethanol or N,N′-dimethylaminoethanol did not improve the growth rate of the chicks. Continuation of the chicks on the diet with the monomethyl compound for a week after the two-week experimental period led to observations of poor growth and feathering, and several instances of perosis and death. It is tempting to speculate that the depression of growth with the realtively high levels of methylaminoethanol might result from an actual methyl

group or folic acid deficiency due to the effort to detoxify this amine. Thus the action of methylaminoethanol in the chick is perhaps similar to that of the methyl deficiency induced by dietary guanidoacetic acid (Stetten and Grail, '42), nicotinamide (Handler and Dann, '42) or pyridine (Dinning et al., '50) in the rat.

Earlier studies with the weanling rat indicate that feeding dimethylaminoethanol prevented the formation of fatty livers, but did not support normal growth. The monomethyl compound was found to be toxic for young rats, but by tracer studies was demonstrated to be a precursor of choline (du Vigneaud et al., '46a). Aminoethanol and its monomethyl and dimethyl derivatives stimulated the formation of both choline-containing and non-choline-containing phospholipids (Artom and Cornatzer, '48). These nutritional observations along with isotopic tracer studies (Stetten, '41; Stetten, '42a; Arnstein, '51; Pilgeram et al., '53) support the sequence of successive steps of aminoethanol to form monomethylaminoethanol, dimethylaminoethanol and choline in the rat.

In contradistinction to the rat, the chick is unable to methylate aminoethanol. By the criteria of growth and prevention of perosis on a methyl-deficient diet, aminoethanol is devoid of biological activity for the chick. On the other hand, methylaminoethanol and dimethylaminoethanol are effective under the same experimental conditions (Jukes, '41; Jukes et al., '45; Schaefer et al., '51). More recently Young et al. ('55) found that either methylaminoethanol plus betaine (1:2) or dimethylaminoethanol plus betaine (1:1) were just as effective as choline for growth or prevention of perosis in chicks. Further evidence that the chick is unable to methylate aminoethanol is found in *in vitro* studies where chick liver slices converted radioactive aminoethanol to phosphatidyl choline at only 4% of the rate for the same reaction in rat liver slices (Pilgeram and Greenberg, '54). Thus all of these studies point to the inability of the chick to add the first methyl group to aminoethanol.

The remarkable fact found in the present studies is the growth-promoting action of aminoethanol for chicks on a glycine-deficient, choline-containing basal ration. This must represent an overall conversion of aminoethanol to glycine. On the basis of the evidence presented and that cited in the literature, this observation cannot be explained on the basis of conversion of aminoethanol through its methylated derivatives to choline and betaine and then the loss of the methyl groups of the latter. Thus the pathway of deamination of aminoethanol, oxidation of the aldehyde carbon of glycolaldehyde, oxidation of the carbinol carbon of glycolic acid, and then transamination to produce glycine must be a quantitatively important reaction in the chick. (fig. 1).

General comments. All of the above nutritional observations probably represent a species difference in the balance between the rate of formation of tissue metabolites and the rate of utilization of these building units in the growth of protoplasm. When the rate of synthesis of a body component is inadequate for the maximum rate of growth, as in the case of glycine for the chick or arginine for the weanling rat, then the presence of this component in the diet is required in order to maintain optimal growth. Figure 1 is an effort to characterize several of the differences between the chick and the rat. As reviewed earlier, all of these reactions are known to be present and to occur readily in rat tissue. By contrast, under the conditions of nutritional studies, the chick cannot convert sarcosine to glycine or aminoethanol to methylaminoethanol. Furthermore, the magnitude of both the interconversion of serine and glycine and the direct oxidation of aminoethanol to glycine are shown in the figure. These differences may also reflect the difference in tissue demands for glycine synthesis in the two species. In addition to the many known reactions for glycine in the rat (*cf.* Arnstein, '54), the chick needs some preformed dietary glycine for the synthesis of muscle creatine, the synthesis of tissue protein due to a faster rate of body growth and feather formation, and the synthesis of uric acid for nitrogen excretion.

Owing to the cyclic nature of the reactions in figure 1, there must be some pathway, other than those shown, for the *de novo* formation of either serine or glycine from carbohydrate. Because of the facile interconversion of serine and glycine (Wixom et al., '55), some earlier biosynthetic reaction must be the rate-limiting step in the manufacture of glycine in order to prevent the attainment of the maximum rate of growth without dietary glycine in the chick. Present evidence indicates that the formation of serine by transamination of hydroxypyruvate, or its phosphate derivative, is probably the major pathway for formation of these two amino acids in the rat (Arnstein, '54; Ichihara and Greenberg, '55; Arnstein and Keglevic, '56; Friedmann et al., '56; Sallach, '56). On the other hand glycolaldehyde, which is related to carbohydrate metabolism by the transketolase reaction, has been considered as another primary source of glycine (Friedmann et al., '56). However, further work is needed to clarify the pathway(s) for *de novo* synthesis of these two amino acids in the chick.

CONCLUSIONS

The specificity of the glycine requirement of the chick and the presence of certain metabolic reactions were examined by adding various test substances to a glycine-deficient, casein-containing basal diet and observing the change in the rate of growth. As the supplements of diammonium citrate, alanine or glutamic acid did not stimulate the growth rate, it is concluded that the chick's inability to form glycine is primarily due to a slow rate of formation of the carbon skeleton of glycine, rather than an inadequate supply of utilizable nitrogen.

Under the conditions of these experiments, the addition of serine to a glycine-containing ration did not improve the growth rate. In conjunction with earlier experiments, this observation indicates the presence of a facile metabolic interconversion of glycine and serine in the chick.

The addition of betaine, dimethylglycine or sarcosine to the glycine-deficient diet did not improve the growth rate of

chicks. In contrast to the rat, the chick evidently cannot readily transform these N-methyl compounds to glycine.

Supplementation with aminoethanol increased the growth rate to almost the same extent as glycine, whereas monomethylaminoethanol, dimethylaminoethanol, glycolic acid and glyoxylic acid did not improve the growth rate. Taking into consideration the known toxic nature of high tissue levels of these latter two acids and the findings of other investigators, the observation with aminoethanol probably represents a nutritional demonstration in the chick of the following metabolic reactions: aminoethanol → glycolaldehyde → glycolic acid → glyoxylic acid → glycine.

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THE ABSORPTION OF RADIOLYSINE BY THE CHICK AS AFFECTED BY PENICILLIN ADMINISTRATION

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INTRODUCTION

The observation that the weight of the intestine is reduced in germ-free and antibiotic-fed chicks (Gordon, '52; Coates et al., '53; Pepper et al., '53) has led to the speculation that a thickening of the intestinal wall occurs as a reaction to the presence of unidentified pathogenic microorganisms in the lumen of the gut. The effect of feeding antibiotics on the histology of the gut wall has been described (Jukes et al., '56a). It has not been established clearly, however, whether this thickening is associated with any specific change in physiological activity. As expressed by Baumann ('56), "the question of importance in nutrition is whether the thin intestine is a better organ of absorption than the thick one."

Experiments have been reported in which an improvement was observed in the "digestibility" of protein nitrogen by animals receiving an antibiotic (Forbes, '54; Huang and McCay, '53), but the conventional digestion trial fails to distinguish between differences in the degree of protein hydrolysis in the gastrointestinal tract and differences in absorption of the end products of digestion. The increase in Ca^{45} uptake by penicillin-treated chicks observed by Migicovsky and associates ('51) and the increase in liver vitamin A cited by Burgess et al. ('51) offer firmer evidence of enhanced absorption. Baumann ('56) has reported that the diffusion of xylose across isolated rat

and chick intestines may be increased by prior feeding of antibiotics.

The purpose of the present study was to compare the absorption of a standard oral dose of radiolysine by penicillin-treated and control birds. A radioactive free amino acid was used in order to circumvent any possible effect of the antibiotic on protein digestion and to provide a sensitive means of detecting small differences.

EXPERIMENTAL

Experiment 1. Nine female chicks of New Hampshire x Columbian breeding, 34 days old, were selected at random from each of two groups of 16 birds which had been maintained from one day of age on a practical diet. The percentage composition of the basal diet was as follows: ground yellow corn 69.85, soybean meal (50% protein) 26.40, bone meal 2.00, ground limestone 1.00, iodized salt 0.50, manganese sulfate (monohydrate) 0.05, vitamins A and D oil (3000 A, 600 D) 0.10, DL-methionine 0.10. The following vitamins in milligrams per kilogram were also included: riboflavin 4, calcium pantothenate 6, menadione 1, nicotinic acid 10, choline 200, cyanocobalamin (0.1% triturate) 15. Group 1 was maintained as a control; group 2 received, in addition, 100 mg of procaine penicillin per kilogram of basal diet.

Each chick was given an oral dose of 0.39 mg of uniformly labelled C^{14}-L-lysine (specific activity 1.40 μc/mg) equivalent to 1,211,300 disintegrations per minute (dpm). One milliliter of an aqueous solution of the labelled compound was placed in the anterior portion of the esophagus by means of a syringe fitted with a blunted hypodermic needle. The birds were sacrificed 150 ± 1 minutes later, during which time access to the diet was permitted. The small intestines and ceca were removed, incised longitudinally, and the contents were flushed out with the aid of hot distilled water and a small spatula. The length of the relaxed small intestine was measured in the horizontal position and the weights of the intestines and ceca were recorded after blotting to remove excess moisture.

Coarse feed particles were removed from the intestinal contents by filtering through a heavy layer of glass wool with subsequent washing. The volume was reduced on a steam bath to about 100 ml and the suspension was agitated vigorously for 5 minutes, using an Alpine counter-current stirrer-homogenizer. Aliquots of the resulting fine suspension could be plated with excellent duplication. The cecal contents were handled similarly, except that they did not require filtration. Counting was carried out using a Tracerlab windowless Q-gas counter and Nuclear scaler.

A preliminary experiment was conducted to determine the distribution of radioactivity throughout the alimentary tract in two untreated birds two and one-half hours after administration of radiolysine.

Experiment 2. A second test was carried out according to the same general plan but with certain modifications. In order to preclude the possibility that penicillin might affect the amount of residual radiolysine in the digestive tract by altering the rate of food passage, the birds were sacrificed 60 ± 1 minutes after dosing, at which time no radioactivity had reached the lower gut. The chicks were fed the same basal diet as those of experiment 1 except that a suspension of raw chick feces was added. The feces were collected from a farm flock, agitated in three volumes of water, and mixed into the diet at a concentration of 1% by weight. The birds were fasted for 10 hours before dosing and were allowed no food subsequently. After one hour, the crop, proventriculus, gizzard, small intestine, ceca, liver and combined excreta were collected. Twelve chicks were used in each group.

Except for liver, the samples were prepared for counting as already described. The livers were treated according to the procedure of Pearce and associates ('56). The whole livers were cut into small pieces with scissors, dried at 95°C for 72 hours and then digested with hot formamide for one and one-half to three hours until dissolution was complete. The dark solutions were cooled, diluted to 100 ml with 95% ethanol, and 250 µl aliquots were plated for counting. All samples were

counted to a 5% coefficient of variability. Radiolysine added to liver and carried through this procedure could be recovered quantitatively.

RESULTS AND DISCUSSION

Experiment 1. The results of the preliminary experiment, shown in table 1, indicate that at least 90% of the dose of radiolysine had disappeared from the digestive tract after two and one-half hours.

TABLE 1

Distribution of radioactivity in the alimentary tract of two chicks two and one-half hours after an oral dose of C^{14}-L-lysine [1]

ORGAN CONTENTS	CHICK A	CHICK B
	% of dose	*% of dose*
Crop	0.4	0.7
Proventriculus	0.1	0.1
Gizzard	1.3	1.8
Small intestine	4.7	6.1
Ceca	0.7	0.4
Large intestine	0.1	0.7
Total residual activity	7.3	9.8

[1] The dose was 0.39 mg, equivalent to 1,211,300 dpm. Chicks A and B weighed 375 gm and 410 gm, respectively.

A summary of the data on the dimensions of the intestines and ceca is given in table 2. In conformity with the findings of other workers, the weights of the small intestine and ceca were significantly less in the group which had received penicillin. No significant change was observed in the length of the small intestine.

The results of the radioassay for residual C^{14} in the intestinal and cecal contents are given in table 3. The data show that there was 15 and 41% less activity in the intestinal and cecal contents of the penicillin-treated and control birds, respectively. The difference was highly significant ($P = <0.01$) for the cecal contents and not significant for the intestinal contents ($P = 0.2$).

These findings imply that penicillin caused a reduction in the weight of the chief organs of digestion and absorption and increased the uptake of free lysine, but the *prima facie* conclusion that the second event was a direct result of the first is subjected to certain qualifications. The occurrence of a shift in bacterial populations in the gut under the influence of

TABLE 2

Intestinal and cecal measurements of penicillin-treated and control chicks of experiment 1

	TREATMENT		AVERAGE DIFFERENCE IN FAVOR OF PENICILLIN GROUP	P VALUE
	None	Penicillin		
			%	
No. chicks	9	9	—	—
Body wt., *gm*	524 ± 19 [1]	535 ± 16	+ 2	>0.05
Wt. of small intestine, *gm*	9.8 ± 0.3	8.1 ± 0.3	−18	<0.01
Length of small intestine, *in.*	36.6 ± 0.5	35.5 ± 0.7	− 3	>0.05
Wt. of ceca, *gm*	1.9 ± 0.07	1.5 ± 0.05	−18	<0.01

[1] Mean and standard error.

TABLE 3

Residual C^{14} in the intestinal and cecal contents of penicillin-treated and control chicks two and one-half hours after an oral dose of C^{14}-L-lysine (1,211,300 dpm)

	TREATMENT		AVERAGE DIFFERENCE IN FAVOR OF PENICILLIN GROUP	P VALUE
	None	Penicillin		
			%	
No. chicks	9	9	—	—
Residual radioactivity (dpm) in:				
Intestinal contents (x 10^{-3})	61.0 ± 3.9 [1]	51.7 ± 4.8	−15	>0.05
Cecal contents (x 10^{-2})	78.0 ± 5.1	46.3 ± 4.1	−41	<0.01

[1] Mean and standard error.

penicillin, with a consequent loss of the isotope as $C^{14}O_2$ arising from fermentation, or an acceleration of food passage, could also account for the results. The former possibility seems unlikely, since in the course of many reported studies on the effect of antibiotics on dietary requirements of various species for protein or individual amino acids, no clear evidence for an increased requirement has been found. Such experiments in general have indicated either the absence of any significant relationship or a "sparing" effect on the required dietary amino acid concentration. On the other hand, several

TABLE 4

Intestinal and cecal measurements of penicillin-treated and control chicks of experiment 2

	TREATMENT		AVERAGE DIFFERENCE IN FAVOR OF PENICILLIN GROUP	P VALUE
	None	Penicillin		
			%	
No. chicks	12	12	—	—
Body wt., *gm*	381 ± 4 [1]	389 ± 5	+2	>0.05
Wt. of small intestine, *gm*	7.9 ± 0.2	7.3 ± 0.3	−8	>0.05
Length of small intestine, *in.*	38.1 ± 0.6	40.1 ± 0.6	+5	0.03
Wt. of ceca, *gm*	1.79 ± 0.10	1.70 ± 0.09	−5	>0.05

[1] Mean and standard error.

workers have reported an increase in rate of food passage through the digestive tract of birds under the influence of antibiotics (Hillerman et al., '53; Stokstad et al., '53; Jukes et al., '56b). Accordingly, the chicks of experiment 2 were sacrificed before sufficient time had elapsed for the radiolysine dose to be excreted in the feces.

Experiment 2. Table 4 summarizes the measurements of the small intestines and ceca of the 12 chicks in each group of this experiment. The weights of these organs, respectively, averaged 8 and 5% less in the penicillin-fed groups, neither difference being significant.

The distribution of residual radioactivity in the digestive tract of these birds is indicated in table 5. The absence of activity in the cecal contents demonstrates that the labelled compound had not traversed the entire intestinal tract and that the small quantity of radioactivity in the excreta was of urinary origin. A reduction of 23% ($P = <0.01$) in the radioactivity found in the small intestinal contents of the penicillin group was observed. Much variability was encountered in the counts on the livers and contents of other organs and no significant difference was found. An average decrease of 18%

TABLE 5

Distribution of C^{14} in the liver and contents of digestive organs of penicillin-treated and control birds one hour after an oral dose of C^{14}-L-lysine [1]

	TREATMENT		AVERAGE DIFFERENCE IN FAVOR OF PENICILLIN GROUP	P VALUE
	None	Penicillin		
			%	
No. chicks	12	12	—	—
Residual radioactivity (dpm) in:				
Crop contents ($\times 10^{-3}$)	7.8 ± 1.8 [2]	14.2 ± 2.7	+82	>0.05
Proventriculus and gizzard contents ($\times 10^{-3}$)	67.5 ± 18.9	50.3 ± 12.4	−25	>0.05
Small intestinal contents ($\times 10^{-3}$)	65.1 ± 4.0	50.0 ± 2.2	−23	<0.01
Cecal contents	none detectable	none detectable	—	—
Composite contents of digestive organs ($\times 10^{-3}$)	140.4 ± 4.8	114.5 ± 3.2	−18	<0.01
Excreta [3] ($\times 10^{-3}$)	2.6 ± 0.9	3.2 ± 0.9	+23	>0.05
Liver ($\times 10^{-3}$)	53.9 ± 11.4	72.5 ± 13.4	+34	>0.05

[1] The dose was 0.67 mg, equivalent to 888,000 dpm.

[2] Mean and standard error.

[3] One treated bird and 6 controls voided no excreta during the one-hour collection period.

($P = <0.01$) was observed in the activity of the combined gastrointestinal contents of the treated birds (140,400 vs 114,500 dpm). In terms of the dose administered, 12.9 and 15.8% were recovered in the digestive tract of the penicillin and control chicks, respectively.

The composite results of experiments 1 and 2 indicate that the feeding of penicillin was associated with a decrease in weight of the primary organs of digestion and absorption, and with an increase in the efficiency of absorption of lysine. Presumably a similar effect on other amino acids might be observed. As might be anticipated from the failure of most workers to observe any marked effect of feeding antibiotics on the requirements of animals for protein or individual amino acids, the differences encountered were relatively small, and might evade detection by a less sensitive analytical method. Nevertheless, under feeding conditions, an improvement of this magnitude in the efficiency of absorption of amino acids might well be reflected in increased growth or feed efficiency. The marked growth-stimulating action of antibiotics on rats fed diets containing poor quality proteins, as compared with that observed when these proteins are supplemented with their limiting amino acids (Sauberlich, '54), may be partly explicable in terms of an improved absorption of those amino acids present in the diet in growth-limiting concentrations. When all the essential amino acids are provided in adequate amounts, no growth increment would be expected to arise from absorption of additional quantities of these nutrients.

Evidence has been presented by other investigators which indicates that an enhancement of amino acid absorption may occur in conjunction with antibiotic administration. Carroll and associates ('53) have reported that the rate of disappearance of several amino acids from the lower intestine of the rat was increased by feeding chlortetracycline, and Ferrando et al. ('53) found that the absorption of nitrogen per unit of time from a casein hydrolysate placed in an isolated portion of rat ileum *in situ* was increased when penicillin and chlortetracycline were present.

SUMMARY

A study has been made of the effect of feeding penicillin on the disappearance of radioactivity from the digestive tract of the chick following an oral dose of C^{14}-L-lysine. In each of two experiments, antibiotic administration was associated with a decrease in the weight of the small intestine and ceca, and a decrease in the amount of radioactivity recovered from the digestive tract two and one-half and one hour, respectively, after administration of the labelled amino acid. The results imply that absorption was more efficient from the lighter intestinal tract of the penicillin-fed birds than from the heavier tract of the controls.

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THE EFFECT OF THE INGESTION OF ASCORBIC ACID AND DEHYDROASCORBIC ACID UPON THE BLOOD LEVELS OF THESE TWO COMPONENTS IN HUMAN SUBJECTS

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Previous studies have shown that man is capable of reducing dehydroascorbic acid. Todhunter et al. ('50) and Johnson and Zilva ('34) fed orange juice treated with norit or iodine as a source of dehydroascorbic acid and found the amount of ascorbic acid in the urine was comparable to that obtained when untreated orange juice was given. Borsook et al. ('37) found similar increases in ascorbic acid in plasma following ingestion of dehydroascorbic acid or of ascorbic acid. Todhunter et al. ('50) reported that 65 mg of dehydroascorbic acid maintained ascorbic acid in serum at levels comparable to those found when 65 mg of the reduced vitamin were fed. In all the above studies dehydroascorbic acid was obtained by oxidation of natural L-ascorbic acid.

Studies comparing the concentration of the two forms of the vitamin in blood after oral dosages of crystalline dehydroascorbic acid and ascorbic acid have not been made.

The present experiment was designed to measure the extent and rate of reduction of dehydroascorbic acid by determining the ascorbic acid and total ascorbic acid (ascorbic

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plus dehydro) in serum before and at various intervals following ingestion of dehydroascorbic acid and ascorbic acid.

EXPERIMENTAL

Seven women ranging in age from 25 to 40 years participated in the study. Subjects reported to the laboratory at 8:00 A.M., and a fasting blood sample was taken, after which the subjects were given either dehydroascorbic acid (50, 150, 300, or 500 mg) or ascorbic acid (150 or 500 mg) and breakfast of buttered whole wheat toast and coffee. No other food was eaten until 1:00 P.M., and only foods low in ascorbic acid were allowed for that day. Blood was analyzed one, one and one half, three, 5 and occasionally 24 hours after ingestion of the vitamin.

Blood was taken by finger tip puncture in capillary tubes which were then sealed [2] and centrifuged. After breaking the tubes to separate serum from cells, the serum was removed by micro pipettes and analyzed by the dinitrophenylhydrazine and the indophenol procedures. The former method (Bessey, Lowry, and Brock, '47) as modified by Lowry, Bessey and Burch ('52) involves the oxidation of ascorbic acid with copper sulfate and the colorimetric estimation of the reaction product with dinitrophenylhydrazine. Total ascorbic acid measured in this manner is believed to represent the dehydroascorbic acid produced by the oxidation of ascorbic acid plus any preformed dehydroascorbic acid and diketogulonic acid.

The indophenol procedure was applied to a sample of the same serum in order to estimate ascorbic acid. The Bessey ('38) and Mindlin and Butler ('38) methods were modified, and readings were made in the Beckman quartz spectrophotometer at 520 mμ. The galvanometer was set at 100% transmission with 0.06 ml of 2.5% HPO_3 and 0.16 ml indophenol-acetate solution which had been completely decolorized with ascorbic acid. A blank (G_b) was read 15 seconds after the indophenol-acetate solution was blown into the 2.5% HPO_3

[2] Pyseal was used.

and was stable for an additional 15 seconds. Equal amounts (0.08 ml) of serum and 5% HPO_3 were placed in 6 by 50 mm culture tubes. The tubes were corked, and the serum and acid were mixed thoroughly by agitation.[3] If the serum contained a high concentration of ascorbic acid, further dilutions were made with 2.5% HPO_3. Protein was removed by centrifugation. Small particles in the supernatant solution were removed by a second centrifugation when necessary. Aliquots were pipetted into micro quartz cells which were then placed in the instrument. The galvanometer was set at 100% transmission with the solution described above. Then the indophenol-acetate solution was blown into the sample and the two were mixed for 10 seconds by gently blowing through the micro pipette. Readings were taken 15 seconds (G_{s1}) and 30 seconds (G_{s2}) after the beginning of the addition of the dye and a final reading (G_{sr}) after added ascorbic acid had completely reduced the dye. Cells were washed by filling with water and drying by suction without touching the outside optical surfaces. A blank reading was taken after each washing to insure complete removal of ascorbic acid from the cells. The amount of ascorbic acid in the aliquot was calculated by use of the following equation:

$$C = K\ (\log\ G_s + 2 - \log\ G_b - \log\ G_{sr})$$

The constants K and G_s were calculated according to Bessey ('38). The reproducibility of this method was checked by 18 determinations on a solution of pure ascorbic acid. The mean result was 1.00 ± 0.021 mg/ml, and the range was 0.97 to 1.04 mg/100 ml.

Dehydroascorbic acid was calculated as the difference between total ascorbic acid as determined by the dinitrophenylhydrazine method and ascorbic acid as determined by the indophenol method. Stewart, Horn and Robson ('53) demonstrated that the difference between total ascorbic acid and ascorbic acid in plasma as measured by these two methods represented a quantitative estimate of dehydroascorbic acid.

[3] A Handee Grinder was used.

The specificities of the two reactions used in the analyses ruled out the possibility that part of the difference might be due to the presence of diketogulonic acid.

RESULTS

When fasting blood samples were analyzed, it was found that total ascorbic acid values as determined by the dinitrophenylhydrazine method were significantly higher than ascorbic acid values as determined by the indophenol method. The total ascorbic acid values in the fasting samples ranged from 0.47 to 1.70 mg/100 ml serum. Values for dehydroascorbic acid were obtained by subtracting the ascorbic acid values from the total. The amount of total ascorbic acid found in the dehydro form ranged from 7 to 41% and averaged 21%. The average total ascorbic acid in the serum was 1.28 mg/100 ml, and of this 1.28 mg, a mean of 0.27 mg occurred in the dehydro form. In 22 samples dehydroascorbic acid accounted for 16 to 26% of the total ascorbic acid concentration; in 5 samples the values were 10% or less and in 7 samples 30% or more. Serum with high concentration of total ascorbic acid usually had a correspondingly high concentration of dehydroascorbic acid. The concentration of dehydroascorbic acid averaged 0.15 mg/100 ml of serum for samples with a total ascorbic acid concentration of less than 1.00 mg/100 ml; samples with a total ascorbic acid concentration of 1.50 mg or more contained an average of 0.39 mg dehydroascorbic acid.

Variance analyses according to the method of Snedecor ('56) were made to test the significance of the differences between subjects, supplement levels and time intervals following ingestion of 50 mg, 150 mg and 300 mg of dehydroascorbic acid and after ingestion of 150 mg of ascorbic acid.

Time was a statistically significant factor in the variance analyses. Following ingestion of either form of the vitamin, there was a rapid increase in ascorbic acid and total ascorbic acid in serum (table 1). The peak in concentration occurred at one and one half hours. Apparently the time required for

maximum concentration was unaffected by the form of the vitamin given. There was a significant decrease in the blood levels between one and a half and three hours after the dosage. The data show that three hours after an intake of 50 mg dehydroascorbic acid, the concentration of the vitamin had returned to the original fasting level. On intakes of 150 mg or more the increased concentration was elevated above the fasting level for 5 hours or more and was restored to the original level within 24 hours.

Figure 1 shows the average increase in ascorbic acid and total ascorbic acid one and a half hours after various intakes for all subjects who had a fasting total ascorbic acid level of at least 1.00 mg/100 ml. The increment in total ascorbic acid after ingestion of 50, 150, 300 or 500 mg dehydroascorbic acid was 0.19, 0.54, 0.81, and 1.02 mg/100 ml serum in the order named, while the corresponding values for the ascorbic acid were 0.13, 0.36, 0.60 and 0.98 mg. After ingestion of 150 or 500 mg ascorbic acid the increase in total ascorbic acid was 0.55 and 1.17 mg/100 ml and in ascorbic acid was 0.44 and 0.98 mg, respectively. The variance analyses showed that equal intakes of either form of the vitamin resulted in comparable increases in both dehydroascorbic acid and ascorbic acid. This is in agreement with results of Clayton et al. ('54) who reported that in human subjects oral dehydroascorbic acid behaved quantitatively like ascorbic acid.

There was an actual increase in the concentration of dehydroascorbic acid in serum one and a half hours after an oral intake of either form of the vitamin and this increase was independent of the form given. The increase in dehydroascorbic acid was 0.11 mg/100 ml serum after ingestion of 150 mg ascorbic acid and 0.18 mg after ingestion of 150 mg dehydroascorbic acid. However, an intake of 500 mg ascorbic acid raised the serum dehydroascorbic acid by 0.19 mg/100 ml, while an intake of 500 mg dehydroascorbic acid caused only a slight increase (0.04 mg/100 ml).

Significant individual differences were noted in the subjects. At the fasting level, the total ascorbic acid concentration

TABLE 1

Ascorbic acid (R) and total ascorbic acid (T) content [1] of serum (mg/100 ml) before and after ingestion of ascorbic acid (AA) or dehydroascorbic acid (DHA)

SUBJECT	SUPPLEMENT		HOUR									
			0		1		1½		3		5		24	
			R	*T*	*R*	*T*	*R*	*T*	*R*	*T*	*R*	*T*	*R*	*T*
H. M.	50 mg DHA		0.97	1.37	1.23	1.60	1.17	1.60	1.02	1.35				
		SD [2]	0.21	0.03	0.13	0.21	0.20	0.07	0.28	0.32				
	150 mg DHA		0.93	1.10	1.16	1.51	1.27	1.64	1.28	1.50	1.23	1.37		
		SD	0.28	0.21	0.19	0.15	0.21	0.30	0.23	0.26	0.33	0.25		
	150 mg AA		0.62	0.92	1.05	1.15	1.06	1.30	1.00	1.07	0.89	1.15		
		SD	0.10	0.15	0.10	0.15	0.10	0.09	0.04	0.07	0.09	0.15		
J. T.	50 mg DHA		0.96	1.31	1.09	1.44	1.05	1.50	0.93	1.34				
		SD	0.07	0.08	0.00	0.01	0.00	0.06	0.03	0.12				
	150 mg DHA		0.91	1.06	1.32	1.70	1.17	1.45	1.03	1.35	0.98	1.35		
		SD	0.10	0.06	0.01	0.09	0.15	0.06	0.15	0.07	0.18	0.08		
	300 mg DHA		0.79	1.23	1.68	2.30	1.27	2.00	1.24	1.80	1.26	1.80	1.03	1.40
		SD	0.18	0.09	0.17	0.13	0.27	0.25	0.13	0.15	0.18	0.17	0.12	0.05
G. B.	50 mg DHA		1.18	1.61	1.32	1.85	1.28	1.75	1.20	1.65				
		SD	0.09	0.03	0.06	0.07	0.09	0.14	0.21	0.14				
	150 mg DHA		1.26	1.57	1.45	1.96	1.39	1.89	1.32	1.67	1.27	1.69	1.13	1.33
		SD	0.02	0.11	0.11	0.15	0.12	0.01	0.06	0.11	0.09	0.09	0.09	0.02
	150 mg AA		1.17	1.48	1.60	2.02	1.54	2.03	1.34	1.82	1.49	1.77		
		SD	0.04	0.04	0.03	0.18	0.15	0.16	0.25	0.01	0.00	0.11		
	300 mg DHA		1.18	1.45	1.75	2.40	1.80	2.28	1.59	1.97	1.42	1.75	1.17	1.30
		SD	0.30	0.08	0.21	0.27	0.22	0.20	0.17	0.16	0.13	0.16	0.16	0.10

B. D.	150 mg DHA		1.05	1.36	1.51	1.99	1.57	2.08	1.48	1.92	1.37	1.60	1.06	1.47
		SD	0.05	0.21	0.11	0.09	0.07	0.04	0.09	0.13	0.23	0.49	0.09	0.08
	150 mg AA		1.43	1.70	1.68	2.25	1.82	2.30	1.75	2.25	1.55	2.25	1.50	1.70
		SD	0.12	0.14	0.15	0.19	0.17	0.27	0.16	0.26	0.14	0.43	0.18	0.08
	300 mg DHA		0.97	1.65	1.64	2.40	1.67	2.47	1.55	2.35	1.50	2.20	1.25	1.50
		SD	0.11	0.35	0.13	0.42	0.10	0.25	0.03	0.11	0.18	0.33	0.41	0.17
L. N.	150 mg DHA		1.15	1.55	1.36	1.97	1.39	1.95	1.34	1.70	1.12	1.90	1.03	1.27
		SD	0.21	0.28	0.21	0.29	0.17	0.22	0.21	0.40	0.11	0.43	0.11	0.18
	150 mg AA		1.08	1.27	1.51	1.71	1.63	1.83	1.46	1.87	1.36	1.53		
		SD	0.21	0.23	0 30	0.23	0.18	0.30	0.12	0.23	0.13	0.10		
K. M.	500 mg DHA		1.42	1.68			2.51	2.85	2.37	2.73				
		SD	0.11	0.25			0.13	0.14	0.11	0.11				
	500 mg AA		1.35	1.58			2.16	2.65	2.18	2.54				
		SD	0.09	0.10			0.06	0.23	0.25	0.06				
A. K.	500 mg DHA		0.43	0.52			1.49	1.56	1.20	1.25				
		SD	0.04	0.13			0.07	0.09	0.17	0.14				
	500 mg AA		0.87	0.90			2.04	2.19	2.08	2.51				
		SD	0.16	0.15			0.09	0.14	0.11	0.27				

[1] Each set of figures represents the average for two or three experimental days.
[2] Standard deviation.

ranged from 0.47 to 170 mg/100 ml. The percentage of total ascorbic acid found in the dehydro form ranged from 7 to 41%. Subjects given the same intake did not respond to the same degree, as evidenced by subject x treatment interaction in the statistical analysis. Insufficient data are available to determine a statistically significant difference in the same subject from day to day; however, certain subjects differed markedly

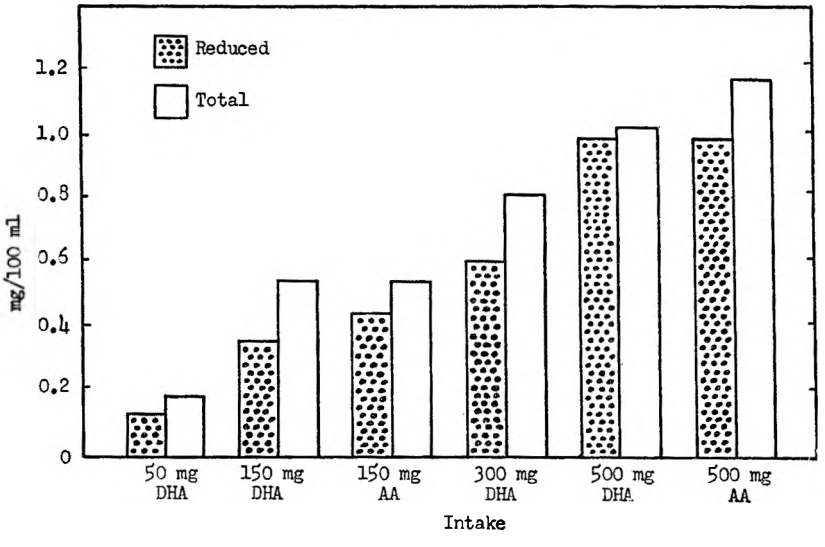

Fig. 1 Increment in ascorbic acid and total ascorbic acid in serum one and one-half hours after various intakes of ascorbic acid (AA) or dehydroascorbic acid (DHA).

in their response from one day to another. This would indicate that not only is there a difference between subjects, but that the same subject may vary from day to day in his response.

A constant ratio between dehydroascorbic acid and ascorbic acid often was maintained for several hours after an oral intake of either form of the vitamin; however, such was not always true. To indicate more clearly the extreme individual differences that were found, the results for three subjects with different fasting concentrations of dehydroascorbic acid

are shown graphically in figure 2. The dehydroascorbic acid (0.65 mg/100 ml) for subject A was extremely high and a considerable fraction (41%) of the total. After an intake of 300 mg dehydroascorbic acid, this high percentage dropped only slightly (31–33%) during a period of 5 hours. In sharp contrast to this, the initial value of 7% for subject C was maintained even though an intake of 500 mg dehydroascorbic acid had more than doubled the fasting total ascorbic acid.

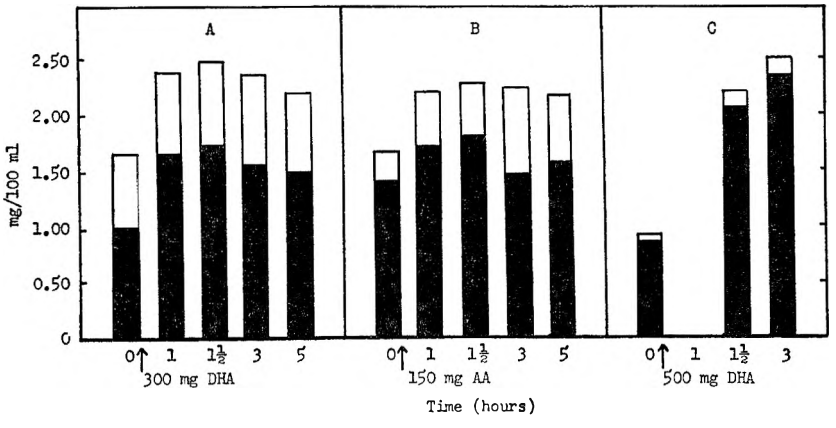

Fig. 2 Ascorbic acid (black) and dehydroascorbic acid (white) concentration in serum before and after certain intakes.

In subject B, however, the initial concentration of dehydroascorbic acid (16% of the total) increased to 31% after an intake of 150 mg ascorbic acid. Furthermore the total ascorbic acid concentration of subject B failed to show the expected decrease within 5 hours.

DISCUSSION

Davey et al. ('52) reported that total ascorbic acid levels were higher than those for ascorbic acid in both serum and plasma of adult women. Stewart, Horn and Robson ('53) presented data which strongly indicated that a significant part of total ascorbic acid in human plasma is present as dehydroascorbic acid and that adrenocortical steroids influence

the ratio between dehydroascorbic acid and ascorbic acid. The latter investigators reported that both ascorbic and total ascorbic acid in plasma were elevated in normal human subjects by the administration of adrenocorticotropic hormone (ACTH); the larger increases in ascorbic acid resulted in a diminution of dehydroascorbic acid. Cortisone caused a complete disappearance of dehydroascorbic acid without changing the total ascorbic acid concentration. These changes occurred within 4 hours after hormone administration. McSwiney et al. ('54) found no consistent effect of ACTH administration on plasma ascorbic acid levels of patients with rheumatoid arthritis; however, there was a tendency for the oxidation product (s) to rise for a few days and then to fall. Clayton et al. ('54) reported that ACTH did not inhibit the reduction of intramuscularly or intravenously administered dehydroascorbic acid in patients suffering from various illnesses, including rheumatoid arthritis; however, similar studies were not reported for normal subjects.

The results of the present study indicate that dehydroascorbic acid is present in serum and that the ratio between ascorbic acid and dehydroascorbic acid varies considerably from day to day. This study also indicates that dehydroascorbic acid in serum is increased after ingestion of either dehydroascorbic acid or ascorbic acid and that the amount of increase is independent of the form given.

Although it has generally been assumed that ascorbic acid exists in the human body almost entirely in the reduced state, the results of this study and other recent studies indicate that this assumption may not be valid. While it is evident that man is capable of reducing large quantities of ingested dehydroascorbic acid, it also appears that when ascorbic acid is consumed, some is immediately oxidized. It appears that the body has a mechanism for maintaining a fairly constant ratio between ascorbic acid and dehydroascorbic acid. Therefore, regardless of the form given, the existing ratio is reached within a few hours after ingestion. The amount of dehydroascorbic acid in serum after an oral intake of the vitamin

seems to be affected not by the form given, but by the existing ratio between the two forms of the vitamin present in serum.

The report that cortisone causes a complete disappearance of dehydroascorbic acid from plasma (Stewart et al., '53) makes the well known relationhsip of ascorbic acid and the adrenal gland assume new importance. Two-thirds of the subjects in the present study had serum dehydroascorbic acid concentrations which accounted for 16 to 26% of the total vitamin; one-fifth, 30% or more; and the remainder, 10% or less. The extreme variation in concentration of serum dehydroascorbic acid in the same subject as well as in different subjects might well be due to differences in adrenal activity. While it is possible that any existing ratio between the two forms may be maintained for several hours even after a large intake of ascorbic or dehydroascorbic acid has doubled or tripled the original serum concentration, it is possible that any alteration in the secretion of adrenal hormones could change the ratio between the two forms.

SUMMARY

Total ascorbic acid values of fasting serum were consistently higher than ascorbic acid values. The difference gave a dehydroascorbic acid concentration which varied markedly from day to day and ranged from 7 to 41% of the total ascorbic acid values. In general, however, serum with a high concentration of total ascorbic acid (av. 1.50 mg) had a correspondingly high concentration of dehydroascorbic acid (0.39 mg). Similarly, serum with an average of less than 1.00 mg/100 ml total had an average of 0.15 mg dehydroascorbic acid.

Following ingestion of either ascorbic acid or dehydroascorbic acid there was a rapid rise in total ascorbic acid and ascorbic acid in serum. The increase was independent of the form of the vitamin given. Total ascorbic acid increased an average of 1.02 mg/100 ml serum after ingestion of 500 mg dehydroascorbic acid and 1.17 mg after 500 mg ascorbic acid. Corresponding values for the increment in ascorbic acid in serum were 0.98 and 0.98 mg/100 ml.

Serum dehydroascorbic acid increased after an oral intake of either ascorbic acid or dehydroascorbic acid, and this increase was unaffected by the form of the vitamin given. The original fasting ratio existing between the two forms often remained remarkably constant after ingestion of either form of the vitamin.

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EXUDATIVE DIATHESIS IN CHICKS

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The work of Scott et al. ('55) has stimulated interest in the possible role of vitamin E in protein metabolism and hematopoiesis. Using a diet containing torula yeast as the protein source, these workers demonstrated that symptoms of vitamin E deficiency in the chick could be produced in approximately three weeks. These symptoms were apparently identical with exudative diathesis as reported by Dam and Glavind ('38). The manifestations of the deficiency condition were edema and profuse hemorrhaging. These symptoms were prevented with either 5 mg α-tocopheryl acetate per pound or 10% dried brewers' yeast added to the diet. Addition of a variety of antioxidants did not alleviate the deficiency. In a later study, Goldstein and Scott ('56) studied plasma and exudate fluid from vitamin E-deficient chicks and found that the deficiency resulted in a decrease in total plasma protein, particularly albumin. The exudates were found to be similar to the plasma as determined electrophoretically. Oral administration of pure α-tocopheryl acetate completely alleviated these symptoms and produced a rapid restoration of plasma albumin level. These workers suggest a possible role of vitamin E in plasma protein anabolism.

Employing a diet similar to that of Scott et al. ('55), turkeys have also been shown to exhibit symptoms of exudative diathesis (Creech et al., '57). Under the conditions of these experiments, only a mild edema occurred and no differences in serum protein concentration were noted. An anemia was

observed, but was found to be macrocytic; whereas the anemia has been reported to be microcytic in chicks. The symptoms could be prevented in turkey poults by supplementation of the torula yeast diet with α-tocopheryl acetate. In addition, a protective effect was found when the maternal diets of the poults studied were supplemented with vitamin E or dried brewers' yeast. Electrophoretic studies of the serum of day-old poults hatched from dams fed diets supplemented with vitamin E or dried brewers' yeast or both indicated that a maternal deficiency results in reduced albumin/globulin ratios. Apparently, this reduction was in albumin as was shown to occur in chicks (Goldstein and Scott, '56).

The present studies with chicks were initiated to study further the production of exudative diathesis using a torula yeast diet and to investigate the serum and exudates of birds suffering from this deficiency symptom.

EXPERIMENTAL

For studies on exudative diathesis in chicks, White Rock chicks [1] were used in all experiments. The birds were randomly distributed into groups of equal numbers and placed on study in electrically heated batteries with raised wire floors. Feed and water were supplied ad libitum. The basal diet used in these studies was identical to that used in previous studies (Creech et al., '57), except that it was adjusted to the nutrient requirements of chicks and cerelose was substituted for lard in experiments where the effect of lard was determined. The ration was calculated to contain 22% of protein and considered to be adequate in all known nutrients except vitamin E. When dried brewers' yeast was added to the diet, it was made on an equi-protein basis for torula yeast. All diets were mixed fresh weekly to preclude the possibility of rancidity being a factor in the production of the deficiency symptoms. As a control measure and to test the possible antioxidant nature of dried brewers' yeast, rancidity indices were determined on

[1] Arbor Acre Farms.

the diets at mixing time and after one week of storage. This index is the milliequivalents of peroxide present in 100 kg of feed. It was determined by extraction of a sample of feed, representing 1 gm of fat, with peroxide-free ethyl ether; followed by evaporation of the ether and subsequent determination of the peroxide number by titration with 0.002 N sodium thiosulfate.

Blood was taken by cardiac puncture for serum analysis and allowed to coagulate at room temperature for one hour. The serum was then separated by centrifugation at 5,000 RPM for 15 minutes. Blood for hematological studies was taken by wing-vein puncture. These measurements consisted of erythrocyte count, hematocrit and hemoglobin determinations. Mean erythrocyte volume was calculated according to Wintrobe ('42).

The serum obtainel by heart puncture was used for electrophoretic analysis.[2] The procedure employed was that recommended for serum by the manufacture of the apparatus. It consisted of stripping 0.01 ml volumes on Whatman 3 MM filter paper strips saturated with barbiturate buffer at pH 8.6 and ionic strength of 0.075. Running time was 6 hours at a current of 15 ma. Quantitative estimation of the percentages of each fraction present was accomplished by scanning the stained strips [3] and calculation of the area under the curve. Total protein determinations were made using the biuret reaction following the method of Kingsley ('42).

The data were treated statistically by analysis of variance according to Snedecor ('56).

RESULTS AND DISCUSSION

Inasmuch as the production of encephalomalacia has been dependent primarily on the inclusion of dietary stress factors, such as unsaturated fats and fish oils, it was considered of prime importance to ascertain the necessity of such supple-

[2] A Spinco Model R paper electrophoretic apparatus was used. Spinco Division, Beckman Instruments, Inc., Belmont, California.

[3] Spinco Analytrol was used.

ments in the production of exudative diathesis. This is particularly true if exudative diathesis is to be considered the primary condition resulting from a metabolic deficiency of vitamin E. The results of such studies are seen in table 1. Production of exudative diathesis was not dependent upon the inclusion of dietary fat. The incidence and severity of the condition was just as high and developed just as quickly on the diets containing no added fat as on those which had been supplemented with vitamin E-free lard.

TABLE 1

Effect of vitamin E, dried brewers' yeast and lard on chick weight, incidence of exudative diathesis, erythrocyte count, hemoglobin and mean erythrocyte volume

SUPPLEMENT TO BASAL DIET	WEIGHT	EXUDATIVE DIATHESIS	RBC IN MILLIONS	HEMOGLOBIN	M. E. V. M^3
	gm	%		*gm* %	
None	199	100	1.39	5.07	155
Vitamin E, 20 mg/lb (DBY)	273 [1]	0	2.19 [1]	8.35 [1]	146
Dried brewers' yeast 10%	282 [1]	0	2.21 [1]	8.37 [1]	143
Butylated hydroxy toluene (BHT), 0.025%	212	100	1.37	4.64	153
Lard, 5%	213	100	1.42	4.93	160
Lard, 5% + vitamin E, 20 mg/lb	286 [1]	0	2.06 [1]	8.09 [1]	142
Lard, 5% + DBY, 10%	294 [1]	0	2.05 [1]	8.30 [1]	138
Lard, 5% + BHT, 0.025%	218	100	1.50	5.00	162

[1] Significant at the 0.01 level of probability.

Supplementation of the diet with 20 mg/lb of *d*-α-tocopheryl acetate or 10% of dried brewers' yeast completely prevented the development of the condition in the presence or absence of lard in the diet. These results also indicate that exudative diathesis is not a symptom of vitamin E deficiency arising from its function as an antioxidant. The addition of 0.025% of butylated hydroxy toluene (BHT) both in the presence and absence of added fat was without effect in preventing the occurrence of the disorder. The values for average weight exactly paralleled those for appearance of the deficiency symptoms. Addition of vitamin E or dried brewers' yeast

significantly increased the weights while BHT supplementation yielded an average weight which was not significantly different from that of the basal group. These data are in complete agreement with the work of Scott et al. ('55). The effect of these supplements on the occurrence and type of anemia is seen in table 1. As before, the inclusion of lard in the diet made little difference in the resultant anemia. Gross reductions in the number of erythrocytes were noted when the birds were maintained on the deficient diet with or without lard; and the antioxidant BHT was not effective in the prevention of the anemia. Vitamin E and dried brewers' yeast were effective in the prevention of the anemic condition. Hemoglobin values followed the same pattern as the erythrocyte counts with significant increases resulting from supplementation of vitamin E and dried brewers' yeast. Calculation of the mean erythrocyte volume showed that no significant differences existed between the groups in cell size. However, these data indicate that the anemia may have been macrocytic as was previously reported in turkeys. In all groups which had received vitamin E or dried brewers' yeast, the cell volumes were smaller. These results do not agree with those reported by Scott et al. ('55), but in all succeeding experiments with chicks, no difference could be found in cell volume.

Although exudative diathesis appears not to be associated with the antioxidant functions of vitamin E as seen in previous data, determination of the rancidity index of feed stored for the maximum of one week sheds more light on, this aspect of the problem as well as the nature of brewers' yeast in the prevention of this condition (table 2). The amount of peroxide in these diets at time of mixing and after storage was so small that the values had to be calculated on the basis of 100 kg of feed to be meaningful. As seen in table 2, only very little oxidation had taken place in the lard used in these studies prior to the time of mixing in the diets. After a period of storage of one week, the rancidity index had increased but not to an excessively high level. Vitamin E and BHT effectively retarded oxidative rancidity of the fat when added to

the diet, but dried brewers' yeast was totally ineffective as an antioxidant.

Examination of the serum and exudates from vitamin E-deficient birds in comparison with those receiving supplemental vitamin E showed gross changes in albumin, globulin and albumin/globulin ratio (table 3). Total protein values

TABLE 2

Rancidity index of feeds containing vitamin E, dried brewers' yeast and butylated hydroxy toluene after storage for one week at 80°C.

SUPPLEMENT TO BASAL DIET	RANCIDITY INDEX [1]	
	0 days	7 days
Lard, 5%	0.30	44.05
Lard, 5% + vitamin E, 20 mg/lb	0.29	32.93
Lard, 5% + dried brewers' yeast, 10%	0.25	45.69
Lard, 5% + butylated hydroxy toluene, 0.025%	0.21	30.87

[1] The milliequivalents of peroxide in one hundred kilograms of feed.

TABLE 3

Serum protein changes in chicks affected with exudative diathesis as determined by paper electrophoresis

SERUM COMPONENT	LEVEL IN SERUM		LEVEL IN EXUDATE
	Without vitamin E	With vitamin E	Without vitamin E
gm %			
Total protein	4.27	4.58	2.40
Albumin	0.49	2.31 [1]	0.65
Globulin	3.78	2.27 [1]	1.85
A/G ratio	0.15	1.04 [1]	0.38

[1] Significant at the 0.01 level of probability.

were somewhat higher in the groups receiving vitamin E, but these differences were not found to be significant. A highly significant reduction in albumin was noted in the deficient birds which could be increased by the addition of vitamin E. Decreases in albumin were accompanied by increases in globulin probably in attempts by the body to counteract the effect of the albumin reduction. Globulin increases and albumin

decreases resulted in drastically reduced albumin/globulin ratios in the deficient birds, while those receiving the supplemented diet maintained a normal ratio. Examination of the exudates was complicated by the fact that a large proportion of these samples solidified within a few minutes after removal from the bird. This observation explained why liquid exudates were extremely hard to find in birds even severely affected. In most cases, examination of sacrificed animals or those which died of the deficiency showed a thick gelatinous mass covering the breast just under the skin. However, in some cases, exudates were obtained in sufficient quantities to allow chemical and edectrophoretic investigation. The average composition of 5 of these fluids is seen in table 3. They contain a relatively higher proportion of albumin than does the serum from vitamin E-deficient birds. The exudate and serum were quite similar in composition. There was a gross reduction in the albumin peak in the deficient serum sample, while the normal bird serum exhibits a large, well-defined albumin peak. This reduction was not completely acccounted for by the level in the exudate and it seems likely that albumin synthesis may be affected in vitamin E deficiency even though the primary factor in appearance of exudates is probably that of increased capillary permeability.

To test further the possibility of the involvement of vitamin E in albumin synthesis, an experiment was set up which involved analysis of blood samples at close intervals during the development of exudative diathesis. In addition to following albumin level in the serum, total protein and globulin concentration were also followed. Blood hemoglobin has been seen previously to be a reliable index of the anemia accompanying vitamin E deficiency; therefore, determination of this factor was carried out on each blood sample to ascertain if the anemia is progressive or a result of hemorrhaging at the time of appearance of exudative diathesis. The results of these studies are seen in table 4. Up until the 7th day on the diets, no differences could be detected in any of the protein components or in blood hemoglobin. At some time between the

7th and the 10th days, a slight difference in total protein appeared. With minor fluctuations, the total protein of both the deficient and supplemented groups increased up to the 18th day at which time exudative diathesis symptoms first appeared. The difference seen at the 10th day was evident throughout the rest of the feeding period, but was not significant until the 19th day. Calculation of the concentration of albumin from the total protein value and electrophoretic patterns showed that the reduction in total protein of the

TABLE 4

Effect of vitamin E on the concentration of serum albumin and blood hemoglobin

DAYS ON DIET	BASAL DIET			BASAL + VITAMIN E		
	Total protein	Serum albumin	Blood hemoglobin	Total protein	Serum albumin	Blood hemoglobin
	gm %	*gm %*	*gm %*	*gm %*	*gm %*	*gm %*
7	3.02	1.27	7.50	2.93	1.29	7.63
10	3.04	1.21	6.95	3.48	1.50	7.10
11	3.41	1.46	7.15	3.69	1.70	7.20
12	3.40	1.53	7.92	3.61	1.77	7.49
13	3.24	1.46	6.63	3.48	1.71	7.05
14	3.05	1.22	7.39	3.57	1.66	7.74
15	3.50	1.37	7.07	3.84	1.77	6.71
16	2.90	1.19	7.53	3.26	1.50	7.61
18 [1]	3.17	1.14	6.48	3.30	1.45	7.04 [2]
19	2.90	0.94	6.30	3.52 [2]	1.69 [2]	7.23 [2]

[1] First appearance of exudative diathesis symptoms.

[2] Significant at the 0.05 level of probability.

deficient birds was due to a reduction in albumin. These reductions were significant at periods during the deficiency production, though not consistently so. The difference in albumin at the 19th day was significant. During the course of the experiment, the globulin level increased with time, but only very small differences were seen between groups. No difference was noted in hemoglobin values until the 18th day, when symptoms of exudative diathesis first appeared. At this time, a low hemoglobin value which proved significant was observed in the deficient group. This finding suggested that the anemia present was due primarily to hemorrhaging.

Varying lengths of time were required by individual birds to develop symptoms of exudative diathesis probably due to an inherent difference in ability of hens to deposit vitamin E in the egg resulting in differing levels of the vitamin stored for use by the chick. It was felt that this might affect the results of experiments where random selection of birds for analysis was employed. Since the values for the 19th day represented 10 birds each, the birds were re-examined and classified as severely affected and slightly affected. The results of the analysis based on this selection method were informative concerning the etiology of exudative diathesis.

TABLE 5

Comparison of exudate and serum protein components of birds severely and slightly affected with exudative diathesis

COMPONENT	SLIGHTLY AFFECTED		SEVERELY AFFECTED	
	Serum	Exudate	Serum	Exudate
gm %				
Total protein	3.18 [1]	3.32	2.62	3.53
Albumin	1.27 [2]	1.56	0.60	1.42
Globulin	1.91	1.76	2.01	2.11
A/G ratio	0.66 [2]	0.89	0.30	0.67
Hemoglobin	7.39 [2]	...	5.21	...

[1] Significant at the 0 05 level of probability in comparison with the severely affected birds.

[2] Significant at the 0 01 level of probability in comparison with the severely affected birds.

These data are presented in table 5. The composition of the serum of slightly affected birds deviates very little from that of the normal birds at the 19th day as shown in table 4.

There were significant differences in total protein, albumin and hemoglobin concentration between the severely and slightly affected birds (table 5). The large difference in albumin may be due to impaired synthesis or to the fact that the albumins are the first protein molecules to escape the blood with increased capillary permeability.

Comparison of serum and exudate from severely affected and slightly affected birds indicates further the possibility of

loss of the smaller albumin molecules to a greater degree than the other serum proteins (table 5). In both severely and slightly affected birds, the albumin concentration of the exudate was higher than in the serum.

SUMMARY

Exudative diathesis is a condition resulting from a deficiency of vitamin E in chicks. In birds fed a diet utilizing torula yeast as the protein source, this condition was the primary symptom. The condition was totally unrelated to the function of vitamin E as an antioxidant, and its appearance was not dependent upon the addition of stress-causing agents, such as fats, to the diet. This condition could be prevented by supplementation with vitamin E or dried brewers' yeast. The severity of the exudative diathesis was found to exert a marked influence on the blood and serum composition of the birds tested.

Development of gross symptoms of exudative diathesis was found to be paralleled by a significant reduction of hemoglobin, total serum protein values, and serum albumin. The anemia accompanying exudative diathesis was found to be due primarily to hemorrhaging.

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Yeast Council; and the vitamin D_3 by Bowman Feed Products, Inc., Holland, Michigan.

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NUTRITIONAL STUDIES WITH THE HYPERTHYROID RAT [1]

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Variations in the dietary regimen which impose additional or increased requirements for nutrients in experimental animals have been studied for a number of years. One approach has involved the feeding of vegetable protein diets to successive generations of animals which presumably results in the depletion of the body stores of essential nutrients. A recent report by Sherman, Schilt and Schaefer ('55) illustrates this approach. Depleted weanling rats from females receiving vegetable protein rations during gestation and lactation were found to require for optimum growth dietary sources of vitamin B_{12} and unidentified factors present in whole liver, fish solubles, or dried whey.

Another approach has employed "stress" conditions extensively in the rat (Ershoff, '47; Betheil et al., '47; Overby et al., '53) and in the mouse (Bosshardt and Huff, '53). "Stress agents," such as dinitrophenol or thyroxine, presumably act by impairing the ability of the tissues or microflora to synthesize nutrients not ordinarily required in the diet for optimal growth or by increasing the requirement for an essential nutrient or both. The stress situation should be alleviated by the administration of larger amounts of the substance(s) involved.

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Such a hypothesis does not appear to provide a satisfactory explanation for all the situations where dietary means have been successful in reversing the toxic effects of certain chemical agents. For example, it has been reported that growth retardation induced by cortisone can be reversed in the rat by feeding dried liver preparations (Meites, '52); however, the growth curves appear to reflect an adaptation to toxicity rather than development of a nutritional deficiency. Feeding liver partially abolishes the growth retardation observed during the first several weeks of cortisone toxicity; after this time growth rates are nearly normal with or without liver residue added to the cortisone-containing ration.[3]

The afore-mentioned hypothesis has been especially appealing in the case of hyperthyroidism. Liver preparations have been reported to reverse growth retardation almost completely and to prevent high mortality rates caused in the rat (Ershoff, '47) by feeding thyroid-active substances in a casein-sucrose ration. Levels of known vitamins above those usually fed in purified casein-sucrose diets do not improve growth significantly (Ershoff, '47). Quantitative requirements for certain of the B-complex vitamins are elevated in the hyperthyroid rat. Betheil and Lardy ('49) found that crystalline vitamin B_{12}, or highly purified liver extracts rich in this vitamin, promoted growth in the hyperthyroid rat. However, dried whole liver preparations promoted growth to an extent that could not be accounted for by their vitamin B_{12} content. Several extractions of whole liver powder, which should have removed vitamin B_{12}, were not successful in solubilizing the growth factor(s) present. In view of these observations it should be recognized that a bioassay for unidentified growth factors for the hyperthyroid rat must employ a basal ration containing an optimal concentration of vitamin B_{12}.

Several years ago work was initiated in this laboratory in an effort to purify the active factor(s) in liver responsible for growth stimulation in the thyrotoxic rat. To facilitate evaluation of fractions it was first found necessary to shorten the rat

[3] Henderson ('57), unpublished data.

growth assay; a "curative assay" method was devised and is reported here. In the course of these investigations considerable data have accumulated which do not agree completely with those reported in the literature cited above. The evidence obtained lends support to the conclusion that there remains an unknown factor in liver which is beneficial to the hyperthyroid rat maintained on the usual purified casein-sucrose diets.

EXPERIMENTAL

Male, weanling rats of the Holtzman strain, approximately three weeks old and weighing 50 to 60 gm, were obtained from the local stock colony, housed in individual cages in an air-conditioned room and given food and water ad libitum. Bacterial infection was reduced to a minimum by the use of raised wire mesh cages, frequent removal of excreta and sterilization of the entire housing units with each new rat population.

The basal rations were similar to those of Overby and associates ('53). Their compositions are described in table 1. The vitamin mixture was prepared at least every 30 days by grinding individual crystalline vitamins with sucrose in the quantities listed in table 1. The complete rations and the vitamin mixture were stored at 5°C.

All rats were first fed the corn oil basal ration (table 1) for one week during which time the average weight gain was approximately 30 gm. This served as a period of adaptation (Overby et al., '53) to a synthetic type diet and resulted in more consistent growth rates in the experimental periods which followed.

Growth studies utilized two experimental designs, the preventive assay and the curative assay. For preventive assays, adapted rats were equitably distributed in groups of 4 to 10 animals on the basis of body weight attained during adaptation but no group contained more than one from the same litter. One group (negative control) received the Crisco basal ration; another group (positive control) received the same diet except that iodinated casein [4] was omitted. Materials

[4] Protamone, 3.07% thyroxine, Cerophyl Laboratories, Inc., Kansas City, Missouri.

under study were added to the Crisco basal ration and these supplemented diets were fed to other groups of animals from the beginning of the preventive or curative assay period. The curative assays employed rats which had been depleted for two weeks following the adaptation period. During this depletion, the animals all received the Crisco basal ration employed in the preventive assay. After depletion the distribution of animals among the experimental groups of 4 to 12 animals each was made on the basis of the body weight gain during the depletion period. Positive and negative control groups were fed the same diets as were the corresponding groups in the preventive assays. Weights were recorded for each three-day period for three weeks for the curative assay and 5 weeks for the preventive assay.

TABLE 1

Composition of rations

COMPONENT	CORN OIL BASAL	CRISCO BASAL
	gm	*gm*
Labcocasein [1]	220.0	282.0
Salts IV [2]	40.0 [3]	44.2 [3]
L-Cystine	2.0 [3]	2.2 [3]
Vitamin mixture [4]	40.0 [3]	44.2 [3]
Protamone mixture [5]		12.15
Corn oil	10.0	11.0
Crisco		110.4
Sucrose	688.0	493.85
	1000.0	1000.0

[1] Vitamin-free Labcocasein, The Borden Co., New York, N. Y.

[2] Salts IV: a salt mixture patterned after the ash of milk by Phillips and Hart ('35).

[3] These three components are present in Corn oil basal and Crisco basal rations in equivalent amounts on a caloric basis.

[4] Sucrose containing in each 40 gm: 30 mg thiamine · HCl, 30 mg riboflavin, 50 mg niacin, 50 mg calcium pantothenate, 30 mg pyridoxine · HCl, 5 mg folic acid, 0.2 mg biotin, 200 mg *p*-aminobenzoic acid, 15 μg vitamin B_{12} and 1.0 gm choline. Two drops of a fat-soluble vitamin preparation were administered orally each week. This preparation consisted of 0.4 gm 2-methyl-1, 4-naphthoquinone and 1.0 gm α-tocopherol dissolved in 50.0 ml A and D Percomorph Liver Oil (Abbott Laboratories, North Chicago, Illinois).

[5] One part iodinated casein and 4 parts sucrose ground to a fine powder prior to incorporation into the ration.

Values for "total cholesterol" content of various starting materials, and fractions obtained from each, were determined according to the method of Schoenheimer and Sperry ('34), after hydrolysis of the sample with 10% alcoholic KOH for 10 hrs. at 60°C.

Carbon dioxide expiration rates were measured under conditions of minimal activity, usually sleeping. The apparatus used provided for passage of carbon dioxide-free air through the metabolism chamber. Expired carbon dioxide was collected for one-hour periods once each week in standard sodium hydroxide solution and determined by titration.

RESULTS

It has not been possible to demonstrate a distinction between growth and survival factor(s) for the hyperthyroid rat. For this reason and because the survival results complicate the expression of growth rates, a single composite measurement incorporating elements of both growth and survival was adopted. This expression which has been termed simply "growth rate" was derived by averaging weight gains for all animals during the entire experimental period. Animals which did not survive the entire period were included as though they had survived and had maintained throughout the experimental period the weight recorded the last time they were weighed before death. This method of expressing results appeared to reflect more accurately the effectiveness of supplements than other methods tested.

Comparison of assay methods. Growth responses under curative and preventive assay conditions are shown in figure 1. Ten per cent pork liver residue was added to the Crisco basal ration at the expense of an equal quantity of casein and sucrose, 1:1. In the curative assay, as the name implies, the supplement was administered after a depletion period. It is emphasized that the concentration of iodinated casein remained constant throughout the depletion period and the ensuing curative assay period in all groups except the positive control. In the latter, iodinated casein was removed from the

ration at the end of the depletion period resulting in rapid recovery from thyrotoxicity during the curative period.

Depletion period conditions influenced markedly subsequent growth responses to pork liver residue supplementation. When the depletion period was extended to three weeks, or the dietary iodinated casein level was raised to 0.35%, no more

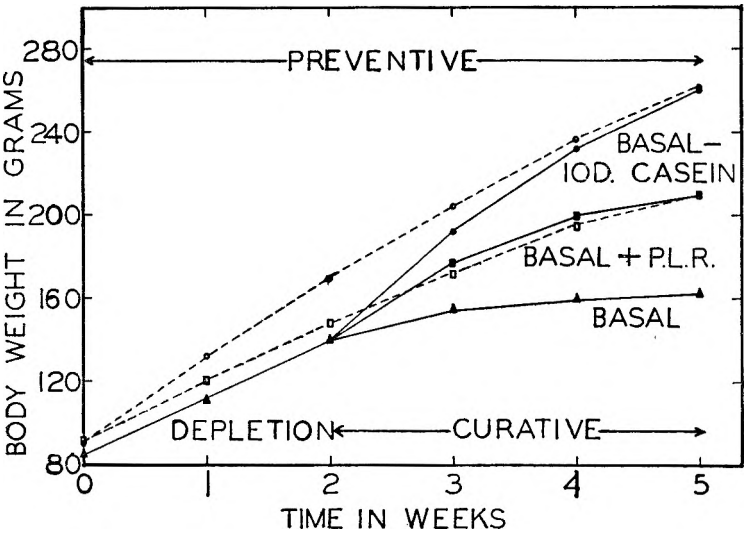

Fig. 1 Rat growth responses during preventive and curative assays of 10% pork liver residue with the Crisco basal ration. Curves represent the following groups: ○---, preventive, basal without iodinated casein, 6 rats; □---, preventive, basal + 10% pork liver residue, 6 rats; ▲—, preventive and curative, basal, 21 rats; ●—, curative, basal without iodinated casein, 13 rats; ■—, curative, basal + 10% pork liver residue, 15 rats.

than a 20% increase in growth could be obtained with 10% pork liver residue supplements during a three-week curative assay.

Under preventive assay conditions the first reliable indication of growth promotion by 10% pork liver residue was given by growth data at the end of 4 weeks (fig. 1). Growth responses showed so much variation from one experiment to the next that little confidence could be placed in the results. In contrast, the curative assay consistently showed growth pro-

motion to the extent of 60 ± 10% within two weeks of the addition of 10% pork liver residue to the ration thus giving a quicker and more reliable assay than the preventive type. Among the animals used in the experiments described in figure 1, deaths occurred only in the negative control group. The mean survival was 27.4 days of the 35-day experimental period. Autopsy showed that all deaths were due to cardiac failure.

Sources of the growth factor. Many naturally-occurring materials were tested for growth-promoting activity in the thyrotoxic rat under curative assay conditions. Some of the results are summarized in table 2. Of all the materials tested, water-extracted pork liver residues were consistently the most active. Three pilot plant lots elicited essentially equivalent growth responses. Dried whole pork livers have been tested and found to vary greatly in activity. One preparation was almost completely inactive. Water-extracted beef liver residues were significantly less active than the corresponding pork liver residues. The results summarized in table 2 were obtained before the growth-promoting action of cholesterol or corn oil under these conditions was recognized. Materials listed in table 2 were subsequently reassayed using the cholesterol and corn oil-containing ration. These preparations, which exhibited only slight activity in the absence of added cholesterol, were equally inactive when both cholesterol and more corn oil were added to the diet. Those which were definitely active in the absence of cholesterol showed similar or enhanced activity when tested with the improved basal ration.

Ten per cent supplements of dried beef kidney, spleen, or lung were almost as effective as 10% pork liver residue in promoting growth. Materials found to be almost completely inactive, when fed at 10% levels, included dried beef pancreas, dried egg yolk, soybean meal, fish meal, ground corn, cottonseed meal, and defatted cottonseed meal. Slightly better survival resulted from feeding 10% yeast, but no significant improvement in growth occurred. Survival was no better when yeast was fed at a 25% level. Nearly all materials tested improved survival slightly.

TABLE 2

Curative growth response with supplements to the Crisco basal ration

GP.	SUPPLEMENT [1]	AV. WT. GAIN IN 3 WKS.	% GROWTH PROMOTION [2]	SURVIVAL	
				Ratio [3]	Av. Duration
		gm			*days*
1	None, iodinated casein removed	116.1 ± 2.5 [4]	100.0	50/50	21.0
2	None	20.8 ± 3.2	0.0	12/47	14.9
3	5% pork liver residue [5] (1.32%) [6]	43.1 ± 11.5	23.5	3/7	16.0
4	10% pork liver residue	67.8 ± 4.2	49.5	42/52	19.7
5	2.7% lipid fraction [7] of pork liver residue (4.50%)	50.5 ± 5.8	31.1	11/19	17.7
6	3.65% defatted pork liver residue [7] (0.36%)	52.3 ± 4.8	33.1	7/7	21.0
7	7.3% defatted pork liver residue	58.6 ± 5.0	39.3	16/20	20.0
8	2.7% lipid fraction and 7.3% defatted pork liver residue	73.5 ± 13.8	55.5	4/4	21.0
9	2.7% lipid fraction and 7.3% defatted pork liver residue	48.9 ± 14.0	28.6	4/6	16.5
10	0.27% lipid fraction and 7.3% defatted pork liver residue	86.7 ± 8.3	69.1	7/7	21.0
11	10% beef liver residue (1.08%)	46.7 ± 14.6	27.2	3/4	18.5
12	10% wheat germ oil [8] (4.08%)	47.6 ± 9.1	28.2	5/7	18.9
13	3% pork liver lipids [9] (6.72%)	17.9 ± 9.6	−3 0	1/6	10.2
14	10% Drackett C-1 soybean protein [10] (0.09%)	32.3 ± 2.1	12 1	0/7	15.8
15	10% dried brewers' yeast [11]	26.8 ± 2.5	6 3	4/8	17.8
16	25% dried brewers' yeast	21.5 ± 5.4	0 7	2/9	17.8

[1] Supplements incorporated in the ration at the expense of an equal quantity of casein and sucrose (1:1).

[2] Per cent growth promotion $= \frac{\text{Wt. gain—Wt. gain of negative growth control}}{\text{Wt. gain of positive growth control—Wt. gain of negative growth control}}$.

[3] Survival ratio $= \frac{\text{rats surviving at 21 days}}{\text{rats starting and surviving at three days}}$.

[4] Standard error of the mean.

[5] Produced by aqueous extraction of whole liver powders at pH 5 and 80°C, dried in Bartlett rotary drier at 120°C, kindly supplied by Eli Lilly and Co., Indianapolis, Indiana.

[6] Figures within parentheses represent "total cholesterol" content of the material.

[7] Fractions produced by chloroform-methanol extraction of pork liver residue.

[8] Rex wheat germ oil, Viobin Corp., Monticello, Illinois.

[9] Pork liver lipids, LP-150-F, Viobin Corp., Monticello, Illinois.

[10] Obtained from the Animal Nutrition Laboratories, University of Illinois, Urbana, Illinois.

[11] Primary dried yeast, Biol. Lab. No. 5836, strain No. 200. Anheuser-Busch, Inc., St. Louis 18, Missouri.

Fractionations. Fractions described here were prepared from a single large lot of pork liver residue. Early attempts, which resolved this material into active lipid and non-lipid portions, employed extraction with a chloroform-methanol (2:1) solvent system by a modification of the method of Folch and co-workers ('51). One kilogram was extracted 9 times, each time with 2 l of solvent under reflux conditions (52°C, 1 hr.). Each extract was removed by filtration and all filtrates were combined and concentrated by vacuum distillation at low temperatures to obtain a dark brown tar-like substance (lipid portion) weighing 270 gm. After drying *in vacuo* the "defatted pork liver residue" weighed 730 gm. Both the extract and defatted material were found to have growth-promoting activity. A recombination of these fractions supported growth as effectively as the original pork liver residue (compare gps. 4 and 8, table 2). The best growth response observed was obtained with a combination of these two fractions in different proportions than those present in the original liver preparation (gp. 10, table 2).

Since activity was consistently retained in the lipid portion, further study of this fraction was undertaken. It is now possible to account for its activity largely on the basis of the cholesterol and fatty acid content (Page et al., 56). Several other extraction procedures were used on the water-extracted pork liver residue. Invariably the residue resulting from the extraction was equivalent to the starting material in activity when tested with the Crisco basal ration. These procedures included exhaustive aqueous (neutral, 0.1 *N* HCl or 0.1 *N* NaOH) extractions at room temperature, neutral aqueous extration under reflux conditions and dry acetone extraction at room temperature or under reflux.

Growth stimulation by known nutrients. Studies in this area were confined to higher levels of combinations of crystalline vitamins, cholesterol, purified forms of several hormones and various purified proteins. Also a higher level of corn oil was tested alone and in conjunction with one or more "known nutrient" supplements. Table 3 summarizes these data.

TABLE 3

Curative growth response with "known nutrient" supplements to the Crisco basal ration

GP.	SUPPLEMENT [1]	AV. WT. GAIN IN 3 WKS.	% GROWTH PROMOTION	SURVIVAL	
				Ratio	Av. Duration
		gm			*days*
1	None, iodinated casein removed	111.2 ± 2.5 [2]	100.0	24/24	21.0
2	None	15.5 ± 3.3	0.0	2/24	12.0
3	5.5% corn oil [3,4]	37.8 ± 7.8	23.1	4/9	16.3
4	0.2% cholesterol [5]	15.7 ± 7.4	0.2	1/9	9.6
5	0.5% cholesterol	18.4 ± 10.8	3.0	2/5	17.8
6	11.8% casein [6]	30.4 ± 7.4	15.4	4/9	15.1
7	5.5% corn oil, 0.2% cholesterol and 11.8% casein	34.8 ± 6.7	20.0	5/16	14.1
8	5.5% corn oil and 0.2% cholesterol	42.6 ± 4.1	28.0	9/27	15.2
9	5.5% corn oil and 0.622% cholesterol	50.1 ± 6.1	36.1	4/7	16.7
10	"Nutrient Mix" [7]	43.7 ± 6.2	29.2	6/12	17.8
11	0.03% aureomycin · HCl [8]	63.1 ± 5.8	49.3	9/15	18.3
12	10% pork liver residue	74.5 ± 10.6	61.1	11/15	18.5
13	10% pork liver residue, 0.2% cholesterol and 5.5% corn oil	94.1 ± 7.6	81.4	23/27	19.6
14	2.7% lipid fraction of pork liver residue, 0.2% cholesterol and 5.5% corn oil	66.5 ± 2.7	53 3	12/15	19.7
15	7.3% defatted pork liver residue, 0.2% cholesterol and 5.5% corn oil	71.6 ± 2.9	58 7	11/14	18.6

[1] Supplements added to the ration at the expense of an equal quantity of casein and sucrose (1:1).

[2] Standard error of the mean.

[3] 1.45% "total cholesterol" in corn oil used.

[4] Corn oil is already a component of the Crisco basal ration at a level of 1.1%.

[5] Cholesterol U.S.P., Nutritional Biochemicals Corp., Cleveland, Ohio.

[6] Casein is already a component of the Crisco basal ration at a level of 28.2%.

[7] "Nutrient Mix" supplied the following additions per kilogram of ration: ascorbic acid, 2 gm; DL-lipoic acid, 25 mg; cholesterol, 2 gm; corn oil, 55 gm; sperm desoxyribonucleic acid (Nutritional Biochemicals Corp., Cleveland, Ohio), 5 gm; yeast ribonucleic acid (Schwarz Laboratories, Inc., Mt. Vernon, N. Y.), 5 gm; Salts IV Mix was supplied at 1.5 times the level already present in the Crisco basal ration; and Vitamin Mix at 2 0 times the level already present in the Crisco basal ration.

[8] Crystalline aureomycin · HCl was a gift of the American Cyanamid Co., Lederle Laboratories Division, Pearl River, N. Y., through Dr. B. L. Hutchings.

Cholesterol alone had no appreciable growth-promoting activity under these conditions, but it had a small effect when fed with 5.5% corn oil. Casein supplements alone showed slight activity, but did not enhance growth further if corn oil and cholesterol were present in the ration. Growth promotion by the "Nutrient Mix" has been attributed to its corn oil and

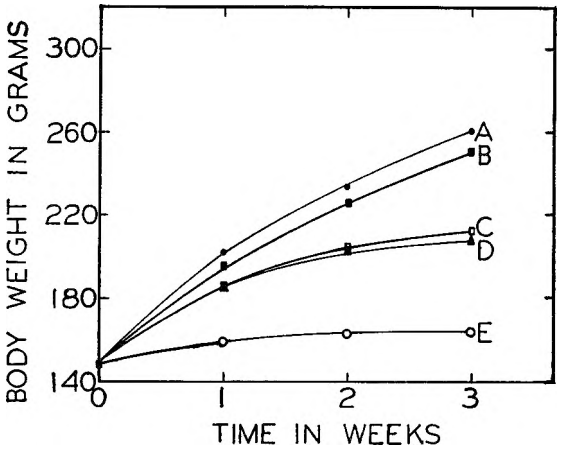

Fig. 2 Rat growth during the curative assay period with supplement combinations added to the Crisco basal ration at the expense of sucrose as listed below.

A: Basal without iodinated casein, 24 rats, 21.0 days survival.

B: Basal + 5.5% corn oil + 0.2% cholesterol + 0.03% aureomycin · HCl + 10% pork liver residue, 14 rats, 19.5 days survival.

C: Basal + 10% pork liver residue, 24 rats, 19.6 days survival.

D: Basal + 5.5% corn oil + 0.2% cholesterol + 0.03% aureomycin · HCl, 13 rats, 16.3 days survival.

E: Basal, 24 rats, 12.0 days survival.

cholesterol content. It was observed that increasing the vitamin level 5-fold or lowering it 10-fold had no effect on growth.

In these studies significant growth stimulation was consistently obtained with crystalline aureomycin (table 3). As will be seen in figure 2 this growth-promoting effect is observed also in the presence of added dietary cholesterol and corn oil.

Combined effects of known nutrients and pork liver residue. The results reported above show that many materials have some beneficial effects on the growth and survival of hyper-

thyroid rats under curative assay conditions. The reports in the literature attribute the growth-promoting effect of natural materials under similar experimental conditions to a number of known nutrients. It would be desirable to know whether a combination of cholesterol, corn oil, and aureomycin would be equivalent to pork liver residue. Figure 2 summarizes data which indicate that this is not the case under the experimental conditions used here. Supplementing the ration with a combination of aureomycin, corn oil and cholesterol elicited growth responses approximately equal to those given by 10% pork liver residue alone. Increasing the level of these three supplements did not result in further growth promotion. Supplementing the diet with a combination of all four of these materials gave a growth rate approaching that obsesrved when iodinated casein was removed from the ration. The marked growth response observed upon feeding defatted pork liver residue with or without cholesterol or corn oil (table 3) supports the hypothesis that pork liver residue contains growth factor(s) distinct from cholesterol or fatty acids. The digitonin-precipitable steroid in this defatted liver residue was less than the lower limits of the analytical method used.

Basal metabolic rates. The possibility of liver containing a factor directly antagonistic to the metabolic action of the thyroid hormone has been considered by several workers (Betheil and Lardy, '47; Ershoff, '47) and tested experimentally by Ershoff who found oxygen consumption rates to rise on administration of 0.5% thyroid powder and 10% yeast to rats. No significant difference could be observed in the rates of oxygen consumption upon feeding 10% liver in place of yeast. Similar studies have been conducted in this laboratory. Carbon dioxide expiration was measured over a period of one hour at weekly intervals throughout the curative assay period. The results expressed on the basis of body surface are summarized in table 4. Average carbon dioxide expiration rates were approximately equal for all 4 groups at the end of the depletion period. Average carbon dioxide expiration rates for the two groups not receiving iodinated casein decreased

TABLE 4

Basal metabolic rates during the curative assay period [1]

SUPPLEMENT [2] TO CRISCO BASAL RATION	AV. BODY WT. GAIN	SURVIVAL RATIO	AV. DURATION OF SURVIVAL	MEQ. CO_2 EXPIRED [3] PER HR. PER 100 CM^2 OF BODY SURFACE [4] AT			
				End of depletion	1 wk.	2 wks.	3 wks.
	gm.		*days*				
Iodinated casein removed. 5.5% corn oil, 0.2% cholesterol.	119.4 ±7.2 [5]	5/5	21.0 ±0.0 [5]	5.23 ±0.20 [5]	3.47 ±0.09	3.33 ±0.27	2.66 ±0.42
Iodinated casein removed. 5.5% corn oil, 0.2% cholesterol, 10% pork liver residue.	111.2 ±3.0	4/4	21.0 ±0.0	4.89 ±0.09	3.60 ±0.04	3.13 ±0.05	2.80 ±0.64
5.5% corn oil, 0.2% cholesterol	39.4 ±6.7	6/8	19.1 ±1.3	5.28 ±0.35 (8) [6]	6.80 ±0.91 (8)	5.30 ±0.34 (7)	5.05 ±0.51 (4)
5.5% corn oil, 0.2% cholesterol, 10% pork liver residue	88.3 ±6.7	7/7	21.0 ±0.0	4.80 ±0.08	5.62 ±0.57	5.62 ±0.54	5.18 ±0.52

[1] This series of experimental groups was made up of male rats whose body weights were 103 to 112 gm before depletion and whose average gain in the depletion period was 23 to 36 gm.

[2] Supplements added at the expense of an equal quantity of sucrose.

[3] Determined according to the procedure described in the text.

[4] Body surface = (12.54) (Body wt.)$^{0.6}$ cm^2.

[5] Standard error of the mean.

[6] Figures within parentheses indicate the number of animals upon which CO_2 expiration measurements were performed to obtain these data.

significantly after the first measurement. However, the average rates remained essentially constant for the group receiving iodinated casein but no liver residue. The group receiving iodinated casein and liver residue expired carbon dioxide at about the same rate as the third group thus confirming Ershoff's conclusion that pork liver does not act by reversing the effect of the thyroid hormone on the basal metabolic rate.

DISCUSSION

It has been recognized for some time that variations in the type and quantity of dietary protein, carbohydrate or lipid affect profoundly the growth response of the hyperthyroid rat. Replacing sucrose with dextrin or cornstarch (Emerson, '49) or substituting soybean protein for casein (Lewis et al., '50) intensified growth retardation which may be alleviated by administration of vitamin B_{12}. Dietary cholesterol has been reported to promote survival (Ershoff and Marx, '48) and to enhance growth slightly (Page et al., '56). Ten per cent cottonseed oil, fed in conjunction with high levels of B vitamins was almost as effective as 10% liver supplements in reversing growth retardation caused by 0.5% U.S.P. thyroid powder in the ration (Ershoff, '52). Emerson et al., ('56), using 0.1% iodinated casein, observed growth stimulation when 5% corn oil was added to the diet and somewhat better growth upon adding a combination of 0.1% cholesterol, 5% corn oil and 10% casein to their 24% casein-sucrose basal diet. Casein also had slight activity alone. Little improvement in growth resulted from adding liver to diets already containing these three supplements.

Our data confirm the findings of Page et al., ('52) and of Emerson and co-workers ('56) in that both cholesterol and corn oil, or the lipid portion of liver, are active in promoting growth in the hyperthyroid rat. However, under curative assay conditions, it is possible to obtain further growth promotion with pork liver residue or defatted pork liver residue when optimum supplies of cholesterol, corn oil and aureomycin are present in the diet. These observations indicate the

presence in liver of still unidentified, non-extractable growth factors for the hyperthyroid rat. O'Dell et al. ('55) have reported some solubilization of such growth factors in dried liver preparations by treatment with proteolytic enzymes, sodium hydroxide, or sulfuric acid.

A major difference between the results reported here and those appearing in the literature was the lack of response to additional casein either with or without added cholesterol and corn oil. Many other supplements which are composed principally of protein promoted growth only slightly. Unidentified growth factor(s) appear not to be widely distributed. Of the materials tested pork liver residue was the only one which was consistently active.

Ershoff ('47) reported that aureomycin mash, but not pure aureomycin, was beneficial to the growth of the hyperthyroid rat. Using the curative assay it has been possible to observe significant growth promotion with crystalline aureomycin. The function of aureomycin here may be to eliminate low level infection of a harmful nature or to enrich the microflora in an organism whose presence is beneficial to the hyperthyroid rat. Several such mechanisms have been proposed already to account for the beneficial effects of certain antibiotics in normal animals (Stokstad, '54).

The survival data presented in this paper for the curative assay provide no basis for postulation of a survival factor distinct from growth factor(s). Significant shortening of survival time occurred in the negative controls. Ten per cent yeast improved survival and growth only slightly; 25% yeast was no better in this respect. On the other hand, 10% pork liver residue while improving growth lengthened survival considerably over the three-week curative assay period. Under our conditions the factor(s) responsible for growth promotion may also be indirectly responsible for longer survival.

It should be added that the curative assay has given somewhat variable results from one experiment to the next. This makes fractionation of the active liver residues difficult. The evidence presented here does not permit one to reach a decision

as to whether the stimulation of growth is the result of one or several entities. Further, it does not provide a basis for deciding whether the active substance(s) is vitamin-or hormone-like in its action.

SUMMARY

1. Experiments designed to improve the assay for substance(s) in liver and other natural materials which partially reverse the growth suppression of hyperthyroid rats have been reported. A three-week curative assay which gives more reproducible results than the 5-week preventive assay has been developed and used. The assay involves feeding weanling rats a typical synthetic-type sucrose-casein-corn oil diet for one week and then a sucrose-casein-Crisco diet containing 0.243% iodinated casein for two weeks. These depleted, hyperthyroid rats are then used for the assay of liver or other natural products.

2. Cholesterol, corn oil or crystalline aureomycin stimulated growth slightly under these assay conditions. A combination of these three substances was almost as effective as 10% liver residue alone. The liver residue plus these three substances supported growth and survival nearly equivalent to that obtained by removing the iodinated casein from the diet.

3. Casein and many other proteins or protein-containing supplements were not active. Beef spleen, kidney and lung exhibited some activity. The active substance(s) in pork liver was not soluble in a mixture of chloroform and methanol.

4. Removal of iodinated casein from the diet lowered the basal metabolic rate and caused immediate resumption of growth. A liver residue active in stimulating growth did not alter the basal metabolic rate.

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DIETARY MINERAL INTERRELATIONS AS A CAUSE OF SOFT TISSUE CALCIFICATION IN GUINEA PIGS

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An earlier publication from this laboratory (Smith et al., '49) confirmed the occurrence of soft tissue calcification and stiff wrist joints in guinea pigs on certain diets, as reported in a series of papers by the Oregon workers (Wulzen and Bahrs, '41; van Wagtendonk and Wulzen, '43 and later papers). Their reports that the abnormalities noted were due to the absence from the diet of a specific factor were not confirmed, however. Our evidence suggested that the wrist stiffness and tissue calcification were not parts of the same syndrome. In further studies, therefore, attention was concentrated on the calcifications which seemed to be the more significant abnormality.

Various purified diets and modifications of them were used and all produced some degree of calcification in one or more organs and tissues as shown by chemical analysis or X-ray, or histological study, in contrast to the absence of calcification in animals fed a stock diet. In general, growth was poor on the purified diets and there was considerable mortality. A more suitable basal purified diet was a recognized need. Such a diet became available as a result of the studies of Reid and Briggs ('53). In our hands this diet produced satisfactory growth and livability and thus was chosen as the basis for the studies here reported.

In view of findings with other species such as those of Tufts and Greenberg ('38) with rats, Moore et al. ('38) with calves and Constant and Phillips ('52, '54) with cotton rats, it was thought that the calcifications in guinea pigs might be the result of inappropriate magnesium-calcium-phosphorus relationships in the diet. The studies here reported were carried out to test this hypothesis.

GENERAL PROCEDURE

The diets used are shown in table 1. The stock diet, consisting of pellets, was stated by the manufacturers [1] to contain ground whole wheat, ground whole oats, alfalfa leaf meal,

TABLE 1

Diets and their mineral relations

DIETS		MINERAL CONTENTS AND RELATIONS					
No.	Designation	Ca	P	Mg	Ca/Mg	P/Mg	Ca/P
		%	%	%			
1	Stock	0.98	0.44	0.24	4.1	1.8	2.2
2	Reid-Briggs	1.10	0.70	0.34	3.2	2.1	1.6
3a	Reid-Briggs, Low Mg	1.06	0.72	0.13	8.1	5.5	1.5
3b	Reid-Briggs, Low Mg	1.13	0.72	0.06	18.9	12.0	1.6
4a	Reid-Briggs, Low Mg, Ca, P	0.39	0.48	0.12	3.2	4.0	0.8
4b	Reid-Briggs, Low Mg, Ca, P	0.38	0.27	0.12	3.2	2.2	1.4
5	Reid-Briggs, Low Mg, Ca	0.38	0.72	0.12	3.2	6.0	0.5
6	Reid-Briggs, Low Mg, P	1.14	0.27	0.12	9.5	2.2	4.2

soybean oil meal, soybean oil, irradiated brewers' yeast, bone meal, limestone, iodized salt and additional sources of various vitamins. The diet was guaranteed to contain 18% protein. The Reid-Briggs diet, described in detail in their publication ('53), consists of casein, corn oil, sucrose, cellophane spangles, starch, cerelose, a salt mixture and a supplement containing 15 vitamins. The casein content is 30%. Diets 3a to 6 inclusive in table 1 represent modifications in the Reid-Briggs diet the bases of which are described under the trials in which they were used. The figures for mineral content given in the table

[1] Rockland Farms, New City, Rockland County, N. Y.

were obtained by chemical analysis in the case of diets 1, 2 and 3a. Most of the others were obtained by calculations based upon the changes made in the relative amounts of the c.p. salts used in making up the salt mixture used in the Reid-Briggs diet. After an appropriate preliminary treatment of the samples to be analyzed, the Clark-Collip modification of the Kramer-Tisdall method was used for calcium, the Fiske and SubarRow method for phosphorus and the Denis-modified method for magnesium, as set forth in Hawk, Oser and Summerson ('54).[2]

The animals were produced in our own colony, providing us with much more uniform and healthy ones than we had previously been able to obtain from commercial sources. The young were weaned at approximately one week of age, and started on the stock diet in trials 1 and 2 and on the Reid-Briggs diet in trial 3. They were then transferred to the other diets, 7 animals being placed on each. The experimental period was approximately 13 weeks in trials 1 and 2 and 15 weeks in trial 3. At the close of these periods the animals were slaughtered by stunning and decapitation, the blood was drained out and the serum saved for analysis. Following a gross post-mortem examination, tissue samples were taken for chemical analyses and in some cases for histological examination. The analytical methods used were the same as those previously mentioned for the diets.

Trial I

In this trial diets 1, 2 and 3a were used, to compare the performance on the stock and Reid-Briggs diets, particularly as regards the production of soft tissue calcification, and also to study the effect of lowering the magnesium content of the purified diet. It is noted in table 1 that the original content was lowered to approximately one-third, a change which also markedly altered the Ca : Mg and P : Mg ratios.

[2] Pages 644–646; 630–633 and 648–649, respectively.

The lowering was accomplished by withdrawing MgO and $MgSO_4.7H_2O$.

Growth was satisfactory on both the stock and the unmodified Reid-Briggs diets, being 4.4 and 4.3 gm per day respectively. All these animals were in thrifty condition at slaughter. By contrast, the animals on the Reid-Briggs diet in which the magnesium content had been sharply reduced, made an average daily gain of only 2.2 gm and became very unthrifty. There was a marked loss of hair, lessened activity and poor muscular coordination of the rear legs. None of the animals died while on experiment. The gross post-mortem examination revealed no abnormalities in the animals on diets 1 and 2. By contrast, on the diet low in magnesium, the kidneys were enlarged and white in color. These enlarged kidneys were about double the weight and volume of those of animals of comparable size on the other diets. The livers of some of the animals on all diets showed small white pinpoints and striations. The occurrence was more marked on the low-magnesim diet. On histological examination these changes were found to represent focal necrosis, which in the case of the animals on the low-magnesim diet was accompanied by calcification. The hearts and muscles appeared normal on gross examination.

The results of the chemical analyses made are shown as group averages under trial 1 in table 2. The diet numbers refer to table 1. It is noted that the calcium values for the serum were similar and at a normal level in the case of all diets. This was also true for magnesium in the case of the stock and Reid-Briggs diets, but when the magnesium content of the latter was lowered (diet 3a) its level in the serum was reduced by 40%. The difference was significant at the 1% level.

The data for the tissues reveal similar and apparently normal values for calcium content in the case of both stock and Reid-Briggs diets. When the magnesium was lowered in the latter, however, the calcium content of the kidney was increased some 25 times, reflecting the gross changes pre-

viously noted. Similarly, the liver content showed a 6-fold increase. The differences for both the kidney and the liver were significant at the 1% level. The average data show that the calcium content of the heart was more than doubled on the low-magnesium diet, but the increase is not statistically significant because of wide individual variations reflected in the average figures. No significance can be attached to the small increase recorded for muscle.

TABLE 2

Calcium, phosphorus and magnesium in blood and organs of quinea pigs as related to the intake of these elements

TRIAL	DIET	NO. OF ANIMALS	BLOOD SERUM			KIDNEY	LIVER	HEART	MUSCLE
			Ca	*P*	*Mg*	Ca	Ca	Ca	Ca
			mg/100 ml			*mg/100 gm dry weight*			
I	1	7	12.3		4.09	59	26	39	34
	2	7	12.0		4.16	88	25	36	32
	3a	7	12.4		2.42	2,229	155	87	41
II	1	7	12.7	7.43	4.48	100	11		
	2	7	13.3	7.33	4.56	105	9		
	3b	7	13.4	11.37	1.83	2,599	460		
	4a	7	13.6	5.82	4.59	60	13		
III	3a	6	13.3	8.84	2.64	1,802			
	4b	7	12.8	5.97	3.99	183			
	5	5	12.6	6.53	3.71	1,193			
	6	6	13.0	5.68	3.33	184			

Trial II

Following the completion of the first trial, the question arose as to whether the abnormal calcifications obtained were due to low dietary magnesium *per se* or to the resulting upset relations with calcium and phosphorus. Trial II was set up to answer this question. Diets 1 and 2 were again included. Diet 3b provided the opportunity to check the effect of lowering magnesium only and also to ascertain whether a more drastic lowering than was the case in trial 1 would increase the severity of the blood and organ changes. Diet 4a furnished a mixture in which calcium and phosphorus, as well as mag-

nesium, were lowered. This was accomplished by removing $Ca_3(PO_4)_2$ from the basal purified diet and lowering its content of $CaCO_3$ and K_2HPO_4.

All animals survived over the experimental period with the exception that one on diet 3b died very early from respiratory disease and was replaced. All animals on diets 1 and 2 appeared healthy and apparently normal throughout. The average gains on the two diets were 5.9 and 5.0 gm per day respectively. With diet 3b the animals gained only 2.6 gm per day. They had rough hair coats and there was loss of hair in a few cases, but they were quite active and no muscular incoordination was apparent. Their appearance was poorest during the first part of the trail. They remained emaciated throughout. The animals on diet 4a gained 4.1 gm per day, and with the exception of two animals, were quite similar in appearance to those on diets 1 and 2. These two animals resembled those on diet 3b. Thus, as regards growth rate and general condition the results were much more nearly satisfactory, compared with those with the stock or Reid-Briggs diet, where all three minerals were lowered than where magnesium alone was decreased. At autopsy some liver necrosis was observed in all groups but was more severe with the low-magnesium diet, 3b. Groups 1, 2 and 4a appeared normal otherwise. In contrast, the animals on diet 3b showed markedly white and enlarged kidneys similar to those observed on diet 3a in trial 1.

In this trial determinations of serum phosphorus as well as magnesium were included, but the tissue analyses were limited to the kidney and liver, the tissues showing significant changes in trial 1. It is noted in table 2 that, as was the case in the first experiment, the serum and organ values were similar and apparently normal in the case of both the stock and the basal purified diet. By contrast, marked changes again occurred on the low-magnesium diet. The serum magnesium was greatly reduced and the phosphorus was elevated. In both cases the differences were significant at the 1% level. Again, many-fold average increases in calcium in the kidneys

and liver were found, the differences being significant at the 1% level. The changes noted for this diet containing 0.06% of magnesium were in general more severe than those produced as a result of lowering the content to 0.13% (diet 3a, trial 1). In marked contrast to the severe changes found on the low-magnesium diet, the blood and organ values found where all three minerals were lowered (diet 4a) corresponded to those found on the unmodified purified diet and on the stock diet. These results indicated that the severe blood and organ changes resulting from a large decrease in magnesium were due primarily to upset relations involving the three elements, magnesium, calcium and phosphorus. A further trial was conducted to test this point.

Trial III

The diets used in this trial were 3a, 4b, 5 and 6. Diet 4b was the same as the previously used 4a with the exception that the phosphorus was lowered in the same proportion as were the magnesium and calcium. As a result the calcium-magnesium, phosphorus-magnesium and calcium-phosphorus ratios remained approximately the same as in the basal purified diet, in contrast to the markedly changed relations that resulted from decreasing magnesium only (diet 3a). The withdrawal of the minerals in diet 4b was accomplished as described for diet 4a but the potassium removed as K_2HPO_4 was restored as potassium acetate in view of the evidence which had become available from House and Hogan ('55), that a combined low magnesium and potassium, along with high phosphorus, was a factor in soft tissue calcification in guinea pigs. In diet 5 both magnesium and calcium were lowered to their levels in diet 4b, leaving phosphorus at its original level. Thus the diet had a high phosphorus content relative to the other minerals, as shown by the ratios in table 1. In diet 6, magnesium and phosphorus were lowered, resulting in a relatively high calcium content. It was hoped to find out through the use of these diets whether the calcifi-

cation resulting from lowering the magnesium, but not found when calcium and phosphorus were lowered as well, was the result primarily of a wide P : Mg ratio or, on the other hand, of a wide Ca : Mg ratio. In all of these diets the potassium content was approximately 1.4%, the same figure as in the unmodified basal diet and corresponding to 1.6% in the stock diet. In this third trial diets 1 and 2 were not repeated. It was considered that the previous trials had satisfactorily established the base line data needed for comparative purposes in trial 3.

Although 7 animals were started on each diet data are presented in table 2 for only 6 in the case of diets 3a and 6, and 5 in the case of diet 5, because of deaths midway in the experiment which are discussed later. As had been noted in previous trials, growth performance was much better where all the minerals were lowered (diet 4b) than where the magnesium content alone was reduced (diet 3a) but not as satisfactory as that obtained earlier on the unmodified Reid-Briggs or stock diets. Decreasing the magnesium and phosphorus only (diet 6) which resulted in a wide calcium-magnesium ratio did not affect growth. In the case of diet 5, however, in which magnesium and calcium were lowered and a wide phosphorus-magnesium ratio resulted, the growth rate was less than for the animals on diets 4b and 6 but better than was the case for those on diet 3a. These differences in group growth rate must be considered to represent trends. Individual performances were too variable to attach statistical significance to them.

The animals on diet 3a developed an unthrifty condition, reflected particularly in a large loss of hair. Those on diet 5 were similarly unthrifty, though making a somewhat better growth. By contrast all the animals on diets 4a and 6, with the exception of the one on diet 6 which died midway through the experiment, remained in excellent condition.

On autopsy all the animals on diet 3a showed markedly enlarged and whiteish kidneys as had been noted in previous trials. The one animal which did not complete the experiment was killed midway during the period because of loss of use of

the hind legs and difficult breathing. Its kidneys were pale and somewhat enlarged. All the animals on diet 4b were found apparently normal on autopsy with the exception of some liver necrosis which was noted in all groups. Three of the 5 animals on diet 5 which were left on the experiment to the end showed pale, slightly enlarged kidneys. Two were killed midway in the experiment because of respiratory trouble. One showed a weakened condition of its hind legs and its kidneys were pale and somewhat enlarged. One of the animals on diet 6 died midway in the experiment from respiratory

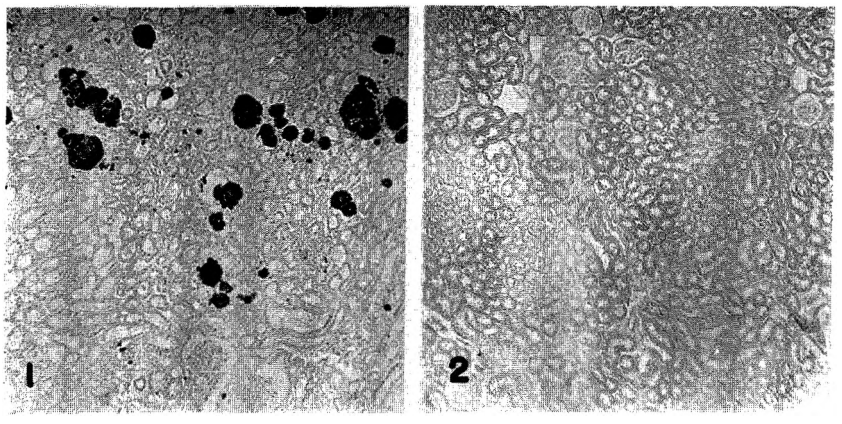

Fig. 1 Section of kidney of animal on diet 3a, showing calcium deposits which occurred throughout the kidney. 100 ×.

Fig. 2 Section of the kidney of an animal on diet 4b. 100 ×.

troubles. Its kidneys and gall bladder were somewhat enlarged. Two of the remaining 6 revealed pale, slightly enlarged kidneys at the close of the trial. On histological examination of the kidneys of the animals in the various groups, those on diet 3a showed calcium deposits in the kidneys, in the form of casts. Two animals on diet 5 showed similar kidney calcification. Other animals in this group and all on diets 4b and 6 were apparently normal in this respect. Other organs and tissues were not examined. In figure 1, the calcium deposits in the kidneys produced on diet 3a are shown, in contrast to the absence of such deposits on diet 4b (figure 2).

The analytical data for the blood and kidneys are shown in table 2. It is noted that, as in the previous trials, the blood calcium levels remained normal whatever the treatment. Again, the lowering of dietary magnesium alone resulted in a statistically significant elevation of serum inorganic phosphorus, though not to the extent obtained in trial 2 in which the dietary magnesium was more drastically reduced. No significance can be attributed to the minor differences in serum phosphorus among the results for diets 4b, 5 and 6. The low-magnesium diet (3a) again produced a marked lowering of the serum magnesium, not apparent where all three minerals were decreased in the diet (4b). The average values for diets 5 and 6, particularly 6, also indicate some lowering but the differences are not statistically significant.

The data for kidney calcium check previous findings in showing a large and highly significant increase on the low-magnesium diet, as compared with the results where all three minerals were lowered (diet 4b). The average figure also reveals a large increase, compared with diet 4b, as a result of lowering the calcium and magnesium and leaving the phosphorus relatively high (diet 5). This large average figure was due almost entirely to two animals, however, and thus the difference between it and the average figure for diet 4b was not found statistically significant. It is worth noting, however, that two of the 5 animals completing the trial on diet 5 showed a high degree of calcification by both histological and chemical findings whereas none of the 7 animals on diet 4b revealed any abnormality in these respects. Clearly, lowering the dietary magnesium and phosphorus and keeping the calcium relatively high (diet 6) resulted in no increase in calcium in the kidneys, compared with the results where all minerals were lowered.

GENERAL DISCUSSION OF RESULTS

The data of the three trials clearly show that decreasing the magnesium content of a satisfactory purified diet results in a lowered serum magnesium, an elevated serum phosphorus

and a high degree of kidney calcification, in contrast to the absence of these findings when calcium and phosphorus are proportionately lowered as well. Thus the deleterious effects of the low magnesium were due primarily to the upset mineral relationships. It was surprising to find that keeping phosphorus alone (diet 5) or calcium alone (diet 6) relatively high had little effect, compared with keeping both (diets 3a and 3b) relatively high. This finding suggests that it is the ratio of magnesium to both rather than to either one alone that is of primary significance. In the case of two out of 5 animals, however, there was clear evidence that the relatively high phosphorus diet was deleterious. Hogan, Regan and House ('50) obtained evidence that a purified diet high in phosphorus in relation to calcium, as was the case in our diet 5, produced calcium phospate deposits in the organs of guinea pigs. In a later study from the same laboratory (House and Hogan, '55) in which magnesium and potassium variables were also introduced, a diet containing 0.9% of Ca and 1.7% phosphorus, but low in magnesium and potassium, produced slow growth, stiff joints and calcium phosphate deposits. These symptoms were greatly reduced when the magnesium and potassium contents of the diet were substantially increased. In our trial 3, the potassium content of all the diets was held at 1.4%, a figure similar to that found satisfactory by House and Hogan. In fact all of our low-magnesium diets contained approximately this level of potassium and thus a relative deficiency of it was not a factor in the calcification we found.

The data here presented raise many questions which might be discussed. It is felt, however, that the primary need is for further studies of them. Mineral interrelationships represent a very complicated field. When, for example, one formulates a diet low in a given mineral he also sets up a diet in which the ratio of this mineral to each of some 12 other essential minerals is changed. In our study the obvious variables have been magnesium, calcium and phosphorus, but changes in their relationships to other essential minerals were also involved. It is possible that herein lies an explanation of the

unexpected results in trial 3 with both diets 5 and 6 as compared with results with diet 3a. Since severe kidney calcification occurred on diet 5, however, in the case of two animals, the need for studies over a longer period is suggested, fully to assess the effects of the diets here used. The animals on the various diets were examined from time to time for wrist stiffness, though not in the quantitative detail of our previous study (Smith et al., '49). Its marked occurrence was noted only rarely and this occurrence was not definitely correlated with the incidence of kidney calcification.

Continuation of the use of guinea pigs for studies of this general problem of soft tissue calcification seems worthwhile. In our hands the Reid-Briggs diet has proven a very satisfactory basal one for the purpose. It results in good growth and in freedom from the calcification under study, in contrast to much less satisfactory results with purified diets we have previously used.

SUMMARY

In contrast to the satisfactory results obtained in guinea pigs with the Reid-Briggs purified diet, lowering the magnesium content of this diet by approximately 70% resulted in poor growth, greatly enlarged and damaged kidneys, decreased serum magnesium, elevated serum phosphorus, a very large increase in the calcium content of the kidney and a lesser increase in the liver. When the calcium and phosphorus contents of the purified diet were lowered along with the magnesium and to the same extent, these deleterious effects did not occur. Neither lowering both magnesium and phosphorus, thus keeping calcium relatively high, nor lowering magnesium and calcium but keeping phosphorus high, produced nearly as marked effects on the average as in the case where magnesium alone was decreased. The high phosphorus diet did result, however, in marked kidney damage in some of the animals.

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NITROGEN BALANCES OF WOMEN MAINTAINED ON VARIOUS LEVELS OF METHIONINE AND CYSTINE[1]

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Observations on self-selected diets of women (Futrell et al., '52; Mertz et al., '52; Reynolds et al., '53) suggest that methionine is the amino acid most apt to be present in an amount less than that designated as the "minimal daily requirement" by Rose ('49). This proposed requirement, 1.1 gm, was based on the needs of young men maintained on a synthetic diet which contained no cystine (Rose et al., '55). Rose and Wixom ('55) later showed that L-cystine can replace 80 to 89% of the methionine requirement. Recently Swendseid

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and co-workers ('56) reported the methionine requirement of young women to be between 150 and 350 mg when a partially synthetic diet containing peanut protein supplied 200 mg of cystine; for some subjects the minimal methionine requirement appeared to be as low as 75 mg in the presence of 100 mg of cystine. The present paper presents nitrogen balance data on 21 women fed semi-synthetic diets furnishing various levels of methionine and cystine.

EXPERIMENTAL

Experimental plan

Five series were conducted according to the general plan reported in an earlier paper (Jones et al., '56). The subjects were fed a diet of natural foods for 8 to 12 days in order to accustom them to the experimental routine and to the level of nitrogen intake used throughout the experiment. During a transition period of three to 4 days, the natural foods were gradually replaced by a semi-synthetic diet which was then fed throughout the remainder of the study. At first the amino acid mixture in this semi-synthetic diet was complete, permitting nitrogen equilibrium; thereafter, variations in methionine and cystine intakes were introduced at the expense of the glycine component, and the effect on nitrogen balance determined.

The lengths of time on the same dietary regimen ranged from 6 to 16 days depending upon the response of the subject, with the exception of one 5-day period in series I, and a 4-day period for one subject in series V. This subject (31) found it necessary to withdraw from the experiment at that time.

In series I, II, and III methionine was omitted from the amino acid mixtures during the periods immediately after the feeding of the complete amino acid mixtures in order to demonstrate the need for this amino acid in the subjects participating in these studies and to demonstrate that the natural foods in the basal diet did not furnish enough methionine to meet the requirement of the subjects In series IV

TABLE 1

Mean daily nitrogen balances of the subjects in series V showing the sequence in which the levels of methionine and cystine were fed to the individual subjects

PERIOD [1] AND DIET		AMINO ACID INTAKE		MEAN DAILY NITROGEN BALANCE OF SUBJECTS					
		Cystine	Methionine	31	32	33	34	35	36
		gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
I	Normal			+ 0.68	+ 0.02	— 0.59	+ 2.10	+ 1.83	+ 0.51
II	Normal			— 0.37	+ 0.77	+ 1.20	+ 1.87	+ 2.13	+ 1.44
III	Transition								
IV	Semi-synthetic	0.26	0.29	— 0.43	— 0.89	+ 0.22	— 0.27	+ 0.09	0.00
V	Semi-synthetic	0.26	0.29	+ 0.06	— 0.39	+ 0.15	+ 0.37	+ 0.83	+ 0.51
VI	Semi-synthetic	0.01	0.29	— 0.35		+ 0.09	— 0.27	+ 0.40	— 0.31
		0.26	0.29		+ 0.19				
VII	Semi-synthetic	0.01	0.29		— 0.72	— 0.49	— 0.39	+ 0.27	— 0.01
VIII	Semi-synthetic	0.01	0.29		— 1.19				
		0.09	0.19					— 0.45	— 0.33
		0.14	0.29			— 0.21	— 0.15		
IX	Semi-synthetic	0.09	0.19					— 0.08	+ 0.12
		0.14	0.29		— 0.68	— 0.04	— 0.05		
X	Semi-synthetic	0.01	0.29					— 0.16	
		0.09	0.19						0.00
		0.14	0.29		— 1.54	+ 0.19	+ 0.21		
XI	Semi-synthetic	0.01	0.29					+ 0.46	
		0.09	0.19						— 0.07
		0.14	0.29			+ 0.69	+ 0.36		

[1] Periods I to X were 4 days in length, period XI, 3 days.

and V various combinations of methionine and cystine which were thought adequate were fed. The order in which the levels of cystine and methionine were altered during the course of a typical series (V) is presented in table 1.

TABLE 2

Vital statistics of the subjects

	SUBJECT NO.	AGE AT START OF STUDY		WEIGHT		HEIGHT
		Years	Months	Initial [1]	Final [2]	
				kg	*kg*	*cm*
Series I	1	47	9	67.7	67.7	160
	2	34	3	54.8	55.2	168
	3	32	8	55.5	56.4	163
	4	30	9	61.3	60.8	168
Series II	10	36	11	58.2	59.1	157
	11	31	2	65.9	66.8	164
	12	30	10	62.2	61.9	171
Series III	13	64	0	74.7	73.9	172
	14	28	6	55.7	56.2	160
Series IV	15	25	11	65.9	65.4	157
	16	21	6	60.8	60.3	175
	17	18	7	70.2	70.5	172
	18	18	7	60.0	60.3	168
	19	21	10	67.9	68.0	164
	20	21	5	60.0	60.0	164
Series V	31 [3]	26	3	63.3	63.1	157
	32	22	5	58.6	57.9	167
	33	19	8	58.3	58.4	175
	34	23	9	51.0	52.7	165
	35	24	2	49.5	50.0	157
	36	22	10	55.0	55.5	156

[1] Mean for the normal diet.

[2] Mean for the final experimental period.

[3] Same as subject 15.

Subjects

The subjects were women students or staff members who maintained their usual academic pursuits throughout the study. They ranged in age from 19 to 64 years (table 2) and were in good health according to physical examinations made at the University Department of Student Health. In series I, II, and III, the subjects were housed in an apartment

under the direction of a graduate dietitian, whereas in series IV and V, they lived in dormitories or private homes, but ate all of their meals in the metabolism unit of the School of Home Economics.

Diets

The daily diet of natural foods (the "normal" diet) used at the beginning of each series supplied about 10 gm of nitrogen (analysis), 1400 mg of methionine (analysis), 700 to 800 mg of cystine (analysis), and about 2000 to 2400 calories (calculated from data in Agricultural Handbook No. 8 by Watt and Merrill, '50). There were slight differences between the foods used in series I to IV (Jones, '56) and those used in series V (Steel, '56).

The semi-synthetic diet consisted of a few low-protein foods [4] plus butter oil, cornstarch, sucrose and vegetable oil,[5] as well as amino acids, diammonium citrate, purified hemicellulose [6] and mineral and vitamin supplements (Jones et al., '56). In series I, the basal portion of the diet supplied about 1.0 gm of nitrogen, 80 mg of methionine and 20 mg of cystine per day. The natural foods in series II, III, and IV contained about 0.5 gm of nitrogen, 30 mg of methionine and 10 mg of cystine per day; in series V the foods supplied 0.6 gm of nitrogen, 40 mg of methionine and 10 mg of cystine. Calories were adjusted to meet the specific energy requirements of the individual subjects by the addition of butter oil, candy (plain fondant), pudding, sucrose or a plain carbonated beverage, or in some instances by the omission of part of the pudding or wafers. The semi-synthetic diet, including the amino acids, supplied from 10 to 11 gm of nitrogen per day.

[4] Natural foods included applesauce, carrots, grape juice, jelly, lemon juice, orange juice, peaches and tomatoes (Jones et al., '56). Series I included more natural foods: 25 gm of lettuce, 100 gm of potato and additional 100 gm of grape juice. In series V, the carrots and grape juice were replaced with small amounts of banana and apricots (Steel, '56).

[5] Wesson oil.

[6] Mucilose Flakes, Winthrop-Stearns, Inc.

In series I, II, and IV, the complete amino acid mixture furnished the essential amino acids plus arginine, cystine, histidine and tyrosine in the amounts found in 20 gm of egg protein. In series III, the cystine was omitted, while in series V, the complete mixture supplied 250 mg of cystine and 250 mg of methionine. In each series, the total nitrogen intake was maintained at a constant level (about 10 gm) by the addition of isonitrogenous amounts of glycine and diammonium citrate. Details regarding the preparation and administration of the amino acids were presented in an earlier paper (Jones et al., '56). In series I to IV, the cystine and tyrosine were mixed with applesauce. In series V, the tyrosine was mixed with the applesauce, but the cystine was administered as a solution which was added to the other amino acids and diammonium citrate.

Samples and analytical determinations

The samples of foods, amino acids, diammonium citrate, urine and feces were collected and prepared for analysis as previously described (Jones and co-workers, '56). Nitrogen was determined by the boric acid modification of the Kjeldahl method (Scales and Harrison, '20). The methionine and cystine contents of the foods were determined by microbiological assay (Jones, '56; Steel, '56). Creatinine was determined daily as an indication of the completeness of collection of the urine samples.

RESULTS AND DISCUSSION

The different levels of methionine and cystine which were fed may be grouped as follows (table 3):

(1) 500 mg of cystine with varying levels of methionine (30 to 850 mg).

(2) 10 mg of cystine with varying levels of methionine (30 to 850 mg).

(3) 290 mg of methionine with varying levels of cystine (10 to 260 mg).

Mean daily nitrogen balances[1] of women ingesting different levels of methionine and cystine

SUBJECT	MEAN DAILY NITROGEN BALANCES ON INDICATED DAILY INTAKES OF CYSTINE AND METHIONINE														
Cystine mg	500						10					10	140	260	90
Methionine mg	30	80	150-180	240-290	500	850	30	150	290	500	850	290[2]	290	290	190
	gm N	gm N	gm N	gm N	gm N	gm N	gm N	gm N	gm N	gm N	gm N	gm N	gm N	gm N	gm N
Series I															
1		— 1.04	— 0.40	+ 0.34	+ 0.48	+ 0.28									
2		— 0.92	+ 0.12	+ 0.21	+ 0.09	+ 0.83									
3		— 0.92	+ 0.05	+ 0.68	+ 1.22	+ 0.66									
4		— 0.79	— 0.31	+ 0.33	+ 0.50	— 0.36									
Series II															
10	— 0.45		+ 0.43 + 0.35			+ 0.56		— 0.49							
11	— 0.63		+ 0.34 + 0.28			+ 0.65		— 0.58							
12	— 0.92		— 0.32	— 0.17		+ 0.01									
Series III															
13							— 1.33		— 0.82	— 0.14	— 0.14	— 0.82			
14							— 0.62	— 0.88	— 0.42		+ 0.02	— 0.42			
Series IV															
15				+ 0.07		+ 0.34									
16				0.00		+ 0.43									
17				+ 0.54		+ 1.27			— 0.20[3]			— 0.20[3]			
18				+ 0.59		+ 0.71			+ 0.48[3]			+ 0.48[3]			
19				— 0.19		+ 0.14			+ 0.08[3]			+ 0.08[3]			
20				+ 0.16		+ 0.27									
Series V															
31[4]									— 0.35			— 0.35		+ 0.06	
32									— 1.19			— 1.19	— 1.54	+ 0.19	
33									— 0.49			— 0.49	+ 0.81	+ 0.15	
34									— 0.39			— 0.39	+ 0.31	+ 0.37	
35									+ 0.27			+ 0.27		+ 0.83	— 0.08
									+ 0.46			+ 0.46			
36									— 0.01			— 0.01		+ 0.51	+ 0.03

[1] Mean daily nitrogen balances are for the final 4 days on a given regimen.

[2] Same as column 3 of section 2, for purposes of comparison.

[3] The amino acid supplement contained 2.92 gm of DL-valine rather than 1.46 gm of the L-isomer.

[4] Same as subject 15.

In addition, two subjects were given 190 mg of methionine and 90 mg of cystine. All subjects were not fed all levels of the cystine and methionine nor was the sequence of feeding the different levels always the same. Nitrogen balances were determined for the entire time on a given regimen, but the data presented in table 3 represent the average for the last 4 days of each dietary regimen.

500 mg cystine with varying levels of methionine

A total of 13 subjects was studied. Seven subjects receiving 30 or 80 mg methionine showed nitrogen losses ranging from 0.45 to 1.04 gm per day. Increasing the methionine intake of these 7 subjects to 150 to 180 mg per day resulted in nitrogen storage for 4 subjects and small losses for the other three subjects. For two subjects (10 and 11) following 8 days on this intake, the cystine was removed for a period of 7 days leaving only the 10 mg furnished by the natural foods. The resulting nitrogen balances were –0.49 and –0.58. When the cystine was replaced in the diet, the subjects again stored small amounts of nitrogen, thus demonstrating the contribution of cystine to the total requirement for sulfur-containing amino acids.

A further increase of the methionine for 5 of the 7 subjects to 240 to 290 mg increased nitrogen retention in all subjects. Subject 12, showing a small loss of nitrogen (–0.17 gm), had been retaining a small amount (+0.13 gm per day) during the 4-day period immediately preceding the final period. Of an additional 6 subjects (series IV) fed these levels of methionine and cystine, 5 were either in balance or were retaining small amounts of nitrogen. Subject 19, with –0.19 gm N balance during the period, showed a +0.03 gm N retention on 290 mg of methionine with only 10 mg of cystine. Subjects 15 and 16 were receiving 400 mg of lysine, subjects 17, 18, 19 and 20, 500 mg — amounts previously found to maintain nitrogen balance (Jones, '56). Larger intakes of methionine (500 and 850 mg) resulted in increased nitrogen storage in most

subjects. Subject 4, series I, showing a slight loss of nitrogen on the 850 mg intake, had stored some nitrogen at lower levels. She had a high energy requirement and presented a problem in providing sufficient calories.

In an experiment in which generous amounts of cystine were furnished (810 mg per day), Rose and Wixom ('55) found that nitrogen balance or storage could be maintained in young men on a methionine intake of 100 to 200 mg per day. The young women in this study apparently required 240 to 290 mg of methionine with a generous cystine supply (500 mg) in order to meet the Rose criterion of adequacy.

10 mg of cystine with varying levels of methionine

Thirteen subjects were studied. With only the 10 mg of cystine furnished by the natural foods of the diet, the 30 and 150 mg intakes of methionine resulted in nitrogen loss by the 4 subjects given these amounts. Of the 11 subjects who were fed 290 mg of methionine, only three stored nitrogen, while the nitrogen losses in the remaining subjects ranged from 0.01 gm to 1.19 gm. Of the two subjects given higher levels of methionine, 500 and 850 mg, one had a balance of +0.02 and the other −0.14. It is possible that subject 14 would have been in nitrogen equilibrium or have stored nitrogen on an intake somewhere between 290 and 850 mg of methionine.

Two nitrogen balances on the 290 mg intake are reported for subject 35. The second value of +0.46 gm is for a period immediately following one in which the subject had been on 90 mg of cystine and 190 of methionine. Thus, in sequence, she showed a −0.27 gm N on 10 mg cystine and 290 methionine, −0.08 on 90 of cystine and 190 of methionine and finally +0.46 on the original 10 mg cystine and 290 methionine.

If one accepts the "zone of equilibrium" proposed by Leverton et al. ('56) as nitrogen balance, the requirement for 9 of our 13 subjects was met with 10 mg of cystine and 290 of methionine or a total of 300 mg of the sulfur-containing amino acids. However, according to the Rose criterion of

adequacy, nitrogen-equilibrium or storage of nitrogen, the 300 mg intake was adequate for only three of the subjects. Consideration of these data leads to the conclusion that 290 mg of methionine with only 10 mg of cystine is on the borderline of adequacy. It represents about one third of the amount reported by Rose ('49) needed to meet the requirement of young men for methionine with no cystine provided. A comparison of the nitrogen balances of subject 13, age 64, with those of the younger subjects suggests the possibility of an increase in the requirement for sulfur-containing amino acids with an increase in age. It would be of interest to secure data on additional older subjects.

290 mg methionine with varying levels of cystine

The effect of altering the cystine intake on the nitrogen balances of subjects receiving 290 mg of methionine is shown in the third section of table 3. Column 3 of the second section has been repeated (column 1) for purposes of comparison. The 260 mg intake of cystine permitted nitrogen storage ranging from 0.06 to 0.83 gm in the subjects of series V. When the cystine was reduced to 140 mg per day for three subjects, one lost 1.5 gm of nitrogen per day. Further reduction of cystine to 10 mg resulted in nitrogen storage in only one of these 6 subjects and in three of the total 11 subjects on these levels.

Subjects 35 and 36, who seemed to have the lowest cystine-methionine requirement of the series V subjects, were given 190 mg of methionine and 90 mg of cystine daily, levels supplying the same amount of sulfur as 290 mg methionine and 10 of cystine. The complete data for series V, as shown in table 1, indicate that a period of adjustment was needed when the source of sulfur-containing amino acids was changed. The nitrogen balances on 10 mg of cystine and 290 of methionine had been +0.27 and —0.01 gm and became —0.45 and —0.33 gm for the first 4 days of the new regimen. The balances for the second 4-day period were —0.08 and +0.12 gm. Subject 36 continued on these levels for two more periods and showed

balances of 0.00 and —0.07 gm. Subject 35, returned to the 290 mg methionine and 10 mg cystine intake, showed balances of —0.16 and +0.46 gm for the two succeeding periods. One might conclude from these limited data that cystine is not quite as effective as methionine in maintaining nitrogen balance at very low levels of intake. It would be of interest to study additional subjects as well as other combinations of these sulfur-containing amino acids.

If one uses the Leverton "zone of equilibrium" as the criterion of adequacy, the requirement for sulfur-containing amino acids for 9 of 11 subjects is as low as 300 mg when a large proportion of the total is in the form of methionine, while 150 to 180 mg of methionine represents the borderline of requirement when generous amounts of cystine are supplied. If, however, the Rose criterion of equilibrium or storage is applied, 240 to 290 mg of methionine with 500 mg of cystine meets the requirement for most subjects. This same amount of methionine resulted in nitrogen storage by all subjects studied at those levels, when the cystine was reduced to 260 mg making a total of 550 mg of sulfur-containing amino acids. These amounts are of the same magnitude as those reported by Swendseid and associates ('56) who found a total requirement of 350 mg or less for 5 women, 450 mg for two others and 550 mg for another when the diet supplied 7 to 8 gm of nitrogen, as peanut protein, the essential L-amino acids and glycine. Results of both studies show a considerable individual variation in subjects. Although the self-selected diets of women in the surveys by Futrell et al. and Mertz et al., furnished less than the 1.10 gm methionine requirement proposed by Rose, they supplied more than the 550 mg requirement suggested in this paper and that of Swenseid et al.

On the normal diet the amounts of food necessary to maintain the weights of the subjects supplied 2017 to 2131 calories daily with a mean of 2068, whereas on the semi-synthetic diet, they ranged from 2163 up to 2839 with a mean of 2341. On the normal diet the mean calories per kilogram of body weight of the subjects varied from 27.4 to 41.6 with a mean of 34.4;

on the semi-synthetic diet, comparable figures were 30.7 to 50.1 with a mean of 38.8. This agrees with the results of others (Rose et al., '54; Pratt et al., '55; Jones et al., '56) who found that subjects on amino acid mixtures require more calories than when on natural foods. In the present study, the average increase in requirement was 13.2% whereas for two of the subjects it was 31 and 33%. The subjects of Swendseid et al. ('56), on the other hand, did not appear to require extra calories when shifted from a natural diet to one high in free amino acids.

SUMMARY

With a cystine intake of 500 mg, 150 to 180 mg of methionine resulted in nitrogen storage in 4 of 7 women, while the balances of the other three were within the "zone of equilibrium." An intake of 290 mg appeared to be adequate for all 13 subjects studied at those levels. A total of 300 mg of the two sulfur-containing amino acids appeared to be marginal while 550 mg resulted in some nitrogen storage in all subjects. From these data it appears that a total of 550 mg of the sulfur-containing amino acids is adequate for the establishment of nitrogen balance in women, under these conditions.

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THE ACCELERATION OF VITAMIN E DEFICIENCY IN THE CHICK BY TORULA YEAST [1]

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The yeast grown in this country on waste sulfite liquor (*Torulopsis utilis*) has been used extensively in vitamin E and Factor 3 studies. Diets low in vitamin E and containing torula yeast as the sole protein source produce liver necrosis in rats which is preventable by vitamin E, Factor 3, or cystine (Schwarz, '52). In testing a vitamin E-free diet containing 58.5% of torula yeast with chicks, Scott et al. ('55) found that a high incidence of the vitamin E-deficiency symptom, exudative diathesis, occurred within three to 4 weeks. Furthermore, when the 5% of vitamin E-free lard (generally considered a pro-oxidant stress) was omitted from the diet, the incidence of symptoms was unchanged. The condition was prevented by either α-tocopherol or dried brewers' yeast. Recently it has been shown that various inorganic or organic selenium compounds show marked Factor 3 activity in preventing liver necrosis in the rat (Schwarz and Foltz, '57). More recently Schwarz et al. ('57) and Patterson et al. ('57) in independent studies with the chick showed that various selenium compounds will also prevent exudative diathesis produced by torula yeast diets deficient in vitamin E. It has thus become apparent that the protective activity of brewers'

[1] A preliminary report of this work was presented before the American Institute of Nutrition, 21st annual meeting, April 15–19, 1957, Chicago, Illinois.

yeast, Factor 3, and of certain other dietary constituents against exudative diathesis produced by feeding torula yeast is due to their content of selenium.

In our studies with diets high in torula yeast we were impressed by two aspects of the vitamin E deficiency produced. First, the exudative diathesis was rapidly precipitated in almost all chicks in 12 to 18 days, whereas little or no exudative diathesis was seen in chicks fed certain diets containing no torula yeast, vitamin E, or Factor 3. Second, a high incidence of symptoms occurred even when the diet was essentially fat-free, contrary to the conclusion of Dam ('44) that unsaturated fat was essential for this syndrome. It was of interest, therefore, to determine why a torula yeast diet so effectively produces exudative diathesis, even in the absence of fat. Evidence is presented in this report for the presence in torula yeast of a substance which accelerates the appearance of vitamin E deficiency symptoms.

EXPERIMENTAL

In all studies 6 or 7 New Hampshire chicks one day old, housed in electrically heated brooders, were started per group. The composition of diets used is shown in table 1. All additions to diets were at the expense of Cerelose. Food and water were given ad libitum. Chicks were examined daily for exudates after the 14th day until the end of the experiment, usually 4 weeks. Chicks which died before the end of the 4-week period without evidence of exudative diathesis at autopsy were not considered in recording the incidence of deficiency symptoms. In addition to the classical green exudate under the skin of the abdomen and breast, some birds (about 9%) developed edema in the wings. Emphasis is placed on the average number of days required for symptoms to appear, since this indicates the relative potential of the diets to produce exudates. Analyses of tissues for vitamin E were performed by the method of Swick and Baumann ('52).

TABLE 1

Composition of vitamin E-free diets for producing exudative diathesis

INGREDIENT	DIET C34	DIET C41	DIET C41A	DIET C46	DIET C47A
	%	%	%	%	%
Torula yeast [1]	60.0	...	...	...	...
Soybean protein [2]	...	...	12.0	12.0	28.0
Gelatin	...	...	...	10.0	...
Amino acid mix 7 [3]	...	18.9	10.0	...	...
Cerelose (glucose)	28.5	8.0	67.5	66.9	61.2
Sucrose	...	62.9	...	...	...
Vitamin E-free lard [4]	4.0	4.0	4.0	4.0	4.0
Salt Mix A [5]	6.0	6.0	6.0	6.0	6.0
DL-Methionine	0.3	...	0.3	0.6	0.6
DL-Phenylalanine	...	...	...	0.2	...
DL-Tryptophan	...	...	...	0.1	...
Glycine	1.0	...	...	...	...
Vitamins [6]	0.2	0.2	0.2	0.2	0.2

[1] Lake States Yeast Corporation – feed grade Torula yeast.

[2] Drackett C-1. Extracted with hot ethanol.

[3] DL-Alanine 1.0, L-arginine HCl 1.3, L-aspartic acid 0.5, L-cystine 0.3, L-glutamic acid 3.5, glycine 1.0, L-histidine·HCl 0.6, DL-isoleucine 2.0, L-leucine 1.4, L-lysine·HCl 1.2, DL-methionine 0.4, DL-phenylalanine 1.0, L-proline 0.5, DL-serine 1.0, DL-threonine 1.0, DL-tryptophan 0.4, L-tyrosine 0.6, DL-valine 1.2. Total 18.9 gm.

[4] Distillation Products Industries.

[5] Briggs et al. ('52).

[6] Fox et al. ('55). (Vitamins D, K, and B complex, including choline; α-tocopherol and vitamin A omitted). Vitamin A acetate given once weekly in the drinking water, 750μg/l (Bieri, '57).

RESULTS

Basal diet C34 with 60% torula yeast and 4% vitamin E-free lard is similar to that used by Scott et al. ('55). In agreement with their results, this diet regularly produced exudative diathesis in 80 to 100% of chicks in an average time varying from 14 to 18 days in different trials (table 2). Because this diet produced a high mortality during the 4th week, only the weights at three weeks are given.

TABLE 2

Effect of differing treatments of the torula yeast diet on the incidence of exudative diathesis

TRIAL	CHANGE IN BASAL DIET C34	AVERAGE WT. AT 4 WKS.	EXUDATIVE DIATHESIS	
			Incidence [1]	Av. days for development
		gm		
1	None	123	4/6	16.2
	Diet pelleted [2]	109	6/6	17.8
	Torula yeast pepsin digested [3]	121	5/7	19.4
	Torula yeast reduced to 45%	114	5/5	17.2
	1% L-cystine added	106	3/3	16.3
	20% Drackett protein added	176	0/6	...
2	None	121	5/6	17.2
	Ethanol-extracted torula yeast	106	6/6	17.3
	Ethanol-extracted torula yeast, lard omitted	120	4/5	24.5
	Ether-extracted torula yeast	106	4/6	21.2
	Ether-extracted torula yeast, lard omitted	119	3/5	26.3
	100 mg dl, α-tocopheryl acetate/kg	170	0/6	...

[1] Incidence – number of affected chicks per number survivors (see text).

[2] Moistened with water, pressed through a large mesh screen, and dried at 60°C.

[3] Contains 40% of nitrogen in amino form. (Kindly supplied by Lake States Yeast Corp.)

Effect of dietary modifications on incidence of exudative diathesis

Numerous variations of the basal diet were made in an attempt to shed light on the exudate-accelerating effect of torula yeast (trial 1, table 2). When the diet, which is powdery, was pelleted, symptoms occurred as usual; growth on this diet was very poor. Substitution of pepsin-digested torula yeast also did not reduce the incidence of symptoms. Decreasing the yeast from 60 to 45% of the diet did not decrease severity of symptoms. The addition of 1% of L-cystine was without protective effect, in agreement with the findings of Scott et al. ('55). The addition of 20% of soy protein[2] to the basal diet completely prevented symptoms, presumably due to Factor 3 in the protein.

[2] Drackett protein.

Effect of a fat-free diet on incidence of exudative diathesis

Attention was next directed toward the possible presence in torula yeast of unsaturated lipids which might aggravate vitamin E deficiency symptoms. Extraction of the yeast in a large soxhlet apparatus with 95% ethanol removed 10% of soluble material; much of this was presumably lignosulfonate. Diethyl ether extraction removed 3% of soluble material. The effect of feeding extracted torula yeast with and without lard is shown in trial 2, table 2. Substitution of the regular torula yeast with either alcohol- or ether-extracted yeast still produced exudative diathesis in about the same time as the unextracted yeast. When the lard was omitted, symptoms were delayed 5 to 7 days. The addition of vitamin E to the basal diet completely prevented symptoms.[3] It is apparent that the presence of fat in the diet is not necessary for the production of exudative diathesis by the torula yeast diet.

Development of an alternative diet low in vitamin E and Factor 3

To eliminate complications arising from the presence of Factor 3 in many proteins, attempts were made to formulate a synthetic diet which would produce exudative diathesis. A protein-free diet (C41, table 1) with an amino acid mixture patterned after that of Fisher and Johnson ('56) proved to be satisfactory for this purpose. The results from two trials with this diet are shown in table 3. These chicks were kept on experiment for 45 days, since symptoms did not begin to appear until the 24th day. In the first trial the incidence of exudative diathesis was relatively high compared to that in trial 2. In both trials, however, an average of 28 to 43 days was required for symptoms to develop. When 30% of torula yeast was added to the basal diet (trial 2), 5 of 6 birds

[3] Selenium compounds also prevent this condition as shown by Patterson *et al.* ('57) and Schwarz *et al.* ('57).

were affected in the usual time of 16 to 17 days. Growth improved considerably by the addition of torula yeast. The addition of vitamin E gave complete protection.

Due to the expense of complete amino acid diets, attempts were made to develop a vitamin E-free diet using a purified protein that would be low in Factor 3. Such a diet would permit the testing of various fractions of torula yeast for possible anti-vitamin E activity. The first diet found to be successful for this purpose was diet C41A containing 12% of alcohol-

TABLE 3

Incidence of exudative diathesis in chicks fed a vitamin E-deficient diet containing amino acids as the nitrogen source

TRIAL	ADDITIONS TO DIET C41	AVERAGE WT. AT 4 WKS.	EXUDATIVE DIATHESIS	
			Incidence [1]	Av. days for development
		gm		
1	None [2]	150	3/11	27.8
	20 mg dl, α-tocopheryl acetate orally, weekly	161	0/5	...
2	None [2]	134	1/6	35.0
	30% torula yeast	176 [3]	5/6	16.2
	100 mg dl α-tocopheryl acetate/kg	172	0/7	...

[1] See table 2.

[2] Basal groups kept on experiment for 45 days.

[3] Three-week weight; all dead but one by 4 weeks.

extracted soy protein (table 1). A representative experiment in which this diet was fed alone and with two levels of torula yeast is given in table 4. No exudative diathesis occurred in the chicks fed the basal diet. The addition of only 7.5% of torula yeast produced symptoms in 6 of 7 chicks in an average of 23.3 days. Fifteen percent of yeast caused a high incidence in the average time of 16.2 days. Addition of tocopherol to the 15% yeast diet gave complete protection.

In order to eliminate the large amounts of amino acids used in this diet, a regimen was found which has served excellently for testing the effects of various substances in accelerat-

ing vitamin E deficiency and producing exudative diathesis. This diet (C46, table 1) is similar to diet C41A with the major difference that 10% of gelatin was substituted for the amino acid mixture. In contrast to the 60% torula yeast diet (C34), chicks grow well on this diet and do not "paste up" around the vent as do chicks fed the C34 diet.

TABLE 4

Incidence of exudative diathesis in chicks fed a soy protein-amino acid diet with varying levels of torula yeast

TRIAL	ADDITIONS TO BASAL DIET C41A	AVERAGE WT. AT 4 WKS.	EXUDATIVE DIATHESIS	
			Incidence [1]	Av. days for development
		gm		
1	None	277	0/6	...
	7.5% Torula yeast	270	6/7	23.3
	15% Torula yeast	233	5/7	16.2
	15% Torula yeast + 100 mg dl-, α-tocopheryl acetate/kg	341	0/7	...

[1] See table 2.

It can be seen in table 5 that in two separate trials the time required for exudative diathesis to appear in chicks fed this diet (C46) was 23 to 26 days and only a few birds (three of 12) developed symptoms during the 28-day observation period. In contrast, either 10 or 20% of torula yeast added to this diet produced a high incidence of symptoms. With 10% of yeast the average time was 20.0 and 17.4 days while with 20% it was 16.6 and 19.8 days, respectively, for the two trials. Evidence that the accelerating effect is a unique property of the yeast is provided by the results from chicks fed two levels of dried whole sulfite liquor,[4] the medium in which the yeast is grown. Although this material contains a considerable amount of inorganic material (14 to 19% ash), it did not produce a significant incidence of exudative diathesis. Dam and Glavind ('42) and Bird ('43) showed that exudative diathesis was increased by additional salt in the diet. The

[4] Lake States Yeast Corporation, Rhinelander, Wisconsin.

addition of 1% of sodium chloride to the basal C46 diet also did not provoke a high incidence of symptoms. When this amount of salt was added to the diet containing 10% of torula yeast, the number of chicks with exudates and the average time for development were similar to those for 10% of yeast alone. The torula yeast used in these studies had an ash content of 7.5 to 7.8%.

TABLE 5

Effect of various treatments on the acceleration of exudative diathesis by torula yeast

TRIAL	ADDITIONS TO BASAL DIET C46	AVERAGE WT. AT 4 WKS.	EXUDATIVE DIATHESIS	
			Incidence [1]	Av. days for development
		gm		
1	None	265	2/6	26.0
	10% Torula yeast	280	6/7	20.0
	20% Torula yeast	219	5/7	16.6
2	None	269	1/6	23.0
	1%NaCl	283	1/6	28.0
	10% Dried sulfite liquor	242	1/6	28.0
	20% Dried sulfite liquor	268	1/6	28.0
	10% Torula yeast	227	5/6	17.4
	10% Heated [2] torula yeast	299	5/6	20.4
	10% Torula yeast + 1% NaCl	194	6/6	17.8
	20% Torula yeast	234	5/6	19.8
	20% Washed [3] torula yeast	361	4/6	19.8
	Torula yeast ash [4] ≅ 10% yeast	243	4/6	22.2
	Torula yeast ash [4] ≅ 20% yeast	283	4/6	19.8
	Oil from torula yeast [5] ≅ 15% of yeast	255	1/6	24.0

[1] See table 2.

[2] Heated at 105°C. for 48 hours.

[3] Washed twice with 20 volumes of tap water; dried at 60°C. in vacuo.

[4] Ashed at 400°C.

[5] Extracted from alcohol-extracted yeast after acid hydrolysis.

Heating the torula yeast at 105°C for 48 hours did not change its ability to produce exudates, nor did reducing the ash content to 3.9% by washing the yeast twice with water (table 5). Both of these treatments, however, improved growth.

When the ash from torula yeast, prepared by dry-ashing at 400°C, was added at levels equivalent to 10 or 20% of the yeast, a high incidence of exudative diathesis occurred. The 20% level produced symptoms slightly quicker than did the 10% level. This experiment shows that at least part of the exudate-producing activity of torula yeast is inorganic. In a repetition of this experiment both a 250°C ash and a 550°C ash produced similar results (data not shown).

Since alcohol or ether extraction of torula yeast does not completely remove bound lipid, the possibility existed that this remaining lipid may be a pro-oxidant which would have an anti-vitamin E effect. To test this, torula yeast which had been extracted with hot ethanol was hydrolyzed with hydrochloric acid and then extracted with petroleum ether. About 0.7 to 0.8% of a light brown oil was obtained. This was added to the basal C46 diet with the results shown in table 5. Since the incidence of exudative diathesis was no greater than in the control group, it would appear that the bound lipid in the torula yeast is not responsible for the acceleration of symptoms.

Effect of torula yeast on liver and serum vitamin E

In order to determine whether torula yeast could possibly have an adverse effect on the tocopherol content of tissues in chicks ingesting the yeast, studies were carried out in which the basal torula yeast diet (C34) was compared with a soy protein diet (C47A, table 1). Groups of day-old chicks were fed these diets supplemented with 100 mg of dl,α-tocopheryl acetate/kg for 16 days and then sacrificed. The results indicated that the level of serum tocopherols was considerably lower in the torula yeast group than in the soy protein group (800 ± 213 and 1380 ± 182 mg%, respectively, 6 chicks per group). There was no difference in the average liver tocopherols. Repetition of this experiment with 4 chicks per group gave an even greater difference between the serum values.

Since the high torula yeast diet (C34) appeared to be poorly utilized, it is very probable that the absorption of the dietary tocopherol was greater in the soy protein group. Consequently, two groups of chicks on these same diets without vitamin E for 13 days were injected intravenously with an aqueous Tween 40 emulsion of d,α-tocopherol. Three days later they were sacrificed and the livers analyzed (table 6).

TABLE 6

Liver tocopherol content of chicks injected intravenously with 7.5 mg DL, *α-tocopherol emulsified in water with Tween 40* [1]

DIET	NO. CHICKS	AV. WT.	TOTAL LIVER TOCOPHEROLS	
			Whole Liver	per gm liver
		gm	*μg*	*μg*
Torula yeast (C34)	6	122	506 ± 98 [2]	101 ± 22
Soy protein (C47A)	5	124	1055 ± 136	143 ± 16

[1] On diets for 13 days; injected and killed after three days.
[2] Mean ± standard error.

Whether expressed as total tocopherols per liver or as micrograms per gram of tissue, significantly less tocopherol was stored by the chicks on the torula yeast diet. Whether this represents an increased rate of destruction of the vitamin or an impaired storage cannot be determined from these studies. The evidence suggests, however, that the chicks fed the torula yeast diet have an impaired utilization of tocopherol.

DISCUSSION

The results of this study indicate that torula yeast contains a nonlipid substance (probably inorganic) which, when present in diets low in vitamin E and Factor 3 (or selenium), accelerates exudative diathesis in the chick. However, alternative explanations for this effect of torula yeast must also be considered. Similar considerations arose in studies of the ability of various yeasts to produce liver necrosis in rats. Diets which produce exudative diathesis in the chick are similar to those which produce liver necrosis in the rat and

the same substances will cure both conditions (vitamin E and selenium, but not cystine for exudative diathesis).

Although György et al. ('50) seriously considered the presence of toxic substances in, or toxic metabolites arising from, certain yeasts, other investigators (McLean and Beveridge, '52; Schwarz, '54b) have discounted this possibility. The primary argument against this theory was based on evidence that increasing amounts of necrogenic yeasts in the diet did not increase the incidence or severity of liver damage. It has been pointed out that other types of diets not containing yeast will also produce liver necrosis, but American grown torula yeast is more satisfactory than any other protein source tested for producing liver necrosis in rats (Schwarz, '54b).

A related consideration in the development of many deficiences is the rate of growth and the accompanying food consumption. Schwarz ('54a) stated that a decreased food consumption will prevent liver necrosis in rats fed a highly necrogenic diet. In the present studies, examination of the records of over 400 chicks fails to show any consistent relationship between growth rate, food consumption, and development of exudative diathesis. With the soy protein diet (C46), although increasing levels of yeast usually gave slightly better growth during the first and second weeks, these differences were not marked. In contrast, with the high torula yeast diet (C34), frequently the poorest growing group developed symptoms in the shortest time (see table 2). Further work is required to clarify the growth-stimulating effect of torula yeast when added to soy protein diets and the possible relationship of this effect to the exudate-producing property.

In our studies of vitamin E in the chick we have found that on various vitamin E-free diets the rate of depletion of the vitamin in the blood and liver is quite uniform despite appreciable differences in the initial total tocopherols in individual chicks. After three weeks on deficient diets almost all chicks have serum tocopherol levels below 100 μg%, and the liver concentrations range from 5 to 15 μg/gm. This is true for birds depleted on diets containing Factor 3, provided

by purified casein (Schwarz et al., '57), as well as on diets without Factor 3. However, if no Factor 3 is present in the diet and a stress is provided, such as unsaturated fat, or as shown here, torula yeast, then exudative diathesis will be precipitated.

In the present studies it has been found that increasing levels of torula yeast in the diet shorten the time for the appearance of exudates. This is particularly evident with the soy protein diet using 7.5 and 15% of torula yeast (table 4). On the other hand, when the torula yeast of the C34 diet (60%) was reduced to 45%, there was no appreciable change in the incidence or time of occurrence of symptoms. It appears that about 15 to 20% of torula yeast contains sufficient activity to precipitate symptoms in the minimum time of 16 to 18 days.

The fact that torula yeast which had been washed thoroughly with water so that the ash content was reduced from 7.8 to 3.9% still produced a high incidence of exudative diathesis would rule out the high mineral content of the yeast as the active agent in the ash. Miller et al. ('55) found that bisulfite accelerated vitamin E deficiency in chicks fed a soy protein diet. Since the water-washed torula yeast had a sulfite content of 0.027% (as SO_2) compared with 0.130% for the crude torula yeast, it would appear that sulfite is not responsible for the exudative effect. Futhermore, the dried whole sulfite liquor, which did not produce exudates, had a SO_2 content of 7.1%.

As a result of these studies, it is evident that the combined deficiency of vitamin E and Factor 3 produced with torula yeast diets is not a simple deficiency state, but may be complicated by an antagonistic substance in the yeast. Elaboration of the nature of this substance is in progress. It should be emphasized that when torula yeast is used as a vitamin and protein supplement in commercial rations containing vitamin E, no difficulties arise in animals fed such rations.

SUMMARY

New Hampshire chicks were fed purified diets essentially free of vitamin E, Factor 3 and selenium in which amino acids or soy protein were the sources of nitrogen. The addition of 7.5 to 30% of torula yeast to these diets accelerated the development of exudative diathesis. This property was not associated with the lipid component of the yeast. Washing the yeast with water did not remove the activity. An ash of the yeast, however, promoted exudates. The storage of injected α-tocopherol was impaired in chicks fed a high torula yeast diet compared with chicks fed a soy protein diet. It appears that the exudate-producing activity is associated with the mineral component of the yeast.

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THE EFFECT OF ETHIONINE FEEDING ON LIVER AND KIDNEY COENZYME A CONTENT IN THE RAT [1]

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INTRODUCTION

It has been shown that coenzyme A and thioctic acid are concerned with pyruvate oxidation and utilization (Littlefield and Sanadi, '52; Olson and Kaplan, '48; Reed, '53). A recent report demonstrated in ethionine-damaged rat livers a concomitant reduction in thioctic acid levels and an interference with mitochondrial pyruvate oxidation (Fischer, '55). Failure to correct the oxidative defect by the replacement of thioctic acid suggested that decreased hepatic coenzyme A levels may have played a significant role. This possibility appeared likely, also, since the hepatic concentration of coenzyme A is known to be diminished in methionine deficiency (Dinning et al., '55), and ethionine has been shown to interfere with the metabolism of methionine. It is postulated that an interference with coenzyme A anabolism may be a consequence of chronic ethionine administration.

In view of these considerations, the present study was undertaken to determine the effect of prolonged ethionine feeding on the coenzyme A content of rat livers and kidneys. The content of this coenzyme was found to be markedly diminished in liver but slightly increased in kidney of ethionine-fed rats.

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[2] Fellow of the National Foundation for Infantile Paralysis.

EXPERIMENTAL

Female Wistar rats weighing approximately 125 gm were divided into three groups. Group I was placed on a vitamin supplemented synthetic diet containing 0.5% of DL-ethionine by weight (Fischer, '55). Group II was given the above diet without ethionine and pair-fed. Both groups were maintained on their respective diets from 21 to 28 days and sacrificed by decapitation. Group III was fed a commercial stock diet ad libitum for one week prior to sacrifice to determine the tissue coenzyme A content of animals not subjected to partial starvation. At necropsy, portions of liver and kidney were immediately excised, weighed and homogenized in 9 volumes of cold distilled water. Homogenization was carried out in a Potter-Elvehjem homogenizer at 0°C. An aliquot of each homogenate was placed in a boiling water bath for 5 minutes, then cooled in crushed ice. The cooled homogenates were centrifuged to remove the precipitated material and the supernatant layers were stored at −10°C until coenzyme A assays were performed. The remainder of each homogenate was frozen and set aside for protein determination by the copper-Folin method of Lowry (Lowry et al., '51).

Coenzyme A analyses were performed with slight modification by the arsenolysis method (Stadtman et al., '51). Dilithium acetyl phosphate was prepared by the reaction of isopropenyl acetate with phosphoric acid and purified by three precipitations with absolute ethanol (Stadtman and Lipmann, '50). Crude phosphotransacetylase was obtained from cells of *Clostridium kluyverii*[3] treated with Dowex-1.[4] Standard coenzyme A, containing 300 Lipman units per milligram, was diluted with distilled water to obtain 10 units per milliliter of solution. Analyses were performed on 0.3 ml of

[3] The authors wish to thank Dr. Arthur Kornberg for kindly donating dried *Cl kluyverii* cells used in the arsenolysis assay for coenzyme A. The extraction and Dowex-1 treatment of the dried cells were carried out by the Sigma Chemical Company, St. Louis, Missouri.

[4] Sections were prepared by the Department of Pathology, Washington University School of Medicine.

each tissue extract and compared with the standard solution containing 3 units of coenzyme A. Coenzyme A assays were performed on both livers and kidneys of 21 ethionine-fed animals, 20 of their pair-fed controls and 10 ad libitum stock-fed controls. Tissue proteins and organ weights were determined on 15, 13, and 10 of the above mentioned animals, respectively. Representative sections from both liver and kidney of the ethionine-fed and pair-fed control rats were fixed in Bouin solution for hematoxylin and eosin and connective tissue stains and in cobalt-calcium-formalin solution for fat staining.[5]

RESULTS

The ethionine-fed rats failed to gain weight during the experimental period, but pair-fed controls gained slightly (table 1). At sacrifice the ethionine-damaged livers were grossly enlarged, firm, pale and granular. Microscopic examination revealed disarrangement of architecture, patchy cellular necrosis, cellular swelling, and fibroblastic proliferation with minimal diffuse fibrosis, as previously described (Koch-Weser and Popper, '52). Kidneys were enlarged and pale but failed to reveal microscopic evidence of renal tubular damage. This latter finding is in agreement with observations of Wachstein and Meisel ('51). The livers of the majority of pair-fed controls appeared normal to gross and microscopic examination, as did all of the kidneys. However, the livers of 6 of the pair-fed rats showed microscopic evidence of moderate periportal fatty infiltration without disturbance of parenchymal architecture.

Based on final body weight, the livers of the ethionine-fed rats were larger than those of the pair-fed controls (table 1), but this difference was not significant ($p > 0.70$). Neither was there a significant difference in liver size of ethionine ($p > 0.25$) nor of pair-fed control rats ($p > 0.4$) when compared to the ad libitum-fed group.

[5] Sigma Chemical Company.

TABLE 1

Organ and body weights as affected by feeding ethionine

DIET	INITIAL BODY WT.	FINAL BODY WT.	LIVER WT. AT AUTOPSY	LIVER TO FINAL BODY WT. RATIO	AVERAGE KIDNEY WT. AT AUTOPSY	KIDNEY TO FINAL BODY WT. RATIO
	gm	*gm*	*gm*	%	*gm*	%
Group I [1]	125	123	7.41	5.86 ± 0.24 [2]	0.701	0.557 ± 0.016
	(21) [3]	(21)	(15)	(15)	(15)	(15)
Group II	126	138	7.37	5.55 ± 0.36	0.572	0.426 ± 0.014 [4]
	(20)	(20)	(13)	(13)	(13)	(13)
Group III	....	127	6.02	4.73 ± 0.16	0.545	0.427 ± 0.009 [4]
		(10)	(10)	(10)	(10)	(10)

[1] Group I, fed a synthetic diet containing DL-ethionine.

Group II, fed the synthetic diet without ethionine, pair fed.

Group III, fed a commercial stock diet for one week prior to sacrifice.

[2] Mean ± standard error.

[3] Number of observations.

[4] Difference from group I significant, $p < 0.02$.

However, kidney size based on final body weight in the ethionine group was found to be significantly ($p < 0.02$) increased by approximately 30% over control groups. Kidney weights in both control groups were closely approximated.

The liver coenzyme A content is summarized in table 2. The coenzyme A concentration, expressed as Kaplan-Lipmann units per gram of wet tissue, was considerably less in ethionine-damaged livers when compared to pair-and ad libitum-fed controls. This difference was highly significant ($p < 0.001$). Of the two control groups, the pair-fed rats displayed a slight ($p < 0.10$) decrease in concentration of coenzyme A. The values obtained in ad libitum-fed controls are comparable to those reported by other investigators (Dinning et al., '55; Tabachnick and Bonnycastle, '54). When coenzyme A concentration was expressed as units per 100 mg of tissue protein, a marked and significant ($p < 0.001$) decrease was still demonstrable in ethionine-damaged livers. However, comparable values were obtained in both pair- and ad libitum-fed groups. The data indicate that moderate fatty infiltration in the liver does not affect the ratio of liver coenzyme A to tissue protein. Total liver coenzyme A content was calculated as units per 100 gm of initial body weight. A total decrease was established at a highly significant level ($p < 0.001$) in the ethionine-damaged livers.

Renal coenzyme A values are presented in table 3. Coenzyme A concentration expressed as units per gram of wet tissue and as units per 100 mg of tissue protein was not significantly ($p > 0.50$) altered in kidneys of ethionine-fed animals. However, the total renal coenzyme A per 100 gm of initial body weight was elevated to a significant degree ($p < 0.05$) in these animals, probably as a result of the increased kidney size. Renal values in ad libitum-fed controls closely approximated those recently reported (Bartlett et al., '56).

Since certain of the coenzyme A analyses were related to tissue protein, it was of some import to determine whether significant changes in tissue protein obtained in the animals

TABLE 2

Coenzyme A and protein content of liver as affected by feeding ethionine

DIET	UNITS/[1] GM WET LIVER	UNITS/ 100 MG PROTEIN	TOTAL UNITS/ 100 GM INITIAL BODY WT.	GM PROTEIN/ 100 GM INITIAL LIVER	GM PROTEIN/ 100 GM INITIAL BODY WT.
Group I[2]	72.2 ± 2.5[3]	50.9 ± 1.9	423 ± 242	14.0 ± 0.23	0.83 ± 0.03
Group II	95.5 ± 3.0[4]	63.5 ± 1.2[4]	571 ± 27.5[4]	14.9 ± 0.56	0.90 ± 0.05
Group III	111.5 ± 9.0[4]	65.4 ± 4.5[4]	.....	16.9 ± 0.32	.....

[1] Lipmann units of coenzyme A.
[2] See footnote 1, table 1.
[3] Mean ± standard error.
[4] Differences from Group I significant, $p < 0.001$.

TABLE 3

Coenzyme A and protein content of liver as affected by feeding ethionine

DIET	UNITS/GM [1] WET WT. KIDNEY	UNITS/100 MG PROTEIN	TOTAL UNITS/ 100 GM INITIAL BODY WT.	GM PROTEIN 100 GM WET WT. KIDNEY	GM PROTEIN 100 GM INITIAL BODY WT.
Group I[2]	40.4 ± 2.4[3]	36.2 ± 2.1	27.2 ± 2.0	13.1 ± 0.17	0.074 ± 0.002
Group II	43.8 ± 1.9	34.5 ± 1.4	21.8 ± 0.9[4]	13.5 ± 0.15	0.064 ± 0.003
Group III	45.7 ± 3.3	33.9 ± 2.2	.....	13.4 ± 0.17	.....

[1] Lipmann units of coenzyme A.
[2] See footnote 1, table 1.
[3] Mean ± standard error.
[4] Differences from group I significant $p << .05$.

studied. Protein values were expressed as grams per 100 gm of wet tissue and as total protein contained in each liver (table 2) and kidney (table 3) per 100 gm of initial body weight. There were no significant differences in protein levels of livers of ethionine and pair-fed rats ($p > 0.60$), a finding in agreement with the observations of Levine and Fopeano ('53). The protein concentrations as grams per 100 gm of liver of the ad libitum-fed rats were slightly but insignificantly ($p > 0.20$) higher than those of the pair-fed group. Although these differences were not statistically significant, it was felt that the slight depression observed in pair-fed rats was due to a combination of fat infiltration and partial starvation (Kosterlitz, '47). Renal protein values in all groups were comparable. The slight elevation in total protein per 100 gm of initial body weight noted in kidneys of ethionine-fed animals was not significant ($p > 0.25$).

To exclude the possibility of deficient pantothenic acid intake contributing to the depression of coenzyme A in ethionine-damaged livers, the average amount of calcium pantothenate consumed daily by each animal was calculated. The synthetic diet contained 0.040 gm of calcium pantothenate per kilogram of diet. The average daily food intake per rat ranged from 5.3 to 9.7 gm, with a mean daily intake of 8.2 ± 0.27 gm. Calcium pantothenate ingestion thus varied from 0.212 to 0.389 mg per day, with a mean daily intake of 0.329 ± 0.011 mg. Therefore, calcium pantothenate intake for all rats in the ethionine and pair-fed groups far exceeded minimal daily requirements reported for rats (Emerson and Evans, '41; Unna and Richards, '42).

DISCUSSION

Diminished coenzyme A values were observed in ethionine-damaged livers. It was first considered that this decrease might be a reflection of cellular necrosis and fibrosis. However, this appears unlikely since the differences persisted when tissue protein levels were used as a standard of reference, or when total coenzyme per liver was determined. At

present, there is no universally accepted standard of reference upon which to base hepatic enzyme or coenzyme concentrations (Waterlow, '52). The validity of using deoxyribonucleic acid (DNA) as a reference standard has been discussed previously (Thompson et al., '53). One might expect in damaged livers containing a variety of infiltrating cells and polypoid nuclei that the DNA-protein ratio would be increased. This has been shown to be the case in ethionine-damaged livers.[6] Thus, if DNA rather than protein were used as a basis of reference in ethionine-fed rats, the hepatic coenzyme A concentration would have appeared even lower. It seems reasonable, therefore, to conclude that the observed decrease of coenzyme A in the ethionine-damaged liver is real and not merely a concomitant of cellular necrosis and fibrous tissue infiltration.

The decrease in liver coenzyme A may be directly related to the biochemical antagonism known to exist between methionine and its ethyl analogue, ethionine. Ethionine has been shown to inhibit the uptake of methionine into rat liver protein (Simpson et al., '50), and may actually be incorporated into abnormal proteins (Levine and Tarver, '51). Methionine has been shown to exert a sparing action on the pantothenic acid requirement of the rat (Ludovici et al., '51). In addition, Dinning et al. ('55) observed a direct relationship between methionine, pantothenic acid intake and the concentration of coenzyme A in the rat liver. They suggested that methionine may supply the thioethanolamine moiety present in the coenzyme A molecule. The diminished hepatic coenzyme A content in ethionine-fed rats suggests that ethionine by antagonizing methionine may actually suppress coenzyme A synthesis. Possible mechanisms by which this could occur include: (1) the formation of abnormal ethionine-containing apoenzymes which could no longer participate in coenzyme A anabolism, or (2) an interference with the transfer of the thioethanolamine radicle from methionine to coenzyme A. The slight elevation of total renal coenzyme A in ethionine-fed rats

[6] Shank, R. E., A. Mendelott and M. Karl, unpublished data.

does not negate this postulated antagonism, since the antagonistic action of ethionine is manifest less in kidney than in liver (Simpson et al., '50). In fact, the rise in renal coenzyme A content may be compensatory to an inhibited hepatic synthesis of the coenzyme, although there is no evidence at present to support this hypothesis.

The question of whether a decreased coenzyme A concentration in itself is responsible for the hepatic lesion observed after ethionine feeding is open to some speculation (Chernick et al., '55; Olson and Dinning, '54). A satisfactory answer cannot be derived from the data presented.

SUMMARY

Coenzyme A concentration and total hepatic coenzyme A content were found to be decreased in rats chronically fed ethionine compared to values obtained in pair-and ad libitum-fed controls.

Total renal coenzyme A content in ethionine-fed rats was slightly, but significantly, increased over that of pair-fed controls.

It is postulated that ethionine may impair coenzyme A synthesis as a consequence of its metabolic antagonism to methionine.

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THE ABSORPTION BY IMMATURE AND ADULT RATS OF AMINO ACIDS FROM RAW AND AUTOCLAVED FRESH PORK

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Decrease in the biological value of most proteins has been shown by many investigators to follow severe heat processing. However, only a few studies of this phenomenon have included observations on the proteins of muscle meat. Morgan and Kern ('34) reported a gradual and progressive decrease of 5 to 30% in protein efficiency of beef and horsemeat when boiled at atmospheric pressure or when autoclaved 7 minutes or one hour. Seegers and Mattill ('35a) found similar effects due to heating or to hot alchol extraction of beef liver, kidney, heart or round along with lowered digestibility both *in vivo* and *in vitro*. Further digestibility studies led these authors (Seegers and Mattill '35b) to conclude that the low biological value of alcohol-extracted or heated liver was due solely to lowered digestibility "such that the resulting amino acid proportions are not representative of the original protein." Poling et al. ('44) found the nutritive value of the proteins of cured pork shoulder slightly but significantly lowered by a commercial canning process. Beuk, Chornock and Rice ('48, '49) reported that autoclaving fresh pork decreased its growth value for young rats and that, although cystine was the only amino acid which appeared actually to be destroyed, so that it could not be recovered after acid hydrolysis of the heated

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protein, all the amino acids determined were released more slowly by enzymatic digestion *in vitro* from the severely autoclaved than from the raw meat.

If decreased availability of amino acids is the cause of the lowering of the biological value of heated proteins it should be possible to demonstrate the different rates of digestion *in vivo* by assaying amino acids in portal blood after feeding raw and overheated proteins. Denton and Elvehjem ('54) have shown differences in the rate of appearance of amino acids in portal blood of dogs after feeding beef, casein, or zein which corresponded roughly with the differences in the amino acid composition of the proteins. This investigation was undertaken in an attempt to correlate the amino acid content of plasma from portal blood taken from rats during digestion of raw or autocleaved fresh pork with results obtained by Beuk et al. from *in vitro* enzymatic digestion, and with changes in the biological value of the proteins.

EXPERIMENTAL

Preparation of samples. The meat of commercial raw fresh hams was freed as completely as possible from fat, then ground thoroughly and packed into half-pint glass jars. Part of it was autoclaved at 120°C for 15, 30, 60, 120, 195 or 240 minutes, then stored along with the untreated meat at –17°C. Small lots were defrosted just before they were offered to the rats.

Treatment of animals. Two types of experiments were done with rats of the Long-Evans strain, a protein efficiency experiment with weanling male rats, and acute digestion experiments with adult male and female rats and with two groups of the young rats at the end of the protein efficiency experiment.

Male rats were divided at weaning into 6 groups of 7 each, caged individually, and offered water and a non-protein basal diet [2] ad libitum. They were fed three times a week approximately one third of a weighed amount of meat raw or auto-

[2] The non-protein basal diet contained in percent: fat (Primex) 20.9, sucrose 74.4, salts 4.7 (Hubbell et al., '37).

claved for 15, 30, 60, 120 or 195 minutes at 120°C, which was calculated to supply them with 5 gm of protein per week. At the same time they were given 2 ml of a standard mixture of B vitamins and 2 drops of oil containing vitamins A, D, and E (Forker and Morgan, '54). After 4 weeks, they were fasted of protein for 48 hours, but allowed free access to the non-protein basal diet and water. On the third day, 4 rats each from the groups which had received the raw pork and that autoclaved for 195 minutes were anesthetized by intraperitional injection of sodium amytal and bled from the portal vein. These served as fasting controls. The other three rats in each group were fed 5 gm each of the meat which they had been fed previously and bled from the portal vein one half hour after the meal. Because of the small size of these rats it was sometimes necessary to pool the blood from two or three for the amino acid analysis.

Other rats were maintained on a satisfactory stock diet until the body weight of the females reached 200 to 270 gm and that of the males 250 to 350 gm. They were weighed, put into separate cages and offered water but no food for 24 hours. They were then offered 10 gm each of either raw fresh pork or fresh pork autoclaved at 120°C for 4 hours. At the end of an hour any uneaten portion of the meat was removed and weighed. The next day they were offered 5 gm of the same meat (unless they were to be used as fasted controls) and portal blood was taken from them under sodium amytal anesthesia at predetermined time intervals. Each rat was used for only one sampling of portal blood. Thus the amino acids levels reported at each time interval represent averages of samples from three to 5 different animals. About 100 adult rats were used.

Tungstic acid filtrates were made immediately from the plasma of heparinized blood and stored at –17°C until they could be analyzed.

Analytical method. Amino acids were determined by quantitative paper chromatography of their 2, 4-dinitrophenyl derivatives by the method of Levy ('54, '55) modified for use

with the tungstic acid filtrates. The reaction was carried out in bicarbonate buffer, pH 9.0, and only the amino acids in the ether phase were determined.

Recovery experiments were done first on mixtures of amino acids made up in proportions similar to those reported by Harper and others ('52) for plasma of normal young men. Then 2 ml of the standard amino acid mixture was substituted for 2 ml of water making 1:5 tungstic acid filtrates of pooled human plasma. This plasma was obtained by pooling the samples remaining after the laboratory work for the San Mateo project on the Nutritional Status of the Aging (Gillum et al., '55) had been completed. Recoveries of $100 \pm 15\%$ were obtained for 15 amino acids; the values for methionine and tryptophan were always very low.

RESULTS AND DISCUSSION

The pooled human blood data. Table 1 shows values obtained from replicate analyses of the pooled human plasma together with values reported by Harper et al. ('52) who used microbiological methods, and Stein and Moore ('54) who used ion exchange columns. Their values have been recalculated to μM/ml. Differences might be expected due to three sources: the long storage of the plasma used in this experiment, the fact that Harper et al. and Stein and Moore reported values for "normal young men" while the subjects of the Nutritional Status Survey were both men and women over 50 years of age, and the fact that blood was taken from them, in most cases, two hours after a meal, whereas subjects used in the other investigations were in the postabsorptive state. Wynne and Cott ('56) found that of 6 amino acids determined microbiologically in unhydrolyzed human plasma only tyrosine and arginine were significantly different in blood obtained in the fasting state or one or three hours after food intake.

However 7 of the values obtained by chromatography were either substantially in agreement with one or the other of those reported by the other methods or intermediate between them. Four were higher than those reported by Harper et al. which,

in turn, were higher than those of Stein and Moore and two were lower than those reported by the latter. Both glutamine and cystine have been found unstable in frozen storage. The frozen plasma contained little of either of these substances but a high level of glutamic acid, presumably a residue of glutamine. The pooled blood analyses were made primarily for testing recoveries of added amino acids and the results shown in table 1 are offered only to indicate the range of free amino acid concentrations obtainable by three different methods.

TABLE 1

Amino acid in human blood plasma as determined by three different methods

AMINO ACID	METHOD USED		
	Dinitrophenol	Microbiological [1]	Ion exchange [2]
	μM/ml	*μM/ml*	*μM/ml*
Lysine	0.262 ± 0.005 [3]	0.252	0.186
Leucine and isoleucine	0.204 ± 0.012	0.342	0.212
Glycine	0.326 ± 0.016	0.388	0.205
Phenylalanine	0.115 ± 0.008	0.121	0.051
Tryptophan	[4]	0.085	0.054
Histidine	[4]	0.136	0.074
Serine	0.158 ± 0.013	0.132	0.107
Threonine	0.130 ± 0.007	0.173	0.117
Valine	0.259 ± 0.007	0.276	0.246
Alanine	0.549 ± 0.028	0.445	0.383
Glutamine	0.272 ± 0.020	0.514	0.568
Proline	0.277 ± 0.012	0.227	0.205
Glutamic acid	0.433 ± 0.018	0.061	0.048
Aspartic acid	0.092 ± 0.006	0.025	0.002
Tyrosine	0.045 ± 0.004	0.073	0.057
Cystine (and cysteine)	0.007 ± 0.002	0.130	0.049
Methionine	[4]	0.038	0.026
Ornithine	0.209 ± 0.006	Not reported	0.054
α-NH_2-butyric acid	0.017 ± 0.004	Not reported	0.029
Glutamine and Glutamic acid	0.715	0.575	0.616

[1] Harper et al. ('52).

[2] Stein and Moore ('54).

[3] Standard error of the mean.

[4] Recoveries very poor.

The growth experiment. Results of the growth experiment confirm those of Beuk, Chornock and Rice ('49) in that the weight gained by the weanling rats fed pork autoclaved for 15, 30, or 60 minutes at 120°C did not differ significantly from that gained by the group fed raw meat, but the weight gains of the groups fed pork autoclaved 120 or 195 minutes were significantly less (table 2). Protein efficiencies of the samples were related in the same way. In this case, the difference cannot be due to differences in the amount of protein consumed by the rats of different groups, since all the animals were fed enough meat to provide 5 gms of protein per week and ate it willingly.

TABLE 2

Growth of weanling male rats in 4 weeks on diets furnishing 5 gm of protein per week as either raw or autoclaved fresh pork (7 rats per group)

DURATION OF AUTOCLAVING	AVERAGE GAIN IN 4 WEEKS	GAIN PER GM PROTEIN	SIGNIFICANCE	LOSS OF EFFICIENCY
min.	*gm*	*gm*		*%*
0	41.7 ± 1.6 [1]	2.08		
15	41.0 ± 2.3	2.05	N.S.	
30	36.7 ± 1.8	1.83	$p < 0.1, > 0.05$	12
60	37.1 ± 1.6	1.85	$p < 0.1, > 0.05$	12
120	34.1 ± 1.6	1.70	Significant, $p < 0.01$	18
195	32.6 ± 1.8	1.63	Significant, $p < 0.01$	22

[1] Standard error of the mean.

Amino-acids in portal blood plasma of young rats. All the amino acids determined showed much smaller increases above fasting levels in portal blood plasma of the young rats fed pork autoclaved for 195 minutes than in that of the young rats fed raw meat. Percentage increases over fasting levels for representative amino acids in plasma of portal blood taken at the half hour interval are shown for these young rats in figure 1. The increases in the young rats fed raw pork were from 101 to 340%, average 208, in those fed autoclaved pork from –33 to 95%, average 48.

Amino acids in portal blood of adult rats Table 3 shows concentrations of leucine + isoleucine (which do not separate by this method), phenylalanine, and alanine in plasma of

TABLE 3

Concentrations in μM/ml of representative amino acids in plasma of portal blood taken from adult rats [1] after feeding raw or autoclaved [2] fresh pork

AMINO ACID	LEUCINE AND ISOLEUCINE				PHENYLALANINE				ALANINE			
SEX OF RATS	Male		Female		Male		Female		Male		Female	
MEAT FED	Raw	Autoclaved	Raw	Autoclaved	Raw	Autoclaved	Raw	Autoclaved	Raw	Autoclaved	Raw	Autoclaved
Hrs. after feeding												
23–28 (fasting)	0.155	0.185	0.189	0.214	0.058	0.054	0.047	0.058	0.484	0.469	0.432	0.545
1/2	0.313	0.337	*0.539	0.377	0.082	0.062	*0.108	0.081	0.846	*0.976	*1.407 [3]	0.955
3/4	*0.557 [3]	0.325	0.315	0.355	*0.111	0.079	0.066	0.082	*1.521	0.882	0.904	*1.032
1	0.292	0.285	0.478	0.402	0.076	0.064	0.083	0.078	1.082	0.762	1.353	0.971
1 1/2	0.316	0.346	0.479	*0.424	0.066	0.075	0.099	0.076	0.887	0.898	1.298	1.010
2	0.273	*0.360	0.345	0.406	0.059	0.081	0.069	*0.086	0.830	0.675	0.995	0.919
3	0.271	0.259	0.387	0.376	0.068	0.080	0.076	0.073	0.680	0.504	0.994 [3]	0.853
4	0.305	0.270	0.362	0.420	0.072	0.078	0.064	0.867	0.867	0.625	0.949	0.859
5	0.307	0.341	0.317	0.388	0.092	*0.096	0.063	0.080	0.904	0.752	0.884	0.808

[1] Three to 5 rats in each group.

[2] Four hours at 120°C.

[3] Significantly higher than for autoclaved.

* Maximum concentration.

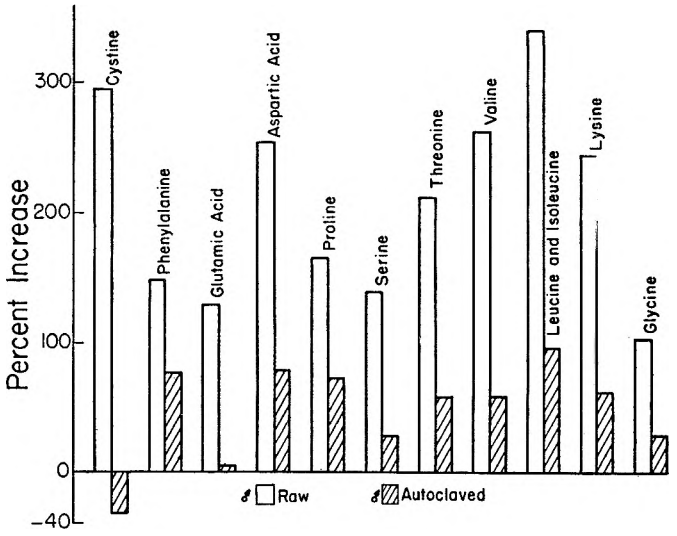

Fig. 1 Percentage increases in free amino acid concentrations in the portal blood plasma of young male rats given 5 gm of raw pork or pork autoclaved 195 minutes one-half hour before sacrifice over corresponding levels in portal blood of similar but fasting rats.

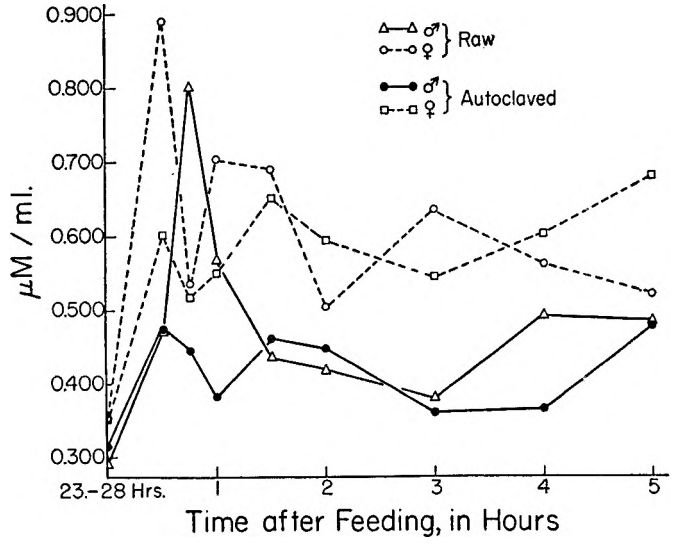

Fig. 2 Concentration of lysine in plasma of portal blood of adult male and female rats fasting 23 to 28 hours or one-half to 5 hours after being given 5 gm of raw pork or pork autoclaved at 120°C for 4 hours.

portal blood taken from adult rats after feeding them raw fresh pork or the same pork autoclaved for 4 hours at 120°C. Figures 2 and 3 present similar values for the portal blood content of lysine, valine and threonine in graphic form. Higher levels of most of the amino acids were found in the blood of females than in that of males at all times except at

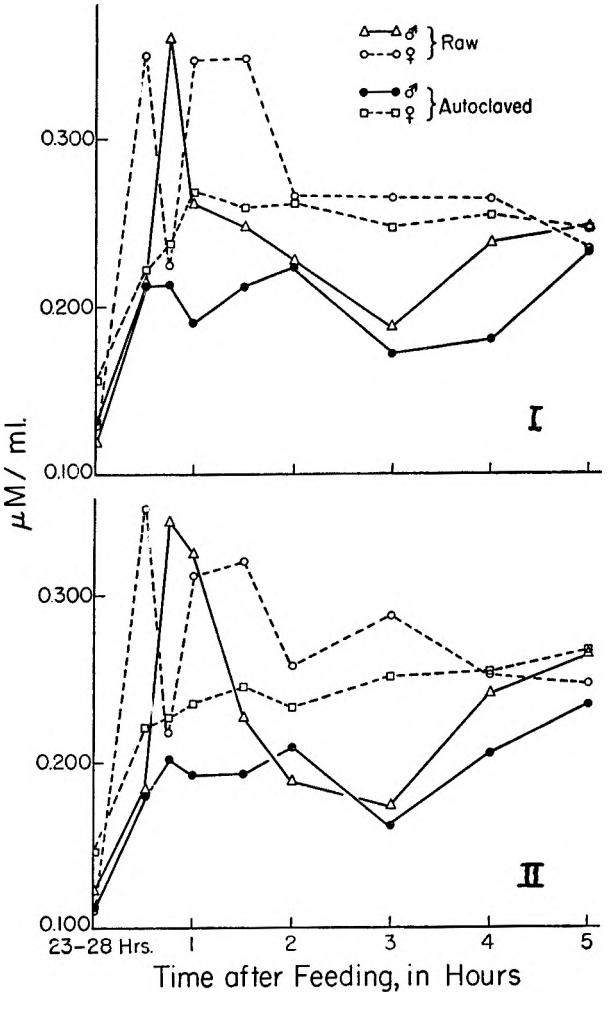

Fig. 3 Concentrations of valine and threnonine in plasma of portal blood of adult rats treated as described in figure 2. I. Valine. II. Threonine.

the peak of absorption in the males, three-fourths hour after feeding the raw meat, and sometimes in the fasting blood samples or at the 5-hour interval. Results for the other 9 amino acids determined show a similar picture. Standard errors have been omitted from the table and the graphs to save space, but in most cases were between 10 and 30%.

TABLE 4

Time of greatest increases over fasting levels in concentrations of individual amino acids in plasma of portal blood taken from adult rats after feeding raw fresh pork or fresh pork autoclaved at 120°C for 4 hours

AMINO ACID	MALES		FEMALES	
	Raw meat fed	Autoclaved meat fed	Raw meat fed	Autoclaved meat fed
	Hours	*Hours*	*Hours*	*Hours*
Cystine	3/4	5	1/2	3/4
Tyrosine	3/4	1/2	1/2	1/2
Phenylalanine	3/4	5	1/2	4
Glutamic acid	3/4	2	1/2	4
Aspartic acid	3/4	5	1/2	3 & 5
Ornithine	3/4	1/2	1/2	5
Serine	3/4	5	1/2	1/2
Threonine	3/4	5	1/2	5
Leucine + isoleucine	3/4	1½	1/2	1½
Valine	3/4	5	1/2,1 1½	1
Proline	3/4	2	1/2 & 1½	1
Lysine	3/4	1/2 & 5	1/2	5
Glycine	3/4	2	1½	3/4
Glutamine	3/4	2	1/2	1½
Alanine	3/4	1/2	1/2	3/4

Although the individual variations among the animals lead to few statistically significant differences when groups of the same sex fed the raw and autoclaved meat are compared, two trends appear which indicate that a biologically significant difference is in fact present. The first is the consistently lower level of all the amino acids in blood from animals fed autoclaved meat for the first two hours after the meal. The second is the fact that whereas the peak of the absorption curve of all

the amino acids is at three fourths hour after the adult males were fed raw meat and at one half hour (except for glycine) for the corresponding group of females, peaks of the curves for both males and females fed autoclaved pork are by no means at the same time for all the amino acids, but vary from one-half hour (e.g., tyrosine) to 5 hours (lysine and threonine) (table 4). This variability in time of peak values and the generally lower levels attained after the autoclaved meat was fed could very well explain the failure of young animals to grow well on overheated proteins. It has been demonstrated (e. g., Geiger, '47) that all the essential amino acids must be present at the same time and in the proper concentrations to promote good growth of young animals or for repletion and maintenance of adults.

If the data for the adult animals are expressed as percentage increase over the fasting level, differences between the groups become even more striking and consistent. In figure 4 average percentage increases of representative amino acids over average fasting levels are shown for the adult rats at the times at which maximum concentrations of all the amino acids were observed in the blood of the animals fed raw meat. These rises were from 48 to 294%, average 187, in the animals fed raw pork, and from −4 to 88%, average 48, in those fed the autoclaved meat.

Comparison of the differences between percentage increases in plasma of portal blood taken from animals fed the two kinds of pork with the data of Beuk et al. ('49), showing amino acids released from autoclaved pork by digestion with trypsin and erepsin as percentages of those released from the raw sample, shows that the differences encountered in the plasma are much greater than those *in vitro*. Threonine, for instance, was liberated from pork autoclaved for 4 hours in 57.5% as great a quantity as from the raw meat, *in vitro*. The percentage increases of threonine in the portal blood plasma of animals fed autoclaved pork were only 27, 44, and 23% of those following the ingestion of raw meat by the immature males, adult males and adult females, respectively (figures 1 and 4).

This variability in time of peak values for the amino acids in plasma of portal blood during digestion of autoclaved pork as contrasted with the constancy in time of peak values after raw pork, along with the consistently lower percentage

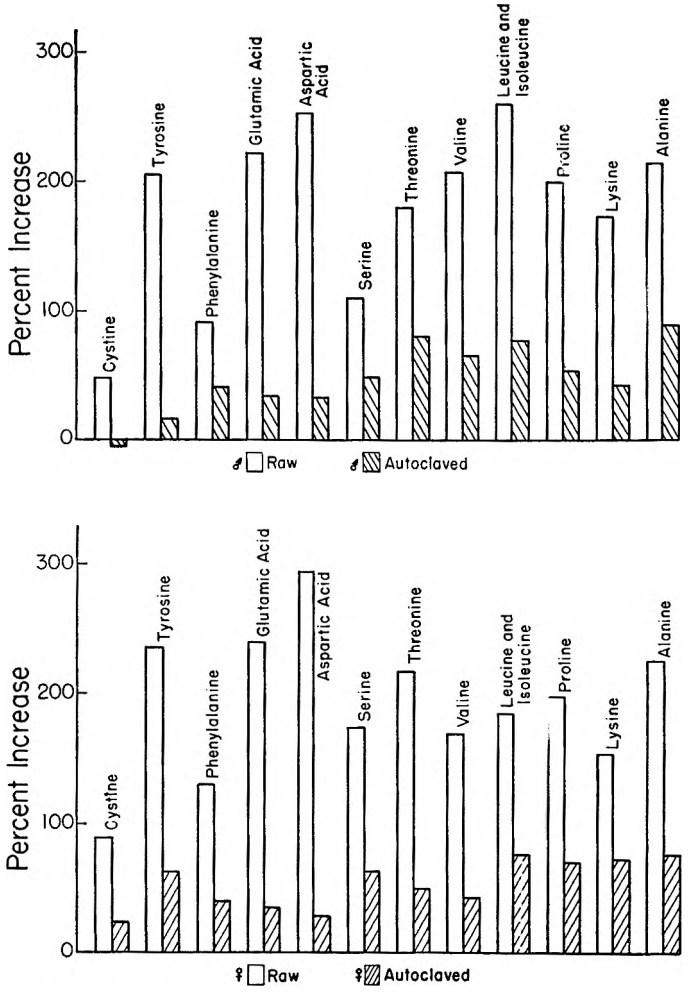

Fig. 4 Percentage increases in concentrations of certain amino acids in portal blood plasma of adult male and female rats given 5 gm of raw pork or pork autoclaved at 120°C for 4 hours one-half hour (females) or three-fourths (males) before sacrifice, over corresponding levels in portal blood of similar but fasting animals.

increases at the same time in the rats fed autoclaved meat, support the theory that overheating, of pork protein at least, causes a decrease in the biological value of the protein because the amino acids are not simultaneously available to the animal in sufficient quantities or in the right proportions for good growth or for the maintenance of tissues.

SUMMARY

Fifteen amino acids have been determined by quantitative paper chromatography of their dinitrophenyl derivatives in plasma of portal blood taken from immature male and from adult male and female rats at various time intervals after feeding them raw or severely autocleaved fresh pork. Rats fed the raw pork exhibited a prompt increase of free amino acids in plasma from portal blood. The highest values of the absorption curve were at three-fourths hour after feeding in the adult males, and, except for glycine, at one-half hour in the adult females. Only the one-half hour time interval was studied with the young rats, after which those fed raw pork showed an increase of 101 to 340% for all amino acids studied. The adult animals had rises of 48 to 294% at the peak of absorption after ingestion of the raw meat. Both young and adult rats fed severely autocleaved pork showed much smaller increases, –33 to 95%, and in the adult rats the highest values for the different amino acids were scattered over the whole time up to and including 5 hours, the last time interval studied.

The results are interpreted as supporting the theory that decreases in biological value caused by overheating proteins are at least in part due to failure of digestive enzymes to hydrolyze the overheated proteins so as to make available simultaneously to the animal an assortment of amino acids favorable to good growth or maintenance.

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THE NUTRITIVE VALUE OF BREAD FLOUR PROTEINS AS AFFECTED BY PRACTICAL SUPPLEMENTATION WITH LACTALBUMIN, NONFAT DRY MILK SOLIDS, SOYBEAN PROTEINS, WHEAT GLUTEN AND LYSINE

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The original observation by Osborne and Mendel (*cf.* Mendel, '23) that lysine is the amino acid which limits the nutritive value of wheat proteins for growing animals has been amply confirmed. Although bread flour contains sufficient total protein (13% of the Calories), less than one-half of this protein is available to the animal for the formation of new tissue (Block and Mitchell, '46–'47). The recognized protein inadequacy of wheat flour is, therefore, not the result of a lack of total protein per calorie but is due solely to the low biological value of the wheat proteins. As early as 1917, Osborne and Mendel said that small additions of the more efficient proteins actually *supplement* an inadequate cereal protein, " . . . instead of themselves furnishing all of the protein used for growth, because equivalent amounts of these proteins alone in a similar ration are incapable of inducing a comparable degree of growth. Small amounts of a superior protein

are often just as efficient for growth as larger amounts of a less adequate protein.'' Thus, for the greatest economy an attempt should be made to achieve a combination of proteins which will be as complete as possible in amino acids at a minimum percentage of protein in the diet. There are often other considerations which make it desirable to feed smaller quantities of a well balanced or ''complete'' protein rather than larger amounts of a poorly balanced pattern of amino acids. Among these are conditions in which the total protein intake must be limited, including low-calorie diets. In the latter instance, sufficient protein must be ingested to replace the ''wear and tear'' losses and to furnish amino acids for new tissue formation. The surplus amino acids which are not utilized by the organism for these specific purposes are potential sources of body fat.

Many of the previous experiments on the supplementary effects on wheat proteins of other proteins and of lysine have been reviewed recently (Allison, '57; Block, '56; Mitchell, '54). Theoretically, the supplementation may be effected by adding sufficient lysine to the flour until a second amino acid deficiency (e.g. isoleucine) becomes the limiting factor. Then lysine and isoleucine may be added until a third deficiency appears and so forth. This procedure is, at the present time, relatively expensive and limited, in practice, to the addition of lysine. Several groups of investigators have deprecated the use of single amino acids to increase the nutritive value of wheat and other food proteins (Jahnke and Schuck, '57). It is quite true that excessive quantities of an added amino acid can actually reduce the nutritive value of a protein (Block et al., '56). However, we are of the opinion that the widespread criticism of the use of amino acid supplementation based upon the above mentioned findings is unjustified. Examination of the experiments in which the addition of a single amino acid depressed growth will show that in every case either the total protein intake was very low (less than 10% of the Calories) or that a very excessive amount of the supplementary amino acid was added. There does not appear to be any doubt that the *pattern*

of amino acids furnished in the dietary is the crux of optimal nutrition and that inferior results are obtained by either a deficiency or an excess of the essential amino acids and in some cases even by an excess of the dietary dispensable amino acids (Block and Bolling, '44; Nasset, '57; Sauberlich, '56). However, much of the criticism of the inclusion of a single amino acid in foods or feeds is unrealistic because no food manufacturer, for economic reasons, would add an excess of the supplementary amino acid. The second approach, generally used, is to add one or more proteins containing an *excess* of those amino acids which are present in suboptimal quantities in wheat proteins. This method of supplementation, which does not, *per se,* exclude simultaneous addition of one or more individual amino acids, has two effects: the first is to increase the total protein of the food; the second is to make a greater proportion of the deficient protein available to the organism for new tissue formation and the repair of metabolic losses.

The foregoing discussion tacitly assumes that what is desired is a protein mixture which will supply the optimal proportions of amino acids. *The composition of this ideally balanced "complete protein" is not known.* However, from the evidence available on the amino acid composition of various adequate and inadequate diets, of animal tissues, of food proteins, especially whole egg, and of the minimal amino acid requirements of many species of animals, the authors have *tentatively* assumed that a "complete protein" should contain 5.3 gm of lysine per 16.0 gm of N (Allison, '57, Block, '56). This value forms the basis for the term "complete protein" used throughout this paper. The secondary and tertiary amino acid deficiencies of wheat flour (Block and Mitchell, '46-'47) will not be discussed as the experimental design was such as to exclude any but a lysine deficiency. [1]

[1] The term "deficiency" has generally been used to express a lack of an essential amino acid or other nutrient, while "imbalance" has been used to indicate a relative surplus of one or several amino acids (cf. Harper, '56). Rerat et al, '56 suggest that an amino acid can be limiting either by deficiency or by excess and is most effective in nutrition at an intermediate level depending on the relative proportion of the other amino acids in the diet.

The supplemental value of a food protein for another food may be considered as arising from two factors: (A) the quantity of "complete protein," and (B) the "extra" quantity of the first limiting amino acid furnished by the supplement.

In the particular case of wheat products, including bread, in which the limiting amino acid is lysine, the supplemental value of a food protein is given by

$$S = Q \frac{L}{5.3},$$

where

S = the supplemental value per 100 gm,[2]
Q = the percentage of crude protein (N x 6.25) in the supplement,
L = the grams of lysine per 16.0 gm N in the supplemental protein food.

The supplementary value of any combination of two food proteins may also be calculated by the following formula:

$$S_t = S_1 (X) + S_2 (1 - X)$$

where

S_t = the supplementary value of the total product,
S_1 = the supplementary value of the first protein,
S_2 = the supplementary value of the second protein, and
X = the fraction of S_1 in the mixture.

Supplemental values for the proteins used in this study are shown in table 1. These calculations show clearly that on a weight basis lysine hydrochloride is, as would be expected, the supplement of choice within the limitations described above. Nonfat dry milk solids have limited supplemental value since

[2] The supplementary value (S) may be illustrated as follows: One part of lactalbumin, used as a supplement to 100 parts of wheat flour, makes an effective contribution of 1.49 parts of "complete protein;" one-tenth part of lysine hydrochloride contributes 1.38 parts of "complete protein;" while one part of wheat gluten contributes only 0.22 parts of "complete protein."

Since wheat flour proteins are about 42% "complete" a flour containing 12.5% crude protein (N x 6.25) would provide 5.2% "complete protein." A mixture of 100 lbs. of such flour supplemented with two pounds of lactalbumin would contain 13.7% crude protein and 8.0% "complete protein." The original values would be increased 10 and 54% respectively. A mixture of two pounds of wheat gluten with 100 lbs. of flour contains 13.9% of crude protein and 5.5% of "complete protein." In this case the crude is increased 13%, but the "complete protein" is increased by less than 6%. This is explained by the fact that whereas the proteins of the original flour were 42% "complete," the addition of gluten has produced a protein combination which is less than 40% "complete."

the protein concentration is low. Isolated soybean proteins provide a concentrated source of "complete protein," but supply so little extra lysine that their supplementary value is also rather limited. Lactalbumin has a very high supplementary value because, in addition to the added "complete protein" which it furnishes, it also provides a relatively large amount of *extra* lysine. The futility of adding wheat gluten to bread from the point of view of protein nutrition is apparent.

TABLE 1

The relative supplementary value [1] *of four food proteins and lysine for wheat flour*

PROTEIN SOURCE	CRUDE PROTEIN (N x 6.25) Q	SUPPLEMENTAL VALUE S
Lactalbumin [2]	75	149
Nonfat dry milk solids [3]	36	58
Soybean protein [4]	89	101
Wheat Gluten [5]	84	22
Lysine monohydrochloride [6]	96	1380

[1] See footnote 2, page 154.
[2] Borden, Labco.
[3] Borden, Starlac.
[4] Glidden, Promine.
[5] A. S. Hoyt, Inc.
[6] du Pont, Darvyl, 95% L form.

The experiments to be described were carried out to test the validity of the assumptions made in this introduction with respect to the supplementation of wheat flour with lysine, lactalbumin, milk proteins, soybean proteins, and wheat gluten.

EXPERIMENTAL

A preliminary feeding trial with weanling rats was conducted in which it was established that the protein in a high-protein flour [3] and that in a standard low-protein, 72% extraction flour [4] were equal in nutritive value when fed at equal levels of nitrogen. This was demonstrated by dilution of the high-protein flour with wheat starch. Both flours were incor-

[3] "Energetic," Electric Steel Company, Duluth, Minn., nitrogen = 2.7%.
[4] "Pillsbury's Best," nitrogen = 2.0%

porated into diets to provide 10.4% protein (N x 6.25) and adequate quantities of vitamins, minerals, etc. were provided as described below.

The results of the feeding tests are in agreement with the finding that the lysine content of the proteins in the two types of flour is the same within the experimental error of the analytical method. (Block and Mandl, '57.)

Diets

Six protein sources were mixed into diets to provide three different levels of dietary protein (N x 6.25) from each source. Each diet contained 4% corn oil, 4% USP XIV salts, and adequate quantities of vitamins.[5] The quantity of wheat starch in each ration was adjusted so that all diets were essentially isocaloric. Thus the only variables in the diets were the source and quantity of protein and the quantity of wheat starch, as shown in table 2.

The composition of the protein sources is shown in table 3.

The lysine values shown in table 3 are in agreement with values calculated from published data for the individual components (Block and Bolling, '51; Block and Weiss, '56).

Experimental plan and results

One hundred forty-four weanling male rats, weighing 45 to 50 gm each, were randomly distributed among 18 groups of 8 rats each. The animals were housed in individual cages. The rats assigned to each group received, ad libitum, one of the experimental diets and water. Individual records were kept

[5] Vitamin Supplement per 100 gm of diet:

2-Methyl-1, 4-naphthoquinone	1.0 mg	*p*-Aminobenzoic acid	4.0 mg
Thiamine·HCl	0.8 mg	Choline bitartrate	400.0 mg
Riboflavin	1.6 mg	Inositol	21.6 mg
Pyridoxine·HCl	0.8 mg	Vitamin B_{12}	3.3 μg
Nicotinic acid	4.0 mg	Folic acid	0.1 mg
d-Calcium pantothenate	4.4 mg	Biotin	30.0 μg

Three drops of oil containing Vitamin A (4000 I.U./ml), vitamin D (800 I.U./ml) and vitamin E (4.0 mg./ml) were fed separately once a week to each rat.

TABLE 2

Composition of diets

CODE	COMPOSITION	PROTEIN SOURCE	WHEAT STARCH	CRUDE PROTEIN	CALCULATED AMOUNT OF "COMPLETE PROTEIN"
		%	%	%	%
F_{15}	Flour	92.00	...	15.2	6.3
F_{12}	Flour	74.05	17.95	12.6	5.2
F_{10}	Flour	59.20	32.80	10.9	4.5
L_{15}	Flour + lactalbumin	86.85	5.15	15.7	8.6
L_{12}	Flour + lactalbumin	69.9	22.10	12.7	7.0
L_{10}	Flour + lactalbumin	55.9	36.10	10.1	5.5
S_{15}	Flour + soybean protein	86.4	5.60	15.5	7.3
S_{12}	Flour + soybean protein	69.5	22.5	12.3	5.8
S_{10}	Flour + soybean protein	55.6	36.4	10.7	5.0
M_{15}	Flour + nonfat dry milk solids	90.05	1.95	15.9	8.4
M_{12}	Flour + nonfat dry milk solids	72.45	19.55	12.5	6.6
M_{10}	Flour + nonfat dry milk solids	57.95	34.05	10.8	5.7
Lys_{15}	Flour + lysine	92.0	...	15.2	8.3
Lys_{12}	Flour + lysine	74.05	17.95	12.5	6.9
Lys_{10}	Flour + lysine	59.20	32.80	10.1	5.5
G_{15}	Flour + wheat gluten	88.15	3.85	15.5	5.6
G_{12}	Flour + wheat gluten	70.95	21.05	12.8	4.6
G_{10}	Flour + wheat gluten	56.75	35.25	10.3	3.7

TABLE 3

Composition of protein sources

CODE NO.	PROTEIN SOURCE	PROTEIN (N x 6.25)	LYSINE [1]
		%	*gm/16 gm N*
F	100 parts high protein flour (HPF)	16.7	2.2
L	100 parts HPF + 2 parts lactalbumin	17.8	2.9
S	100 parts HPF + 2 parts soybean protein	18.2	2.5
M	100 parts HPF + 4 parts nonfat dry milk solids	17.4	2.8
Lys	100 parts HPF + 0.15 parts commercial L-lysine · HCl	16.8	2.9
G	100 parts HPF + 2 parts wheat gluten	18.0	1.9

[1] Determined by quantitative paper chromatography (Block and Weiss, '56).

of weekly weight gains and food consumption. After 28 days on the experimental diets all animals were sacrificed, the entire gastrointestinal tract and contents were removed, and the carcasses frozen. The carcasses of all animals from each dietary group were then pulverized and thoroughly mixed.

TABLE 4

Carcass composition

GROUP [1]	MOISTURE		FAT		PROTEIN	
	%	gm	%	gm	%	gm
F_{15}	64.0	54.6	12.2	10.5	18.5	15.9
F_{12}	64.0	44.7	12.1	8.4	18.6	12.9
F_{10}	65.1	40.6	11.0	6.9	18.1	11.3
L_{15}	61.9	71.5	16.1	18.6	17.3	20.0
L_{12}	62.9	56.0	14.7	13.1	18.3	16.3
L_{10}	63.4	50.4	13.8	11.0	17.6	14.0
S_{15}	62.5	65.6	15.1	15.9	18.5	19.4
S_{12}	62.1	56.1	15.8	14.3	18.0	16.3
S_{10}	64.0	45.4	13.0	9.2	18.4	13.1
M_{15}	63.0	70.3	15.2	17.0	18.1	20.2
M_{12}	63.6	55.4	13.9	12.1	18.5	16.1
M_{10}	63.5	49.4	13.4	10.4	18.0	14.0
Lys_{15}	62.6	77.4	16.1	19.9	17.2	21.2
Lys_{12}	63.8	61.6	14.6	14.1	17.6	17.0
Lys_{10}	65.4	53.2	12.9	10.5	17.2	14.0
G_{15}	65.2	56.9	14.0	12.2	16.7	14.6
G_{12}	64.7	46.5	13.1	9.4	17.5	12.5
G_{10}	66.5	41.8	10.5	6.6	17.7	11.2

[1] See table 2 for composition of the diets for these groups.

Aliquots from each pooled sample were analyzed in triplicate for moisture, fat, and protein (table 4).

Initial and final weights, weight gains and food consumption of each group for the 4-week experimental period are given in table 5, along with the quantities of crude protein and "complete protein" consumed and the average daily gain in per cent.

DISCUSSION

It is apparent from table 5 that there is an increase in weight gain with increasing protein intake and with increase in the level of dietary protein with each protein source. This simple relationship, however, does not hold true for different protein sources.

TABLE 5

Weights, weight gains and food consumption in a four-week period

GROUP [1]	WEIGHT			AV. DAILY GAIN	CONSUMPTION		
	Initial	Final	Gain		Total Food	Crude Protein	Complete Protein
	gm	*gm*	*gm*	*%*	*gm*	*gm*	*gm*
F_{15}	48.4	84.1	35.8	1.93	228	34.4	15.2
F_{12}	48.4	69.5	21.1	1.32	196	24.7	10.7
F_{10}	48.3	62.3	14.0	0.91	185	20.2	8.7
L_{15}	48.1	115.5	67.4	3.06	288	45.2	24.8
L_{12}	48.3	89.1	40.9	2.12	246	31.2	17.1
L_{10}	48.4	79.5	31.1	1.77	223	22.5	12.3
S_{15}	48.4	105.0	56.6	2.64	270	41.8	19.8
S_{12}	48.5	90.4	41.9	2.15	248	30.5	14.4
S_{10}	48.3	71.0	22.8	1.37	208	22.3	10.4
M_{15}	48.3	111.6	63.4	2.81	281	44.6	23.5
M_{12}	48.3	87.1	38.9	2.12	236	29.5	15.6
M_{10}	48.0	77.8	29.8	1.70	229	24.8	13.1
Lys_{15}	48.3	123.5	75.5	3.14	299	45.5	24.9
Lys_{12}	48.9	96.6	47.8	2.35	258	32.3	17.8
Lys_{10}	48.4	81.3	32.8	1.81	228	23.0	12.5
G_{15}	47.8	87.3	39.4	2.08	251	38.9	14.1
G_{12}	48.4	71.8	23.4	1.39	200	25.6	9.18
G_{10}	48.7	62.9	14.3	0.95	180	18.5	6.64

[1] See table 2 for composition of the diets for these groups.

Thus, rats receiving a diet containing approximately 12% protein from flour supplemented with lactalbumin, soybean, or lysine grow more rapidly than those receiving a diet containing 15% protein from flour alone or flour supplemented with wheat gluten.

Since growth rate and food intake are not independent of each other, little is gained by calculating efficiency ratios from

these terms. A more informative approach seems to lie in the correlation of protein dietary levels with growth. A graphic representation of the average percentage weight gain per day versus the dietary protein level for both crude and "complete protein" is shown in figure 1.

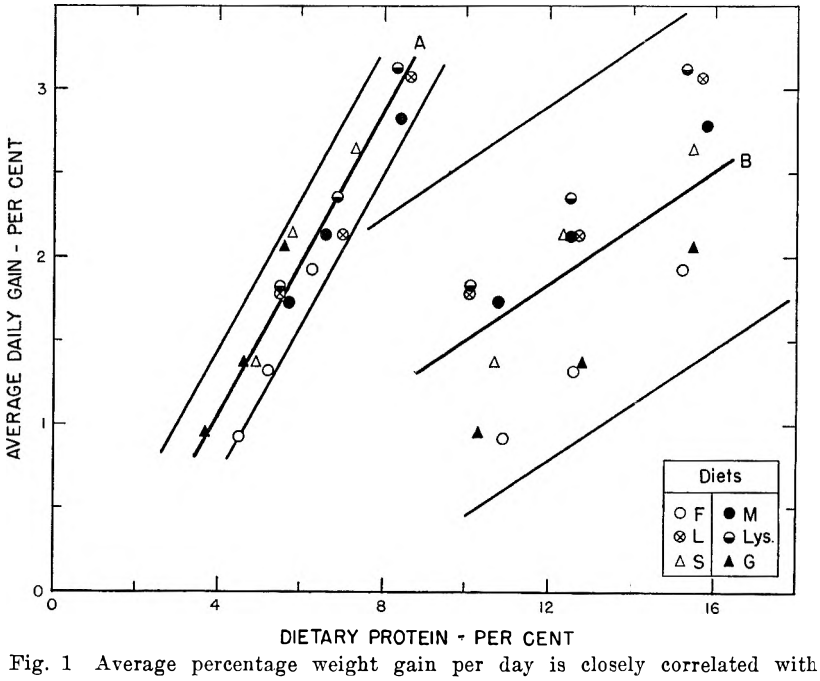

Fig. 1 Average percentage weight gain per day is closely correlated with the dietary level of "complete protein" (A) and to a less extent with the dietary level of crude protein (B).

The regression line for daily gain (y) on dietary level of "complete protein" (x) is given by $y = 0.452\,x - 0.77$, and on dietary level of crude protein (x) by $y = 0.167\,x - 0.16$.

As may be seen from the graph, the correlation between growth rate and "complete protein" is much better than that between growth rate and crude protein. The correlation coefficients(r) are calculated to be 0.96 and 0.55 respectively and the corresponding standard errors of estimate are (Sy) 0.18 and 0.53. The fine lines on the graph have been drawn at

$\pm$ 2 Sy and represent the range within which 95% of the values may be expected to fall.

This high degree of correlation between growth rate and dietary level of "complete protein" is in entire accord with the views expressed in the introductory portion of this paper and confirm that lysine is the primary limiting amino acid in white flour alone and in combination with practical levels of supplementary proteins or lysine.[6]

In other words, the results indicate that the rate of growth of weanling rats is directly related to the dietary level of "complete protein" when white flour supplies the major part of the diet. Furthermore, the inclusion of proteins which provide non-supplementary essential amino acids as well as excess quantities of dietary dispensable amino acids (i.e. all amino acids except lysine) do not *per se* influence the rate of growth.

It is of interest to note that food intake is also closely correlated with the dietary level of "complete protein" and to a far less extent with the dietary level of crude protein. The correlations are given by the regression equations $y = 23.5\ x + 9.16$ and $y = 10.0\ x + 108$ for complete and crude proteins respectively. These relationships are plotted in figure 2 in which the regression equations are represented by the heavy lines and the fine lines are two standard errors of estimate distant from the regression lines.

The regression coefficients (r) for "complete protein" and crude protein are 0.96 and 0.62 and the standard error of estimate (Sy) 10.5 and 26.2 respectively.

[6] It should be noted that the degree of correlation observed between dietary level of "complete protein" and growth rate (food intake, carcass protein content) would remain unaffected by moderate revision of the numerical value chosen for the lysine content of a "complete protein." The growth response obtained in the present investigation can readily be represented graphically as a function of the dietary lysine level; however, the use of the concept "complete protein" makes possible a direct comparison with crude protein since these terms can be plotted on a common axis in the same units.

The concept of "complete protein" also facilitates comparisons between foods which differ in their limiting amino acids. Thus it is possible to compare quantitatively, in terms of "complete protein," dissimilar foods such as bread and bean soup, in which the limiting amino acids are lysine, and cystine-methionine, respectively.

The total carcass protein is plotted against dietary level of protein in figure 3.

In this instance also, a better correlation is obtained when dietary "complete protein" is used rather than crude protein. The regression data for "complete protein" are $y = 2.01\ x + 3.28$, $r = 0.97$, $Sy = 0.69$ and for crude protein, $y = 0.955\ x + 2.9$, $r = 0.71$, $Sy = 1.98$.

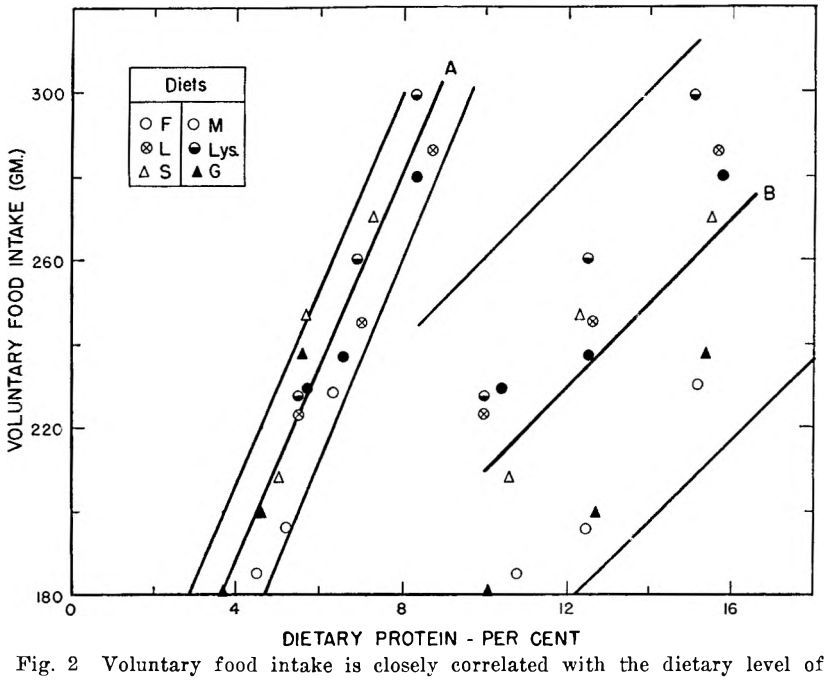

Fig. 2 Voluntary food intake is closely correlated with the dietary level of "complete protein" (A) and to a less extent with the dietary level of crude protein (B).

As is evident from figures 1, 2 and 3, and the data given in the text, it is possible to predict, from the dietary level of "complete protein," the growth rate, the voluntary food intake, and the protein content of the carcass with remarkable precision. In contrast, the dietary level of crude protein lacks predictive value. Thus, the growth rate, food intake and carcass protein content can be calculated ($P < 0.05$) within $\pm$ 18, 9 and 9% respectively from the "complete protein" content

of the diet, while from the crude protein level the same three terms can be estimated only within ± 53, 21 and 19% respectively.

The particular quantitative relationships shown above obviously hold true only under these experimental conditions.

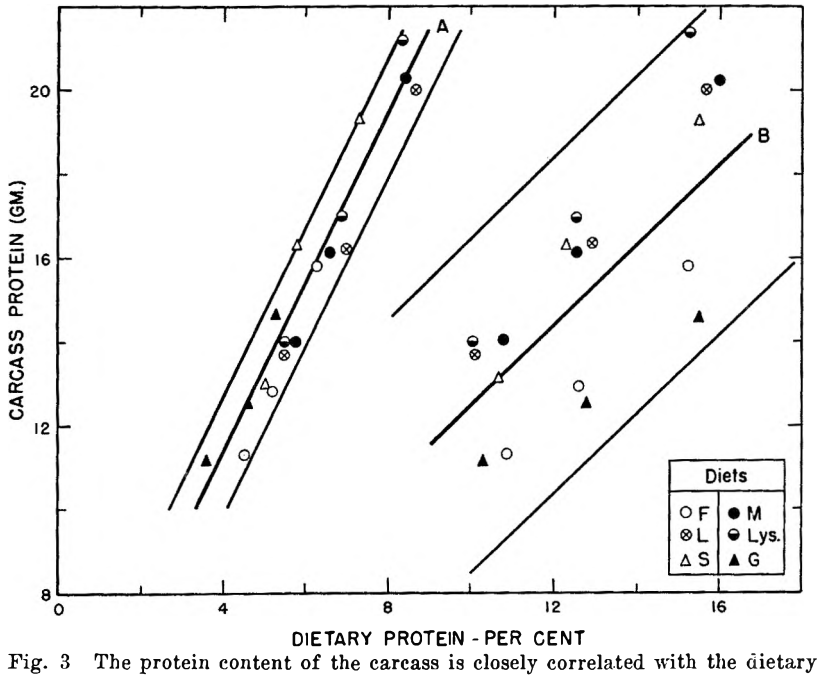

Fig. 3 The protein content of the carcass is closely correlated with the dietary level of "complete protein" (A) and to a less extent with the dietary level of crude protein (B).

Nevertheless, the superiority of "complete protein" over crude protein as a nutritional index would be expected to be generally valid in any situation in which an amino acid is the limiting nutrient of the diet.

Under conditions such that some other nutrient (calories, vitamins, minerals, etc.) is limiting, one should be able to show a similar relationship by which the growth rate, voluntary food intake, and body composition could be predicted from the dietary "level of completeness." [7]

[7] By "level of completeness" is meant the nutrient content expressed as a fraction of the optimum requirement.

SUMMARY

1. The use of "complete protein" as an index of nutritional value is introduced and shown to be superior to that of crude or "total" protein.

2. Experimental results are reported which show a high degree of correlation between the dietary level of complete protein, the growth rate, voluntary food intake, and the protein content of the carcass.

3. Data are presented which confirm that lysine is the limiting amino acid in diets in which wheat flour supplies the major source of protein, and that the rate of growth is not affected by the inclusion of other amino acids furnished by the supplementary protein.

4. A method is proposed for calculating in numerical terms the supplementary value of one or more proteins for a food primarily deficient in a single essential amino acid.

5. The nutritional effects of supplementing wheat flour with lactalbumin, nonfat dry milk solids, soybean proteins, wheat gluten and lysine were investigated. The results accord with the assumption that a well-balanced or "complete protein" contains 5.3 gm of lysine per 16.0 gm of nitrogen.

6. Isonitrogenous diets in which high-protein or low-protein wheat flours supply all the protein, give equal lysine intakes and equal rates of growth.

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