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OTTO FOLIN

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(1867 – 1934)

In Volume xxvii of *Biographical Memoirs,* published by the National Academy of Sciences in 1952, two accounts of the career of Otto Folin will be found. One is the memoir of Folin written by the author of this paper; the other is included in the memoir of Stanley R. Benedict written by E. V. McCollum. The availability of these recently-published accounts of Folin's life and works, supplemented by his Bibliography and references to relevant data, permits brevity in this special tribute in his honor.

There could be no more fitting introduction to a sketch of Folin's life and work, addressed to readers of the Journal of Nutrition, than the following words written by E. V. McCollum in the opening paragraph of his memoir to Benedict:

"It is not possible to give an accurate account of the scientific work of Stanley Benedict without at the same time discussing the parallel history of the researches of Otto Folin. . . . It is the merit of these two men that they succeeded, through many years of intensive investigations, in devising and refining analytical procedures for the determination of minute amounts of the principal non-protein constituents of blood and urine so that, for the first time, chemical analysis became a highly useful technic for the discovery of the chemical processes in the normal functioning of the body. These new methods also advanced to an astonishing degree the effectiveness of studies of the chemistry of pathological metabolism. In the entire history of biological chemistry no two individuals excelled these men in achievement. In the application of chemistry to the solution of biological problems, their work opened a new era, so that, in respect to knowledge of metabolism, historians will discuss the state

of this department of science as it existed before and after Folin and Benedict."

The portrait reproduced as the frontispiece of this paper was painted from life in the spring of 1934, about 6 months before Folin's death. It hangs in the library of the laboratory at Harvard where he worked during the last 27 years of his life. This portrait, bearing an emblem of his many colorimetric methods, has become for historians a symbol of the era above referred to by McCollum.

It is of interest to ponder, in the light of the life history of pioneers who have altered the course of events, what factors were dominant in their success. We shall try to note such influences in the life of Folin.

Otto Knut Olof Folin was born April 4, 1867, in Asheda, a village in a rural province of southern Sweden. His parents were Nils Magnus Folin, the village tanner, and Eva Olson, who married when she was 16 years old. Otto was the last of 12 sons born to this couple; 8 of their children died in infancy or early childhood. The father's business did not prosper. Needs of the family and the toll of sickness and death among her children led the mother when still young to secure training in nursing and midwifery, and later to become licensed as midwife for the district. Her earnings aided much in supporting the home and provided for her youngest son three terms of additional schooling beyond the elementary grades of the village school, which had to suffice for her older sons.

It is clear from Otto's memory of his childhood that his mother was a woman of high courage, devotion and ability, whose character and ideals of usefulness to others left a deep impression on his own personality and ambitions.

At the age of 15, instead of being apprenticed for a trade at the nearest town, as were his brothers, Otto was sent to America to live with his brother Axel, who provided money for his passage and later aided him in emergencies. Axel was a lumberman at Stillwater, Minnesota. There Otto, the young Swedish immigrant, unable to speak English, began the strug-

gle to support himself by working at the St. Croix River log-boom while dreaming of ways to continue his schooling and to learn the English language. At that early age he seems to have acquired a determination to become an educated man and to seek a career of service. One likely source of that ambition was the example of his mother.

Six years later, at the age of 21, and while supporting himself by various jobs — as laborer in harvest fields in summer, night clerk and watchman in hotels at Stillwater during school terms — he was graduated from high school in that town. After securing a job in Minneapolis, he enrolled in the fall of that year (1888) as a student at the University of Minnesota. Four years later he was graduated with the B.S. degree. Reports of his record as a college student state that he did "more than average work in chemistry and showed marked ability in English." In 1890 he became a citizen of the United States. During his senior year he was an editor of the student paper "Ariel," and "was known among fellow students as an advocate of democratic policies in student affairs."

In the autumn of 1892 Folin was accepted as a graduate student at the University of Chicago. He chose chemistry as his major and physiology as his minor subject, perhaps forecasting his intent to become a physiological chemist. Neff was the professor of chemistry; Stieglitz, then a young member of Neff's staff, became Folin's faculty advisor and later directed his research for his dissertation. Jacques Loeb was the professor of physiology.

Doubtless Folin's knowledge of organic chemistry and other subjects was greatly extended and matured by study and laboratory experience during 4 years at Chicago, in close contact with such teachers. But it is the writer's impression from close acquaintance with Folin (beginning three years later) that he had not been as much or as deeply marked by points of view which characterized Neff, Stieglitz and J. Loeb as he was later influenced by his shorter contact with Hammarsten, Salkowski and Kossel. To the extent that this

impression is valid, it may help to explain a quality of freedom from bias, the desire to formulate — and to change — his own plans and the methods and purposes of his research which seem to characterize and largely to account for the pioneering aspect of Folin's achievements.

After writing the manuscript of his dissertation — on a topic the significance of which neither he nor Stieglitz could then have understood — Folin went to Europe for two years to study biochemistry and to try his hand at research of a different sort. Under Hammarsten at Upsala he felt at home; with Salkowski at Berlin he was not altogether happy but became interested in uric acid and began to speak German. As Hilding Berglund, a friend and co-worker of Folin in later years, wrote after Folin's death: "During his (longer) stay in Marburg (in Kossel's laboratory), Folin started getting eager. His interests in the intermediary stages of protein metabolism were started here and he never quite lost sight of his problem. There he also discovered a new practice in colorimetry used in the brewing industry, not without connection with his first colorimetric method."

Following his return to Chicago in 1898, Folin received his Ph.D. degree in chemistry and was anxious to begin an academic career in physiological chemistry. But there seemed to be no demand for a biochemist of his training. That subject was not then represented at the University of Chicago and in only a few universities and medical schools in this country had the need for biochemists been recognized at that time. So Folin found temporary employment as chemist in a flour mill.

In 1899 he accepted an assistant professorship of analytical chemistry at West Virginia University at Morgantown. There he gave a course in elementary quantitative analysis and, at his own request, an even more elementary course in physiological chemistry. That was his first experience in teaching. It was the good fortune of the writer to have been a student in both these courses and in consequence to become Folin's first pupil, for the following 4 years, and a close friend for the rest of his life.

About mid-year Folin was invited to equip and conduct a research laboratory for biochemical studies at the McLean Hospital (for the insane) at Waverly, Massachusetts. The medical director, Dr. Edward Cowles, a veteran of the Civil War and then (1900) advanced in years, was a man of vision who had decided that research in physiology and biochemistry (as well as in pathology and psychology) might in time contribute to better understanding of mental disease. Folin sensed the opportunity afforded in a hospital to conduct the kind of studies he felt qualified to undertake and he promptly accepted. Informed that he might bring a young assistant who would live in the hospital as a junior interne, Folin offered the writer that appointment. So it happened that Folin (accompanied by his youthful assistant) reported at McLean for duty in September 1900 and at once began assembling equipment and formulating plans for his research. The coincidence of Doctor Cowles' dream and Folin's ambitions and talents, if viewed against the delayed recognition, especially in this country, of the usefulness and promise of biochemistry, suggests an explanation for the great influence of Folin's works about 5 years later in awakening wider interest in this field. Full recognition of biochemistry awaited new demonstrations of its usefulness.

How Folin chose the problems and methods of approach which comprise his life work is best told in his own words. The only autobiographical notes left by Folin consist of a document in his own handwriting, dated April 9, 1924 (5 days after his 57th birthday), found among papers in his desk after his death. It is quoted here in full:

"When I was appointed chemist to the McLean Hospital in 1900 it became my duty to do chemical research on problems bearing on mental diseases. As the pathologist wanted all the brain material I took to the field of metabolism.

"It was hopeless to try to find deviations from the normal in the metabolism of the insane without far more exact knowledge of the human waste products than was available. My immediate and comprehensive problem became, therefore, the chemistry of urine. I realized that by thus interpreting my duty to the hospital I could do work of more general interest. I probably also followed my taste, for I

enjoyed the mere puzzle aspect which is always present when one tries to devise a new method.

"My papers on the Laws Governing the Chemical Composition of Urine and a Theory of Protein Metabolism (1904) will probably be considered my best; but the data for those papers came easily and naturally by the help of the new methods for the determination of urea, ammonia and creatinine which I had devised during the preceding three years.

"My later studies — Protein Metabolism from the Standpoint of Blood and Tissue Analysis — in the main represent attempts to pursue experimentally theoretical concepts which I had developed on the basis of urine analysis. My elucidation of amino acid absorption does not stand out well, partly because it was presented in the form of a series of short papers, and partly because Van Slyke soon came into the field with a method of his own. My 'best work' in the field of blood and tissue analysis, aside from the methods developed, should be the work on uric acid, now in press. It will be the starting point for much new metabolism literature."

This brief document reflects some of the qualities of the man: modesty and quiet humor, tranquility and kindliness, candor combined with a distaste for controversy, resulting in a chivalrous attitude toward his competitors. Perhaps aware that he had shot his bolt, Folin nevertheless looked forward with hope to other achievements — in a field in which his career began.

A few comments from the writer's memory may be recorded to supplement Folin's document. One of his first plans at McLean was to search for clues to possible abnormality of metabolism among mentally disturbed patients. The first effort, soon abandoned, was a study of the toxicity of normal urines versus that of mentally disturbed patients by intravenous injection of urine and its known constituents in rabbits; only the already-established toxicity of ammonium and potassium ions was detected. The second effort was more extensive and somewhat successful, but the clue was not appreciated and an opportunity may have been missed. In the course of prolonged study of metabolic discrepancies as revealed by analysis of the urines of normal and disturbed persons (reported in long papers published in 1904), a case was encountered of a patient having a remarkable daily cycle in mental state and a corresponding alternation in amount

of urinary excretion of inorganic phosphate; the data were published in the *American Journal of Physiology* in 1902 (over the protest of the editor, W. T. Porter). Years later that paper was cited as of interest to psychiatrists, and the wide role of phosphate in many metabolic reactions gradually came to be appreciated. In that later development Folin had no part.

Another item concerns "the starting point" of the famous papers, "Composition of Urine" and "Theory of Protein Metabolism." In a footnote to the first of these papers, Folin explains his debt to Professor Bowditch and to Doctor van Someren (associate of Horace Fletcher, the subject of Chittenden's studies). The low values of van Someren's protein metabolism observed by Folin is acknowledged as the "starting point" for interpretation of the data presented in those papers. Timing was again fortunate in providing a new aspect to Folin's ideas of protein metabolism, strengthening the basis for his theory.

The extent to which others of note became interested in Folin's work and served as subjects for his experiments may now be indicated by identifying some of them. "Dr. E. V. S." in the tables of his papers was of course van Someren, and "O. F.," Folin. "H. B. H." was Doctor Howard, then superintendant of the Massachusetts General Hospital; "E. S. A." Dr. Stanley Abbot and "Aug. H." Dr. August Hoch, both well-known psychiatrists. This point is cited to illustrate another of Folin's characteristics — his ability to explain in simple terms his ideas in a quiet manner that enlisted interest and cooperation in his work from those who came to know him well.

It was these papers, published in 1905, that first demonstrated the usefulness of Folin's methods for exploration of fundamental problems of intermediary metabolism. His work was already known to members of the medical faculty at Harvard, among them Dr. Henry Christian, then dean as well as professor of medicine. In 1907 Folin was appointed associate professor of biological chemistry at Harvard, where

L. J. Henderson and Carl Alsberg were already members of the staff. Two years later Folin became the first professor in biological chemistry appointed at Harvard.

Although the knowledge gained from Folin's researches during 7 years at McLean added little to an understanding of metabolic factors in relation to mental disturbances, his personality and achievements set an example which added to the traditions of that institution. The laboratory of two rooms he started, one of the first to be established for research in biochemistry in the hospitals of this country, was continued after Folin left and now occupies a new institute with modern facilities and is affiliated with the Department of Biochemistry at Harvard. During a period when the laboratory was still under Folin's guidance it was the training ground for both biochemistry and psychiatry. Doctor John C. Whitehorn, now the chief of psychiatry at Johns Hopkins, was from 1921 to 1938 the successor to Folin as Director of the McLean Laboratory of Biochemistry.

The work Folin did at Harvard is without doubt the basis for his influence in demonstrating the value of his type of biochemical methods for a variety of clinical and experimental studies in medical and biological fields. About 1912 he began (first with W. Denis, then H. Wu and others) to develop methods to explore protein metabolism by blood and tissue analysis. The object was to learn the paths of amino acid absorption and to locate the organs where tissue proteins and metabolic products were formed, about which there was then little knowledge and much speculation. Two by-products were of greater value than the data and deductions therefrom: one was the obvious value to clinicians of simple methods to measure "n.p.n."; another was the stimulus of Folin's papers to many of his younger biochemical contemporaries to improve upon and supplement his methods, thereby spurring similar investigations in many places here and abroad. That is probably the logical fate and reward of most pioneers.

There seems little point in laboring this theme further. Folin was an interesting and stimulating character who made

his mark on the renascence of biochemistry during the first third of this century. His career and influence recall that of another Swede, Berzelius, who had a similar and wider role in chemistry during the same period of the previous century.

Having introduced this paper by a quotation from Professor McCollum's account of the relations between Folin and Benedict, it seems appropriate to record at its close an excerpt from a letter written by Stanley Benedict soon after Folin's death:

"One of the qualities which so impressed me in Folin, so rare among scientific writers, was the fact that he was able to drop out personalities when it came to a matter of difference of scientific opinion. I have known no one with whom it was possible to have such strenuous differences of opinion or viewpoint in scientific work and have this not interfere one iota in the close personal friendship which lasted over more than twenty-five years."

The sentiments there expressed would doubtless be endorsed by all who knew Otto Folin.

PHILIP SHAFFER

FURTHER STUDIES ON THE ALFALFA FACTOR AND ITS RELATION TO THE LIVER AND WHEY FACTORS

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INTRODUCTION

Alfalfa meal (dehydrated and suncured) has recently been shown to stimulate chick growth (Hansen et al., '53; Scott et al., '53). Menge et al. ('52) have demonstrated separate unidentified chick growth factors in dried whey and liver "L". In the present study the alfalfa factor has been investigated in relation to the separate unidentified factors in whole liver substance and in dried whey, both of which are known to enhance chick growth. In addition, certain well-identified constituents of alfalfa have been tested for their ability to stimulate chick growth.

PROCEDURE

The chicks were the progeny from either a mating of New Hampshire males × Barred Rock females (experiments 1 and 4) or New Hampshire males × Columbian females (experiments 2 and 3). With one exception, the parent stock had been fed an all-mash ration containing liberal amounts of animal protein and alfalfa meal. The depleted chicks used in the first replicate of the second experiment originated from dams maintained on a raised wire floor and fed a corn-soybean meal all-mash ration fortified with B vitamins, in-

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cluding crystalline B_{12}, but devoid of all animal protein and alfalfa meal. The depletion period had been in progress for approximately 4 months before eggs from these birds were saved for hatching purposes.

The chicks were divided as to sex on the basis of down color, and an equal number of males (13 in experiment 4) or males and females (9 of each in experiment 1, 10 of each

TABLE 1

Composition of basal diet

INGREDIENTS	AMOUNT	VITAMINS ADDED	AMOUNT
	%		*mg/kg*
Cerelose	60.16	Thiamine HCl	25
Casein [1]	18.00	Riboflavin	16
Gelatin	10.00	Calcium pantothenate	20
Corn oil	6.00	Niacin	100
Salt mixture (Glista, '51)[2]	5.34	Pyridoxine HCl	6
DL-methionine	0.30	Biotin	0.6
Choline chloride	0.20	Folic acid	4
	100.00	Inositol	1000
		Para-aminobenzoic acid	2
		B_{12}	0.02
		Menadione	5
		Vitamins A, D, E and thiamine were administered weekly by dropper	

[1] Vitamin-free test, General Biochemicals, Inc.

[2] Salt mixture, in per cent:

$CaCo_3$	0.3000	KI	0.0040
$Ca_3(PO_4)_2$	2.8000	$CuSO_4 \cdot 5H_2O$	0.8020
K_2HPO_4	0.9000	H_3BO_3	0.0009
$MgSO_4 \cdot 7H_2O$	0.2500	$CoSO_4 \cdot 7H_2O$	0.0001
$Fe(C_6H_5O_7)_2 \cdot 6H_2O$	0.1400	$MnSO_4 \cdot H_2O$	0.0650
$ZnCl_2$	0.0020	NaCl	0.8800
		Total	5.344

in experiment 2, 7 males and 6 females in experiment 3) was randomly assigned to each experimental group. Following wing banding and weighing, the various lots were assigned at random to compartments of electrically heated battery units. Individual chick weights were recorded at weekly intervals. Feed and water were supplied ad libitum.

The composition of the basal diet is given in table 1. Vitamins A, D and E (α-tocopherol acetate) were administered

orally on the first day and at weekly intervals thereafter. During the course of the experiment (26 to 28 days) each chick received approximately 25,000 units of vitamin A, 12,500 units of vitamin D_3 and 8 mg of alpha-tocopherol. In addition to the thiamine in the basal diet, a solution of thiamine was introduced into the crop at the same time intervals. In this manner each chick received approximately 4 mg of thiamine in addition to that consumed in the basal diet.

Previous supplementation with liver powder had shown that 5% gave a maximum growth response. Likewise, alfalfa meal had given maximum growth response when fed at the 5% level. Menge et al. ('52) showed that 3% whey gave a maximum growth response when added to a purified diet. Consequently, all three products were added at the 5% level, thus supplying an adequate amount for maximum response to each supplement. All supplements to the basal ration were made at the expense of an equal weight of cerelose. In experiments 1, 2 and 3 the same sample of suncured alfalfa meal was used, while a different sample was employed in the 4th experiment.

RESULTS

The experimental design and the final weight averages for experiments 1 and 2 are shown in tables 2 and 3, respectively. Statistical analyses of the data are given in table 4. It may be seen that alfalfa, liver and whey all gave significant growth stimulation. It will be noted that alfalfa supported better growth than either of the other two supplements. This greater efficacy of alfalfa is attested by the consistently better growth obtained on double combinations containing alfalfa as compared with that obtained on the liver-whey combination. In experiment 1 there was no evidence of any interactions between or among the combinations tested, indicating that each supplement contained a separate factor and that the factors were additive. This is demonstrated also by the excellent agreement between the observed averages and expected averages, calculated on the basis of the additive nature of the

TABLE 2

Effect of alfalfa meal, liver powder and dried whey and combinations thereof on chick growth
(Experiment 1)

SUPPLEMENT	MALES					FEMALES				
	Ave. wt. (gm) 28 days					Ave. wt. (gm) 28 days				
	R_1 [1]	R_2	Obs.	Calc.	Deviation	R_1	R_2	Obs.	Calc.	Deviation
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
None	354	294	324	322	+ 2	343	291	317	311	+ 6
5% alfalfa [2]	376	374	375	376	— 1	341	354	347	345	+ 2
5% liver [3]	399	335	367	356	+ 11	358	277	317	329	— 12
5% whey [4]	325	353	339	348	— 9	344	338	341	335	+ 6
5% alfalfa + 5% liver	408	380	394	410	— 16	351	380	365	363	+ 2
5% alfalfa + 5% whey	406	408	407	402	+ 5	367	337	352	369	— 17
5% liver + 5% whey	393	358	375	372	+ 3	348	352	350	353	— 3
5% alfalfa + 5% liver + 5% whey	453	443	448	436	+ 12	406	395	400	387	+ 13

[1] R = replicate.
[2] Suncured sample "D."
[3] Defatted powder — Vio Bin.
[4] Western Condensing Co., Appleton, Wisconsin.

TABLE 3

Effect of alfalfa meal, liver powder and dried whey and combinations thereof on growth of depleted and non-depleted chicks
(Experiment 2)

SUPPLEMENT	MALES					FEMALES				
	Ave. wt. (gm) 26 days					Ave. wt. (gm) 26 days				
	Dep.	N.-dep.	Obs.	Calc.	Deviation	Dep.	N.-dep.	Obs.	Calc.	Deviation
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
None	250	262	256	278	— 22	269	222	245	265	— 20
5% alfalfa [1]	328	345	337	336	+ 1	315	282	299	293	+ 6
5% liver	323	323	323	314	+ 9	306	293	299	291	+ 8
5% whey	303	334	318	308	+ 10	289	292	291	285	+ 6
5% alfalfa + 5% liver	385	381	383	372	+ 9	338	313	325	319	+ 6
5% alfalfa + 5% whey	383	369	376	366	+ 10	338	304	321	323	— 2
5% liver + 5% whey	361	331	346	344	+ 2	329	305	317	311	+ 6
5% alfalfa + 5% liver + 5% whey	396	366	381	402	— 21	340	301	320	339	— 19

[1] Suncured sample "D."

TABLE 4

Analyses of variance for data in tables 2 and 3

SOURCE OF VARIATION	EXPERIMENT 1		EXPERIMENT 2	
	DF	F ratio	DF	F ratio
Treatment	7	10.74 [4]	7	25.42 [4]
Alfalfa	1	41.03 [4]	1	81.64 [4]
Liver	1	12.86 [4]	1	43.32 [4]
Whey	1	15.97 [4]	1	27.98 [4]
A × L	1	1.39	1	6.39 [2]
A × W	1	1.15	1	6.02 [2]
W × L	1	2.61	1	12.57 [4]
A × W × L	1	...	1	...
Sex	1	21.16 [4]	1	62.00 [4]
Replication	1	9.14 [3]	1	9.11 [3]
S × T	7	1.16	7	1.84
S × R	1	...	1	6.74 [3]
R × T	7	2.28 [2]	7	1.38
S × R × T	7	...	7	...
Error	192–1 [1] = 191	...	256–2 [1] = 254	...

[1] Missing values replaced.
[2] Significant at 5% level.
[3] Significant at 1% level.
[4] Significant beyond 0.1% level.

three supplements.[3] These calculated values and their deviations from the observed values are given in columns 4 and 5 of table 2 for the males and columns 9 and 10 for the females.

In experiment 2 there appeared to be significant interactions between several combinations. While this is contrary to the findings of the first experiment, critical appraisal of the data reveals that the discrepancy is due to the failure of the unsupplemented basal ration to support growth comparable to that obtained with the basal diet in experiment 1. The inferior growth of the chicks on the basal diet probably accounts for increased growth differences observed with the three supplements. Nevertheless, reference to columns 4, 5, 9 and 10 of table 3 again demonstrates good agreement between the observed and the expected final weight averages calculated on the asumption that the factors are separate and additive in nature. Since, as previously stated, each product was added at a level known to give a maximum growth response to that supplement, the additive effect cannot be explained on the basis of an increased concentration of the same factor or factors. This was again clearly demonstrated in a trial where the double combinations of supplements (alfalfa + liver, alfalfa + whey, liver + whey), each product added at the 7.5% level, were compared to the triple combination with each supplement fed at the 5% level. If the separate response to each supplement were merely due to a total higher concentration of the same factor or factors, then the double combination supplementation fed at the 15% level (7.5% of each product) should give the same response as could be attained on the triple combination fed at the 15%

[3] For example, the expected average weight of the males (table 2, column 4) was computed as follows: the alfalfa meal effect is the average of all groups containing alfalfa minus the over-all mean of the 8 groups ($406 - 379 = 27$). Similarly, the liver effect is $396 - 379 = 17$, and the whey effect is $392 - 379 = 13$. The calculated weight in the absence of any supplementation (basal group) is the over-all mean minus the sum of the alfalfa meal, liver and whey effects $[379 - (27 + 17 + 13)] = 322$. Similarly, the calculated weight of the group receiving 5% alfalfa meal is the over-all mean plus the alfalfa effect minus the sum of the liver and the whey effect $[379 + 27 - (17 + 13)] = 376$.

level (5% of each product). The 4-week weight averages of two replicates per group were as follows: basal 280, alfalfa + liver 334, alfalfa + whey 332, liver + whey 312 and alfalfa + liver + whey 365 gm. It may be seen that the three supplements again supported better growth than any double combination fed at the same total concentration as the triple combination.

The results of experiment 2 demonstrate that depleted and non-depleted chicks responded equally well to defatted whole liver, alfalfa meal and whey. The interaction term replication × treatment proved non-significant in the variance analysis.

In view of the highly significant sex differences observed in both experiments, it is of interest that the weights attained by the chicks on the triple supplementation compared favorably with weights that might be expected from a very good practical ration. It is such rapid growth which accounts, at least in part, for the large sex difference encountered.

Indirect evidence has been presented by Hansen et al. ('53) showing that the response to alfalfa cannot be explained on the basis of the cellulose contributed by the alfalfa meal. We have investigated this point further by direct means, and in addition have explored the possibility that other known constituents in alfalfa exert a favorable influence on chick growth. Experiment 3 was designed to test: (1) the effect of cellulose [4] when fed in the basal ration supplemented with 5% dried whey, and (2) the hypothesis that the growth inhibition resulting from feeding a high level of alfalfa meal is not solely a question of alfalfa's containing a growth-depressing agent, non-fibrous in nature (Lepkovsky et al., '50; Kodras et al., '51a, b) or a toxic factor (saponin; Peterson, '50a, b). The results of this phase are to be found in table 5.

It is evident that cellulose did not improve the basal diet containing dried whey, whereas 5% of alfalfa meal again showed a marked growth-stimulating effect. The data demonstrate also that 33.5% cellulose and 40% wheat bran depressed

[4] Ruffex.

growth equally as much as did the 40% level of alfalfa meal. An effort was made to make the high cellulose and high alfalfa diets isocaloric by increasing the cerelose in the cellulose diet 6.5%. It will be observed that lowering the density of the diet with high levels of either alfalfa or cellulose greatly increased feed consumption. Since growth on these diets

TABLE 5

The comparative effects of high and low levels of alfalfa, cellulose and wheat bran on chick growth

(Experiment 3)

SUPPLEMENT	RATION DENSITY	AVE. WT. (GM) 28 DAYS			FEED CONSUMED PER CHICK	GAIN/GM FEED CONSUMED
		R_1 [1]	R_2	Ave.	R_2	R_2
	gm/liter				*gm*	
5% whey	609	341	356	349	565	0.56
5% whey + 5% alfalfa [2]	575	382	380	381	597	0.57
5% whey + 5% cellulose	549	347	364	355	629	0.52
5% whey + 40% alfalfa	420	308	298	303	731	0.35
5% whey + 33.5% cellulose	339	305	317	311	754	0.36
5% whey + 40% wheat bran	468	292	292	292	535	0.47

[1] R = replicate.

[2] Suncured sample "D."

was significantly depressed below that of the basal chicks, the data are interpreted as indicating that energy became the limiting factor. If alfalfa contains a toxic factor that depresses chick growth, its influence was not superimposed on the energy effect, since cellulose inhibited growth to the same degree. According to the manufacturer, the cellulose had been exhaustively extracted and should have been free of saponins.

Definite rachitic symptoms were observed in the chicks fed the diet containing wheat bran, although they received the same amount of the antirachitic vitamin as the remaining groups. The nutritional failure of this diet probably stems from the abnormal Ca : P ratio. In any event, increased feed consumption was not observed on the low density bran diet. However, its palatability may also have been a factor in reducing feed intake.

TABLE 6

The effect of alfalfa meal and various constituents of alfalfa meal on the growth of chicks

(Experiment 4)

SUPPLEMENT	AVE. WT. (GM) 28 DAYS		
	R_1 [1]	R_2	Rep. ave.
None	267	306	286
5% Alfalfa [2]	359	312	336
5% Cellulose (Ruffex)	320	320	320
5% Gum arabic	285	283	284
Alfalfa ash ≈ 5% alfalfa	288	281	285
Amino acids ≈ 5% alfalfa [3]	293	322	308

[1] R = replicate.

[2] Suncured sample "H."

[3] L-arginine, 0.65 gm/kg diet.
L-lysine, 0.50 gm/kg diet.
L-cystine, 0.18 gm/kg diet.
DL-tryptophan, 0.22 gm/kg diet.
DL-methionine, 0.22 gm/kg diet.

While high levels of alfalfa and cellulose significantly depressed gross efficiency of feed utilization, 5% alfalfa had no adverse effect in spite of the greater maintenance requirement of the chicks consuming the alfalfa ration. Efficiency was only slightly depressed by 5% cellulose.

Experiment 4 was conducted to determine whether the response to alfalfa can be explained on the basis that this plant material contributes known nutrients that are either lacking in the basal diet or present there in suboptimum amounts. The substances tested included, in addition to cellulose: al-

falfa ash, the critical amino acids and gum arabic, which contains glucuronic acid postulated to be a chick growth factor by Stokstad et al. ('41). The inorganic constituents of alfalfa exerted no influence on chick growth (table 6). This was likewise true for the critical amino acids calculated to be present in 5% alfalfa. The basal diet did not appear to be improved by a uronic acid. Contrary to the results of the previous experiment, cellulose did give a significant response, although to a lesser degree than alfalfa meal.

DISCUSSION

The results of these studies indicate that the factor (or factors) present in alfalfa meal is distinct from the the factor found in either defatted whole liver or dried whey. The factor does not appear to be related to the ash, amino acids or glucuronic acid present in alfalfa. In some, but not all, instances a response was obtained by supplementing the basal diet with 5% cellulose (approximately 4 times as much fiber as is found in 5% suncured alfalfa meal) but in no case did the cellulose response approach that noted for alfalfa meal. In contrast to the work of Menge et al. ('52), who reported that chicks not depleted of their animal protein reserves would not respond to the unidentified factor in liver "L", we were able to show significant responses to whole liver with both depleted and non-depleted chicks. Savage et al. ('50) have found that the residue of a water extract of whole liver substance supported better growth than the water extract. It appears very likely, therefore, that another factor, or factors, occurs in whole liver in addition to those present in liver "L", the alcohol-insoluble fraction of the water extract of whole liver substance. The whey response was obtained with both depleted and non-depleted chicks, which is in agreement with the report by Menge et al. ('52).

SUMMARY

A factorial design was employed in two experiments with chicks depleted and non-depleted in animal protein and alfalfa

meal to test the response to unidentified growth factors present in alfalfa meal, defatted whole liver and dried whey. The data suggest that each of the three supplements contributes separate and distinct factors which are additive in their action. Alfalfa meal gave the greatest and most consistent response of the supplements tested. It was shown that the growth response from alfalfa meal could not be explained on the basis of its content of ash, critical amino acids or glucuronic acid. The results obtained from adding 5% cellulose to the basal diet were inconclusive. Growth inhibition resulting from 40% alfalfa meal cannot be explained solely on the basis that alfalfa contains a toxic factor.

ACKNOWLEDGMENTS

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NITROGEN BALANCE OF YOUNG ALBINO RATS FORCE-FED METHIONINE- OR HISTIDINE-DEFICIENT DIETS

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THREE FIGURES

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Few data have been published on the effect of amino acid deficiency on nitrogen balance, obtained by employing the technique of force-feeding. This method has the advantage of controlling the feed intake of experimental animals at levels sufficient to provids adequate energy for growth, a condition essential for studies of the efficiency with which the body utilizes dietary amino acids for protein synthesis (Mitchell, '43; Allison et al., '46).

Glista ('51), working with chicks, found the force-feeding technique very useful in determinations of amino acid requirement, using as a criterion the least amount of a given amino acid required to promote maximum nitrogen balance under conditions of equal caloric and equal nitrogen intake. Denton et al. ('50) and Bothwell and Williams ('51, '52), however, found the nitrogen balance of young rats resistant to change related to the amino acid adequacy of force-fed diets. The present investigation was originally planned to test the response of rats, in terms of urinary nitrogen excretion, to the presence or absence of methionine under conditions of force-feeding, with the ultimate aim of using this technique for studies of amino acid requirements. Later the objective was altered to a study of the conditions necessary for the

production of negative nitrogen balance in young rats supplied with adequate calories and total nitrogen but deficient in methionine.

EXPERIMENTAL

Three experiments were conducted in these studies. The composition of the various rations used is shown in table 1. Total nitrogen determinations were made on rations and feces by the Kjeldahl-Wilfarth-Gunning method, using mercury as a catalyst, and on urines by micro Kjeldahl, using selenium and copper as catalysts. Weanling male albino rats of the Sprague-Dawley strain were used in all experiments.

Experiment 1

Eleven rats weighing 60 to 80 gm were used. They were given not more than 6.0 gm of stock diet (27% protein) per rat daily for two weeks prior to initiation of the force-feeding procedure. For purposes of feeding, the experimental diets were homogenized with water in proportions of 35 gm diet to 50 ml water. The total volume of this mixture was 78 ml, and by analysis each milliliter contained 11.94 mg of nitrogen. A total of 12 ml of homogenate was fed to each rat daily in 4.0-ml feedings at 8:00 A.M., 2:00 P.M. and 8:00 P.M. The feeding was done with a 10.0-ml syringe to which a curved fine copper tube was affixed by means of a short length of flexible rubber tubing. Post-mortem examination of all animals revealed lesions attributable to the feeding technique in only one instance.

During collection periods, urine was collected daily, acidified, made up to a volume of 250 ml, and a portion of this saved for chemical analysis.

For the first 6 days of force-feeding, period 1, all rats received the complete diet. Animals were weighed each morning before feeding, and urine collections were made during the last 5 days of the period. During this period two rats died, but no evidence of feeding accident could be found.

During the next 8 days (period 2), 5 animals were given the basal diet and the remaining 4 were continued on the complete diet. For the last 5 days (period 3) of the experiment, all animals received the complete diet.

TABLE 1

Composition of experimental diets

INGREDIENT	EXERIMENT 1 Basal [6]	EXPERIMENT 2 Basal [7]	EXPERIMENT 3	
			N-free	Basal [8]
Oxidized casein [1]	18.0	..	..	..
Salts 446 [2]	3.0	4.0	4.0	4.0
Vitamin-starch 849 [3]	1.0	..	..	..
Vitamin-starch 691	..	5.0	..	..
Vitamin-sugar	..	..	5.0	5.0
Cod liver oil	1.5	1.5	1.5	1.5
Wheat germ oil	0.5	..	..	..
Corn oil	18.0	5.0	5.0	5.0
DL-tryptophan	0.4	..	..	..
Sucrose	28.8	68.3	84.5	67.2
Starch	28.8	..	..	..
NEAA [4]	..	8.12	..	8.12
EAA [5]	..	8.09	..	8.09
DL-histidine · HCl · H_2O	..	..	..	1.08

[1] Toennies ('42).

[2] Spector ('48).

[3] The vitamin premixes differed only in the amount or type of diluent and all contributed the following milligrams of vitamin to 100 gm of diet: thiamine HCl 0.25, riboflavin 0.5, pyridoxine HCl 0.25, calcium pantothenate 2.0, niacin 1.0, choline HCl 100, biotin 0.01, folic acid 0.10, inositol 10, para-amino-benzoic acid 5, 2 methyl-1, 4 naphthoquinone 0.10.

[4] Non-essential amino acids, as a per cent of the ration: glutamic acid 2.00, DL-serine 0.50, glycine 0.70, L-tyrosine 1.40, L-cystine 0.20 L-proline 0.90, L-asparagine 1.22 and DL-alanine 1.20.

[5] Essential amino acids, as a per cent of the ration: L-lysine HCl 1.24, L-arginine HCl 0.75, DL-tryptophan 0.20, DL-phenylalanine 0.90, DL-leucine 1.60, DL-isoleucine 1.00, DL-threonine 1.00, DL-valine 1.40.

[6] The complete diet for experiment 1 was made by adding 0.6% DL-methionine to a portion of the deficient diet.

[7] Additions of 0.59% DL-methionine or 1.08% DL-histidine HCl · H_2O, or both, were made to this basal ration, depending on conditions described in the text.

[8] The complete diet contained 0.60% DL-methionine in place of an equal weight of sucrose. The deficient diet contained 0.15% DL-methionine in place of an equal weight of sucrose. The "devoid" diet contained no methionine.

Experiment 2

In the second experiment 14 rats were individually fed a commercial stock ration for a one-week period; all rats received the same amount of feed, amounting to 6 gm daily during the last three days of the week. The animals were then paired on the basis of body weight, and force-feeding of the complete amino acid diet was initiated at the rate of 4.5 gm in three daily feedings. After two days the level of intake was increased to 6.0 gm. The diet was prepared for feeding by adding to the dry basal diet one-third of its weight of water or of amino acid solution as required (footnote 7, table 1). Homogeneity of the suspension was aided by warming it in a water bath and by the use of a few drops of Tween 80. The suspension as fed contained 1 gm of diet per milliliter. After three days on the 6.0-gm intake, individual collections of urine and feces were begun. During period 1, when the animals all received the basal diet supplemented with both histidine and methionine, three consecutive two-day urine collections were made, and the entire feces for the 6-day period were obtained by use of Fe_2O_3 as a marker. Immediately following this period, histidine was removed from the diet of the even-numbered animals and methionine was removed from the diet of the odd-numbered animals. Collections of urine and feces were made as before. Following the 6-day period of depletion (period 2) the complete diet was fed to all animals for a 6-day recovery period, during which urine collections were made over consecutive two-day periods.

Experiment 3

The third experiment differed in plan from the previous ones in that the 18 animals were subjected to a 7-day preliminary period on a nitrogen-free diet. This was done in an effort to determine the effect of depletion of protein stores on nitrogen balance during subsequent feeding of diets varying in methionine content. The diets used in this experiment

were designed to be similar to those fed in experiment 2, although only a methionine deficiency was planned.

After the depletion period, the animals were placed on the complete (0.60% methionine), the deficient (0.15% methionine) or the "devoid" diet (no methionine). They were force-fed in the same fashion as in experiment 2. Continuous two-day urine collections were made during the entire experiment, while feces were pooled in 4-day collections during the force-feeding of the amino acid diets, using ferric oxide and chromic oxide alternately as markers.

RESULTS AND DISCUSSION

The striking effects of amino acid deficiency were clearly seen in all experiments. In general, the animals on the complete amino acid rations gained 1.5 to 2.0 gm daily, while those on the methionine- or histidine-deficient diets no more than maintained their weight and in some cases lost weight. Two of 5 methionine-deficient animals died on the 8th day of deficiency in experiment 1; hence shorter deficiency periods (6 days) were planned for experiment 2. Although the time required for appearance of deficiency symptoms varied from three to 7 days, all affected animals presented a uniform syndrome. The first symptom, other than an abrupt decrease in weight gain, was the appearance of porphyrin-like pigment around the nose and forepaws. This stage was followed by hyperemia and denudation of throat and wrists. During this second stage the animals became quite lethargic and had a generally unkempt appearance. They were also more difficult to feed, and it was frequently necessary to allow them to rest for a few minutes during feeding in order to prevent the feed from welling up into the mouth. At this time the animals uniformly presented a very bloated appearance, which was confirmed at autopsy. In addition to severe bloat and impaction of the stomach and, sometimes, intestines containing undigested food, animals autopsied in the methionine-deficient state exhibited a very fatty liver and in experiment 3 a hemorrhagic condition of the stomach, but no other gross abnor-

malities. Histidine-deficient animals did not exhibit fatty livers.

The rapid sequence of events leading to death in amino acid-deficient, force-fed animals has not been satisfactorily explained. It does not seem that forcing the animal to metabolize large amounts of an unbalanced amino acid mixture is an adequate explanation, although this is thought to account for the early onset of deficiency symptoms. Probably a factor strongly contributing to death is shock produced by distention of the gastrointestinal tract. The mechanism by which such a shock is produced is imperfectly understood (Best and Taylor, '45) but is thought to include dehydration of tissues and loss of chloride, although it has been demonstrated that a nervous element may also be involved.

The response of the deficient animals to refeeding of the missing amino acid was striking. In experiment 2, 4 of 6 histidine-deficient animals and two of 4 methionine-deficient animals died within 24 hours after they were replaced on a complete diet. The 4 survivors responded well, in common with the methionine-deficient animals in experiment 1. A typical example is that of an animal which, at the end of the deficiency period, was almost stuporous, had heavy scabs on throat and wrists, and was very difficult to feed. Within 24 hours after initiating the complete diet he was much easier to feed and was more alert. By the second day he was very alert and the scabbiness of throat and paws was markedly reduced. After 4 days on the complete diet the denuded areas were almost entirely healed and the animal appeared to be normal otherwise. Autopsies of all refed animals failed to reveal abnormalities traceable to the previously existing deficiency or to the feeding technique.

The data on urinary nitrogen excretion show clearly the effect of amino acid deficiency and corroborate completely the findings made on the basis of body weight and physical appearance. In experiment 1 the urinary excretion of nitrogen was 68% higher in those animals receiving the basal diet than the average of the same animals on the complete diet.

Figure 1 shows the average daily nitrogen excretion for the experimental and for the control animals. The control animals excreted in the urine 64% of the nitrogen fed, while the deficient animals excreted 92%. Denton et al. ('50) and Bothwell and Williams ('51), reporting their results in terms of nitrogen balance, obtained about 34% retention in rats of similar age fed a similar caloric and nitrogen intake. The presence or absence of methionine or of histidine was reported not to alter the retention materially in their studies. The

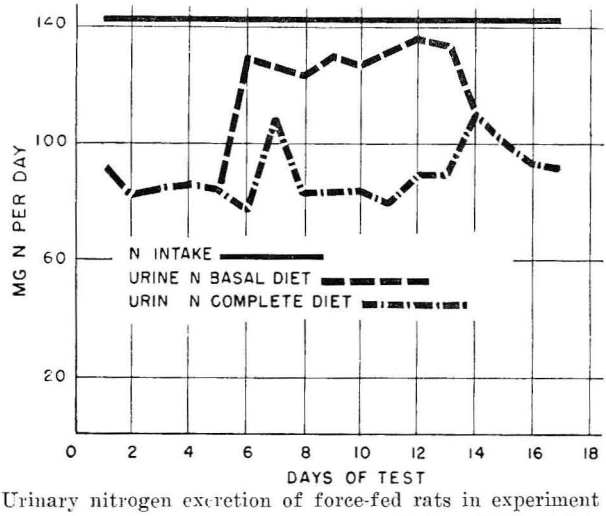

Fig. 1 Urinary nitrogen excretion of force-fed rats in experiment 1 on a diet containing adequate methionine and on the same diet markedly deficient in methionine.

major apparent difference between experiment 1 and the reports of the Wisconsin group is that the sole nitrogen source was oxidized casein supplemented with tryptophan in the present case, while a mixture of amino acids served as the nitrogen source for the rats used by the Wisconsin group. The diets utilized in experiments 2 and 3 were designed to be similar to those of the Wisconsin group, to facilitate comparison of results.

During the 6-day deficiency period of experiment 2 the histidine-deficient animals lost an average of 3.2 ± 0.60 [1] gm,

[1] Standard error.

while the methionine-deficient animals lost an average of 6.0 ± 0.86 gm. The difference is significant at the 2% level. During the recovery period the greatest gains were made by rats previously deficient in methionine.

The data on nitrogen retention for the rats in experiment 2 are shown in figure 2. These show clearly the reduction in nitrogen retention accompanying an amino acid deficiency and with constant nitrogen and energy intake. These results are in entire agreement with the data of experiment 1, in

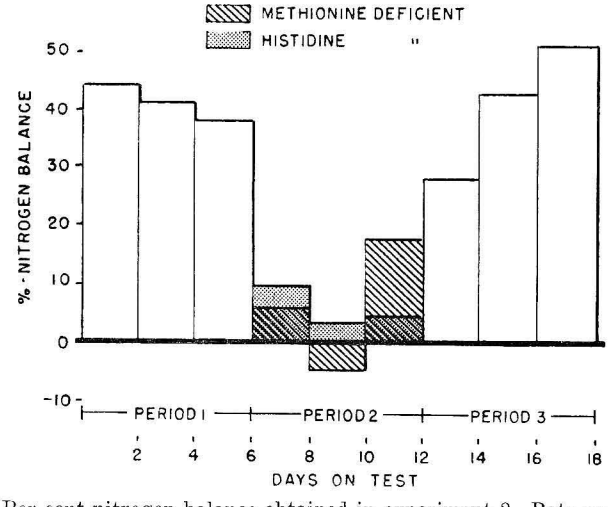

Fig. 2 Per cent nitrogen balance obtained in experiment 2. Rats were force-fed a complete diet in periods 1 and 3, and a diet lacking either methionine or histidine in period 2.

spite of the differences in nitrogen source and fat level of the diets. There was no effect of these deficiencies on the apparent digestibility of nitrogen, which averaged 97.3% during period 1 and 96.3% during the deficiency period. During the latter period, however, it was noted that three to 4 days were required for the marker to appear in the feces, in contrast to the single day required in period 1. This indicates a profound effect of amino acid deficiency on gastrointestinal motility, a finding which is in keeping with the observation of impaction of the tract in autopsied deficient animals.

The average nitrogen balance obtained during period 1 was 41.0%; during period 2, 6.4% for the methionine-deficient animals and 5.9% for the histidine-deficient animals; and during period 3, 40.6%. The difference in response expressed as nitrogen balance is not significant between the two deficiencies.

The fact that consistently negative nitrogen balances were not obtained is at variance with reports to be found in the literature concerned with nitrogen balance in adult animals fed incomplete amino acid mixtures (i.e., Benditt et al., '49), and points to the possibility that labile protein stores were raided, although inefficiently, to provide the missing amino acid. In order to determine the effect of labile protein stores on nitrogen balance during an induced deficiency, the third experiment in this series was conducted, with the following results.

During the protein depletion period of experiment 3 the animals consumed an average of 5.0 gm of feed daily and lost an average of 17% of their body weight; the urinary nitrogen excretion decreased to less than 1 mg per gram body weight $^{3/4}$, thus approximating the endogenous nitrogen excretion and showing that labile protein stores were depleted.

It was planned to have 6 animals on each of the three experimental diets, but within two days after starting the force-feeding two animals on the complete diet, two on the deficient diet, and 5 on the "devoid" diet died. All animals in the last two groups except for one on the deficient diet exhibited a hemorrhagic condition of the pyloric section of the stomach, covering about one-third of the entire stomach wall. Of the 9 animals dying, 6 exhibited severe bloat and impaction of food in the stomach and intestines, and two revealed lung congestion. The remaining animals continued on the experiment with the results shown in figure 3.

A total of 6 4-day fecal collections, with two separate two-day urine samples per collection period, was obtained. The apparent digestibility of nitrogen averaged 97% for the 22 individual collections from the complete diet, 88% for the

10 collections from the deficient diet and 93% for the 11 collections from the "devoid" diet. This difference in digestibility was unexpected and can undoubtedly be attributed, not to the difference in methionine supply, but to the ash content of the diets. Analysis of the diets, in an effort to explain this anomalous result, revealed that the complete diet contained the designated 4% ash, but that through an error in making up the diets the deficient and "devoid" diets contained 11%

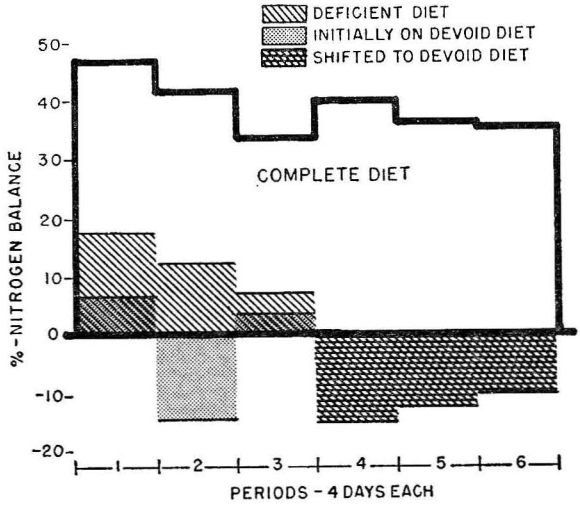

Fig. 3 Per cent nitrogen balance obtained in experiment 3. Rats were force-fed diets either complete, containing a deficient amount of DL-methionine, or devoid of methionine.

ash. Although this resulted in bulkier, greyish feces and a decreased apparent digestibility of nitrogen, it is considered by the authors that the data are not invalid on this account. In a check experiment, rats receiving the "devoid" diet supplemented with methionine exhibited the same bulky, grey feces but grew well for the two-week period the diet was fed.

For the animals on the complete diet, the percentage of consumed nitrogen retained was 47% during the first collection period and averaged 37% during the remaining collection periods, as shown in figure 3. This clearly demonstrates that

the protein-depleted animals were more efficient in utilizing the dietary nitrogen at first than after their protein stores were partially re-built, a finding in agreement with observations of Allison et al. ('46).

Although the animals on the deficient diet made increasingly less efficient use of the dietary nitrogen from the first to the third collection (18% to 6%), they continued to be in positive nitrogen balance.

By the end of the third collection period the single animal on the "devoid" diet died, having been, for the second period, in a definite negative nitrogen balance of —14%. At the time of death, the stomach of this animal was intensely bloated and contained much undigested food, thus accounting for the apparent positive balance of nitrogen in the third period.

The three animals which had received the deficient diet for 12 days were placed on the "devoid" diet, and immediately went into negative nitrogen balances of 15% and 13% during periods 4 and 5. One of these rats died during period 5 and another early in period 6. For period 6, two of the rats from the complete diet were placed on the "devoid" diet and exhibited negative nitrogen balances averaging 11%.

The data of experiment three are interpreted as an indication that labile protein stores may be called upon in the event of a methionine deficiency to provide methionine for synthesis of protein less rich in this amino acid than are the labile protein stores. That the proportion of various proteins within the body can vary under conditions of stress such as are imposed by an amino acid deficiency may be surmised from the studies of Williams and Elvehjem ('49), who have shown changes in enzyme levels in rat livers as a result of amino acid deficiency, and from the studies of Whipple et al. ('47), who have demonstrated a "raiding" of body tissue proteins for synthesis of hemoglobin and blood plasma proteins.

The increase in urinary nitrogen excretion obtained as a result of methionine and of histidine deficiency under conditions of equal caloric and equal nitrogen intake is at vari-

ance with the results of Denton et al. ('50) and of Bothwell and Williams ('51), but is in agreement with the many reports in the literature in which controlled feeding methods have been employed in nitrogen balance procedures to test the adequacy of varied proteins for growing animals (Block and Mitchell, '46). The entire concept of the biological value of protein is based on the inverse relationship between adequacy of a protein source and urinary nitrogen excretion under conditions of equal caloric and equal nitrogen intake.

The extreme measures which must be taken to induce a negative balance of nitrogen in young animals, in contrast to the ease with which this may be accomplished in adult animals, is a new finding whose explanation may lie in the fact that the normal rate of protein anabolism in the young animal greatly exceeds the catabolic rate, whereas these two processes are more nearly balanced in the adult animal. Thus, it appears logical that the young animal will retain protein more efficiently under adverse conditions than will an adult.

SUMMARY

The nitrogen balance of young albino rats was studied under conditions of methionine and of histidine deficiency by a force-feeding technique which assured equal and adequate caloric intake. Under these conditions the amino acid deficiencies resulted in markedly reduced nitrogen balances as a result of increased amounts of urinary nitrogen excretion. If the animals were depleted of protein stores prior to force-feeding the amino acid-deficient diet, the nitrogen balances decreased beyond the point of equilibrium and became negative.

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GROWTH OF THREE- TO FOUR-WEEK-OLD RABBITS FED PURIFIED AND STOCK RATIONS

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ONE FIGURE

(Received for publication August 10, 1953)

A number of efforts have been made to study the growth requirements of rabbits using purified diets of casein, refined carbohydrates, fats, minerals and cellulose. In most of these studies the diets contained natural materials as a source of unidentified and known vitamins. Hogan and Ritchie ('34) and Hogan and Hamilton ('42) added yeast or liver; Swaminathan ('42) added extracted oats and whole cow's milk; Olcese, Pearson and Schweigert ('48) added 30% beet pulp. Wooley and Sebrell ('45) were the first to report the successful feeding of rabbits with a purified diet containing only known "pure" vitamins. They obtained weight gains of 800 to 1,800 gm in 16 weeks. Later, Olcese, Pearson and Sparks ('49) obtained 1,600-gm weight gains in 12 weeks using a purified diet containing 10% wood pulp and pure vitamins. In most of the above experiments rabbits were placed on experiment when 6 to 8 weeks of age.

The following experiments were initiated in an attempt to determine the utility of the rabbit for nutrition experiments and to define its nutritional requirements more clearly. It was found that young rabbits weaned at the age of three to 4 weeks, weighing 350 to 500 gm, could be used for these purposes. The purified diet used in previous experiments

(Wooley and Sebrell, '45) with 8-week-old rabbits did not produce good growth in these younger animals unless supplemented with certain vegetable materials.

EXPERIMENTAL

A pure strain of New Zealand white male rabbits reared at the National Institutes of Health by promiscuous breeding was used. The rabbits were weaned at three to 4 weeks, weighing 350 to 500 gm, and placed directly on experiment at this age. Litters were separated and the animals distributed in such a manner that no two mates were placed in the same experimental group. The rabbits were housed individually in metal cages with one-half-inch wire mesh floors. Each test involved several groups, varying in number from 6 to 25. All groups in a test contained equal numbers of animals, usually 4.

The composition of the various diets is shown in table 1. Diet 1395 is the same as that used by Wooley and Sebrell ('45) in earlier work with 8-week-old rabbits. The other purified diet, 1436, is similar to a diet used by Reid and Briggs ('53) for guinea pigs. Kale has long been added as a supplement to the stock ration of the breeding colony of rabbits at the National Institutes of Health. It was, therefore, decided to feed it as an addition to the purified rations of the experimental animals. The kale was stripped from the stalk, dried in an oven with a forced draft, at 70°C., and ground in a coffee mill. Diets 1407 and 1464 were similar to diets 1395 and 1436, respectively, but with 40% by weight of dried kale added. The protein, fat and cellulose were adjusted to the levels in the diets without kale. Pellets[1] of the same composition as those fed in the stock colony were used to provide

[1] The stock diet consisted of pellets made by a commercial company according to the formula of Mr. Samuel Poiley, the custodian of the animal breeding colony at the National Institutes of Health. The constituents of the diet are ground whole wheat 30.5%, oat groats 15.0%, alfalfa leaf meal 40.0%, soybean oil meal 13.0%, calcium carbonate 1.0%, irradiated yeast 0.25%, iodized sodium chloride 0.25%, and wheat germ oil 8 oz. per ton.

TABLE 1

Diets

CONSTITUENTS OF DIETS	DIET NUMBER			
	1395	1436	1407	1464
	%	%	%	%
Casein, vitamin-free	20.0	30.0	13.76	23.76
Sucrose	55.6	9.25	24.72	. . .
Cornstarch	. . .	20.0	. . .	. . .
Cerelose	. . .	8.0	. . .	6.37
Kale [1]	. . .	. .	40.0	40.0
Cellophane [2]	15.0	15.0	13.08	13.08
Salts	4.0 [3]	6.0 [4]	4.0 [3]	6.0 [4]
Choline chloride	0.2	0.3	0.2	0.3
Inositol	0.2	0.4	0.2	0.4
Ascorbic acid	. .	0.5	. .	0.5
p-Aminobenzoic acid	. .	0.25	. .	0.25
Potassium acetate	. .	2.5	. .	2.5
Magnesium oxide	. .	0.5	. .	0.5
Wesson oil	5.0	. .	4.04	. .
Corn oil	. .	7.3	. .	6.34
Other vitamins	*mg %*	*mg %*	*mg %*	*mg %*
A acetate	. .	0.6	. .	0.6
Carotene	0.45	. .	0.45	. .
Alpha-tocopherol	25.0	. .	25.0	. .
Alpha-tocopherol acetate	. .	2.0	. .	2.0
D_2	(200.0) [5]	. .	(200.0) [5]	. .
D_3 crystalline	. .	0.004	. .	0.004
2-methyl-1,4-naphthoquinone	0.1	0.2	0.1	0.2
Thiamine hydrochloride	0.8	1.6	0.8	1.6
Pyridoxine hydrochloride	0.8	1.6	0.8	1.6
Riboflavin	0.8	1.6	0.8	1.6
Nicotinic acid	20.0	20.0	20.0	20.0
Calcium pantothenate	5.0	4.0	5.0	4.0
Folic acid	. .	0.6	. .	0.6
Biotin	. .	0.06	. .	0.06
B_{12}		0.004	. .	0.004

[1] Dried kale; for preparation see text.

[2] Cellophane spangles secured from the Rayon Processing Co. of R. I., Inc., Pawtucket.

[3] Wesson ('32).

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

[5] Expressed as U.S.P. vitamin D units. Purchased as "Drisdol [R]," in propylene glycol, from Winthrop-Sterns, New York, N. Y.

a basis of comparison in evaluating the adequacy of the experimental diets. Diets were fed ad libitum. Tap drinking water was available at all times. The experiments lasted 4 weeks.

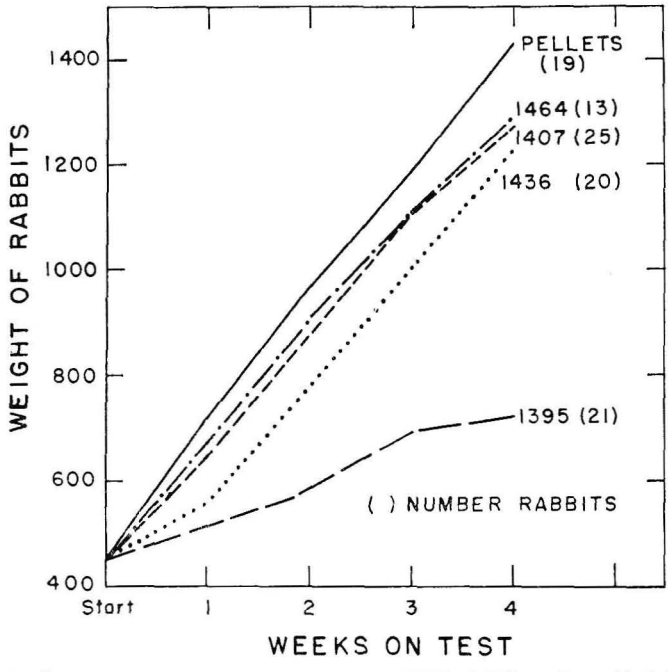

Fig. 1 Growth with pellets, purified diets (1395, 1436) and purified diets plus kale (1407, 1464).

Data from various groups fed the same diet but at different times were analyzed statistically.[2] It was found that variations (for a given diet) from test to test were no larger than expected, considering the variation in response among animals on the same diet on the same test; therefore, the results from all rabbits fed the same diet were pooled.

[2] The author wishes to express his appreciation to Mr. M. A. Schneiderman of the Biometrics Section, National Cancer Institute, for the statistical analyses, and to Dr. Olaf Mickelsen of this laboratory for assistance in the preparation of the manuscript.

RESULTS

The pellets and the purified diets with and without kale were fed with the results shown in figure 1. The diets fall into two categories. The pellets, purified diet 1436, and the purified diets containing kale (1407 and 1464) promoted the

TABLE 2

Growth stimulation resulting from the addition of vegetables to purified diet 1395

	SUPPLEMENTS	AVERAGE WEIGHT GAIN IN 4 WEEKS [1]
		gm
(1)	None	275 (± 25)[5]
(2)	40% Kale leaf meal	827 (± 82)
(3)	40% Alfalfa leaf meal	711 (± 30)
(4)	15% Kale leaf meal	642 (± 87)
(5)	15% Alfalfa leaf meal	519 (± 50)
(6)	8.6% Forage juice [2]	543 (± 57)
(7)	Fresh kale [3]	645 (± 28)
(8)	Spinach [4]	411 (± 87)
(9)	Carrots [4]	366 (± 17)
(10)	Cabbage [4]	436 (± 47)
(11)	Cabbage core [4]	344 (± 32)
(12)	Chicory [4]	349 (± 40)
(13)	Snap beans [4]	418 (± 60)
(14)	Celery [4]	445 (± 40)
(15)	Radish [4]	357 (± 35)

[1] All groups comprised 4 rabbits except 1, 7 and 8, which had 21, 6 and three, respectively.

[2] Juice from forage (80% alfalfa, 20% rye grass) equivalent to 15% of dry forage in the diet.

[3] Fresh kale was fed daily in amounts equivalent to the amount consumed by the rabbits receiving 15% kale leaf meal in the diet.

[4] Amounts equal to the fresh kale supplements.

[5] Standard error computed by the method of Mantel ('51).

same rate of growth (following different initial gains the first week). Diet 1395 gave substantially slower growth than the other 4 throughout the growth period. The contrast between the pellets and purified diet 1436 is of interest. During the first week on the pellets, the animals gained an average of 265 gm. On diet 1436 the average gain was 104 gm. This difference is highly significant ($P < 0.01$). The addition of

dehydrated kale to purified diet 1395 (diet 1407) or 1436 (diet 1464) produced growth during the first week similar to that obtained with the pellet diet; viz., 193 and 227 gm, respectively. During the next three weeks the pattern of growth with each of these 4 diets was somewhat similar.

Tests were made to compare the growth stimulation resulting from kale leaf meal, alfalfa leaf meal, fresh kale and other vegetables. The data in table 2 indicate that dried kale gave slightly better growth than alfalfa at both the 15 and

TABLE 3

Effect on growth of rabbits when potassium was added to diet 1395

SUPPLEMENT	NO. OF ANIMALS	AVERAGE [1] WEIGHT GAIN IN 4 WEEKS
		gm
None	21	275 (± 25)
0.1% Potassium (as acetate)	4	370 (± 32)
0.1% Potassium (as bicarbonate)	4	337 (± 50)
0.2 to 2.0% [2] Potassium (as acetate)	59	449 (± 12)
2.0% Potassium (as acetate)	7	442 (± 44)
0.2 to 0.8% [2] Potassium (as bicarbonate)	13	442 (± 25)

[1] Average and standard error.

[2] These different levels of potassium gave similar growth responses; therefore, the results for all groups were combined. The standard errors for these groups bear out this statement.

40% levels. Fifteen per cent of either kale or alfalfa was less effective than 40%. There was no difference between 15% dried kale and an equivalent amount of fresh kale fed as a daily supplement. Several other fresh vegetables fed in amounts equivalent (dry weight basis) to the 15% fresh kale gave some growth stimulation but less than kale.

Since rabbits in a wild state consume a diet mostly of vegetable material, a relatively large amount of potassium is consumed. The dried kale used in these experiments contained 29 mg of potassium per gram.[3] Addition of 40% dried kale to purified diet 1395 increased the total potassium in the diet

[3] Flame photometer determinations were done under the direction of Dr. R. W. Berliner of the National Heart Institute.

to 1.76%. The superior growth resulting from diet 1436, which contained added potassium, also suggested that the inferior growth on diet 1395 might be due to insufficient potassium. The mineral mixture in diet 1395 supplied sufficient potassium to make a concentration of 0.6% in the complete diet. When this diet was further supplemented with potassium at 8 different levels, varying from 0.2 to 2.0%, significant growth stimulation resulted (table 3). The growth increment from 0.2% potassium was just as great as with larger amounts.

TABLE 4

Effect of altering type of carbohydrate in diets 1395 and 1436

DIET	SUPPLEMENT	NUMBER OF ANIMALS	AVERAGE [1] WEIGHT GAIN IN 4 WEEKS
			gm
1395	None	21	275 (± 25)
	20% Cornstarch for equal amount of sucrose	15	347 (± 39)
1436	None	20	779 (± 38)
	20% Sucrose for cornstarch	4	738 (± 87)
	28% Sucrose for cornstarch and cerelose	4	790 (± 37)

[1] Average and standard error.

When 0.1% potassium was added, smaller and inconsistent growth stimulation resulted. There was no significant difference when potassium was added in the form of acetate or bicarbonate. Potassium acetate had no deleterious effect on the animals, even when added to the diet at a 5% level.

Rabbits in the wild state also consume large amounts of complex carbohydrates such as starch and cellulose. Substitution of 20% cornstarch for an equal amount of sucrose in diet 1395 gave an increase in growth of borderline significance (table 4). Substitution of sucrose for cornstarch, or sucrose for cornstarch and cerelose, in diet 1436 had no significant effect on growth.

The effect of different levels of casein in purified diets 1395 and 1436 is shown in table 5. When casein in diet 1395 was increased from 20 to 30%, at the expense of sucrose, 8 of 11 rabbits in three separate experiments died between the second and 4th weeks of the test. The three rabbits which survived gained an average of 260 gm. However, all the rabbits on the 30% casein diet lived if potassium was added. Substitution of 20% casein (plus 10% sucrose) for 30% casein in diet 1436 caused a significant reduction of weight gain.

TABLE 5

Effect of casein levels in diets 1395 and 1436

DIET	CASEIN [1]	SUPPLEMENT	NUMBER OF ANIMALS	AVERAGE [2] WEIGHT GAIN IN 4 WEEKS
	%			gm
1395	20	None	21	275 (± 25)
	20	0.8% Potassium	18	457 (± 21)
	30	None	11 (8 died)[3]	260 [4]
	30	0.8% Potassium	7	590 (± 83)
1436	30	None	20	779 (± 38)
	25	None	7	756 (± 31)
	20	None	20	505 (± 31)

[1] Casein levels varied at expense of sucrose.

[2] Average and standard error.

[3] Eight rabbits died between second and 4th week.

[4] Average weight of three surviving rabbits.

When diet 1436 contained 25% casein, the resulting growth rate was equal to that obtained with the original diet.

DISCUSSION

It was the opinion of certain commercial breeders that young rabbits taken from their mothers before the age of 8 weeks would not survive. The experiments reported above show that rabbits weaned at three weeks of age, and weighing as little as 350 gm, will survive and can be used in nutritional studies without supplements of milk or other natural foods. When only the stock ration and water were given to rabbits

weaned at three weeks of age, the rate of growth for the next 5 weeks and the final weight approximated the results secured with rabbits left with their mothers until 8 weeks of age; viz., 1,500 to 1,800 gm. In our experience with rabbits weaned at 8 weeks of age, special measures have occasionally been necessary to induce them to consume purified rations. This difficulty has not been encountered with three- to 4-week-old rabbits.

Rabbits fed pellets grew at a significantly greater rate ($P < 0.01$) than those fed any of the purified diets, when judged by their 4-week growth performance. Purified diet 1395 was obviously inadequate for these young rabbits. Purified diet 1436, of quite different composition, supported much better growth but less than the pellets. Diet 1395 was strikingly improved when modified to include 40% kale. This modified diet supported growth equal to purified diet 1436. Diet 1436 was not significantly improved by kale (except for its growth-promoting potential during the first week).

Of numerous vegetable materials tested for their ability to promote the growth of rabbits on diet 1395, kale, either fresh or dry, was most effective. Kohler, Elvehjem and Hart ('36) reported the presence of growth factors in grass juice. In our experiments, "forage juice" derived from alfalfa and rye grass [4] stimulated growth to about the extent of alfalfa but to a lesser extent than an equivalent amount of kale, although this difference was not significant statistically with the small number of animals used.

The nature of the factor or factors in kale and other vegetables which are responsible for the greater weight gains on diet 1395 is not entirely clear. The possibility must be considered that these vegetables have an appealing taste for the rabbit and stimulate growth through non-specific appetite effects. This could explain the observation that rabbits receiving the kale-supplemented diets showed significantly better growth during the first week on experiment, even though in

[4] Kindly supplied by Cerophyl Laboratories, Inc., Kansas City, Mo., through the courtesy of Dr. G. O. Kohler.

the case of diet 1436 their 4-week growth performance was not significantly improved. However, our results demonstrate that potassium added to purified diet 1395 will stimulate growth but only partially duplicates the kale effect. This raises the possibility that the effect of kale is due only to the minerals it supplies, and indicates that purified diet 1395 was deficient in one or more minerals. Booth, Elvehjem and Hart ('49) reported the presence in alfalfa of a growth factor or factors for the guinea pig. Later studies by this group (Roine et al., '49) led to the conclusion that this growth effect could be attributed entirely to potassium and magnesium. Our studies, thus far, have not enabled us to determine whether all of the growth-promoting properties of kale for rabbits is due to minerals, particularly the growth-promoting effect during the first week on the diet. It should be noted that diet 1436 contained added magnesium as well as potassium and liberal amounts of other minerals but failed to stimulate growth during the first week to the extent the pellets did and the kale-supplemented purified diets.

The present work indicates that the potassium requirement of the rabbit may be several times that of the rat. A level of 0.8% potassium in diet 1395 was the minimum amount that produced maximum growth. If this level represents an approximation of the minimum for potassium, then it is considerably higher than that required by the rat (0.17%, Kornberg and Endicott, '46). The possibility exists that the minerals supplied by diet 1395 may have been improperly balanced for the rabbit and that the potassium effect may be more complicated than a straight deficiency phenomenon. This is being investigated.

Our results also indicate that the protein requirement of the rabbit, with casein as the protein source, on diet 1436 is probably between 25 and 30%, but that on diet 1395 30% is toxic unless the potassium level is increased. However, it should be noted that diet 1407, which supplied only 20% protein, 13.76 from casein and 6.24 ($N \times 6.25$) from kale, supported growth equal to that obtained with diets containing

30% protein. Likewise, the pellets, which contained an estimated 23% protein (N × 6.25), supported the best growth of any of the diets. The decidedly unfavorable result when the casein level in diet 1395 was increased to 30% is being investigated.

SUMMARY

It has been shown that rabbits weaned at three to 4 weeks of age can be used in nutrition studies. A purified diet previously used for older rabbits did not support good growth in these young animals. Kale produced considerable growth stimulation when added to this diet. A number of other vegetables also produced growth stimulation but to a lesser extent than kale. The addition of potassium also increased growth with this diet but not to the same extent as kale. A purified diet developed for guinea pigs, which differed in a number of respects from the rabbit diet, supported growth equal to that obtained with the kale-supplemented rabbit diet but less than that attained with stock pellets. Preliminary experiments indicate that at least 25% protein is required by the rabbit with casein as the protein source, but equally good growth was attained with diets containing 20% protein partly or completely from vegetables.

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INFLUENCE OF CARBOHYDRATE, NITROGEN SOURCE AND PRIOR STATE OF NUTRITION ON NITROGEN BALANCE AND LIVER COMPOSITION IN THE ADULT RAT

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Previous studies showed that adult rats fed low levels of amino acids (0.1%) were in negative nitrogen balance when the dietary carbohydrate was sucrose, whereas nitrogen equilibrium was attained when the carbohydrate was corn dextrin or when the essential amino acid intake was increased. Diammonium citrate had no significant effect on the nitrogen balances (Womack, Marshall and Parks, '53). A preliminary investigation of the fat content of the livers of the animals indicated that rats receiving sucrose and 0.1% of the amino acids had more fat in the liver than those fed corresponding diets with corn dextrin. The present study was designed to investigate further the effect of a different source of nitrogen, glutamic acid, upon nitrogen balances when fed as a supplement to diets containing low levels of amino acids, and to determine the effects of the two carbohydrates, sucrose and corn dextrin, on the amount of fat in the liver.

EXPERIMENTAL

Determination of nitrogen balances

The compositions of diets and amino acid mixtures have been previously described (Womack et al., '53). The basal nitrogen-free diet consisted of a source of carbohydrate (su-

crose or dextrin), 80.65%; salt mixture (Jones and Foster, '42), 4%; corn oil, 3%; lard, 12%; vitamin A and D concentrate,[1] 0.05%; inositol, 0.1%; and choline chloride, 0.2%. The following vitamins were added per kilogram of ration: thiamine HCl, 5 mg; pyridoxine HCl, 5 mg; nicotinic acid, 5 mg; riboflavin, 10 mg; Ca pantothenate*(d)*, 25 mg; *p*-aminobenzoic acid, 300 mg; α-tocopherol acetate, 25 mg; 2-methyl-1,4-naphthoquinone, 2 mg; biotin, 100 μg; folic acid, 2 mg; and vitamin B_{12}, 30 μg.

The physiologically available forms of the essential amino acids were fed at levels of either 0.1 or 0.2%. Non-essential amino acids were included at levels of 0.1%. Glutamic acid, when fed as a source of additional nitrogen, was added at levels of either 1.65 or 6.45% of the rations. All substitutions were made at the expense of the carbohydrate. All animals were offered 12 gm food each day, except those fed nitrogen-free rations, which were fed ad libitum. In calculating the essential amino acid intake, no allowance was made for the small amounts of amino acids present in the corn dextrin.

The animals used for these investigations were male rats, housed individually when 21 to 23 days old and given the commercial stock ration and water ad libitum until they attained the desired age and weight. This ration contained approximately 25% protein and 5% fat. Animals to be used in the undepleted series were removed from the stock diet when 70 to 76 days of age and given diets 2 to 5, containing either sucrose (series A) or corn dextrin (series B), for three weeks. Animals to be protein-depleted were removed from the stock ration when 90 to 100 days old, fed the nitrogen-free rations containing either sucrose or corn dextrin for 18 days, and then given diets 2 to 5, containing either sucrose (series C) or corn dextrin (series D), for three weeks. This experimental design was varied for both the undepleted and the protein-depleted animals fed diet 2 and sucrose. At the end of the three-week period, one-half of each group of ani-

[1] Squibb's Navitol, containing 65,000 U.S.P. units of vitamin A and 13,000 units of vitamin D per gram.

mals was transferred to diet 2, containing corn dextrin, for three additional weeks, while the other half continued to receive diet 2 containing sucrose for a total of 6 weeks (series A-1 and C-1). All animals were divided into groups according to litter and body weight before being placed on the experimental diets.

The first week during which amino acids were fed was an adjustment period and no collections of urine and feces were made. Nitrogen balances were determined for each animal during the next two 7-day periods. For most of the animals of series C and D, nitrogen balances were also determined during the last 7 days of the 18-day protein-depletion period.

Weekly weighings were made of the animals and of their uneaten food. When food was scattered during a collection period, the data for the animal for that period were discarded.

The methods of collecting the urine and feces and of the nitrogen analyses have been previously described (Womack et al., '53).

Liver analyses

On the 22nd day of amino acid feeding, all of the animals were anesthetized with 1% sodium amytal except those of series A-1 and C-1, which were killed on the 43rd day. The livers were removed, blotted free of excess blood, placed in dishes and weighed; they were dried under infra-red lamps at a distance of 13 inches, ground, and duplicate samples analyzed for total lipids (ether-extractable material), nitrogen and moisture.[2] When an excessive amount of fat was present in the dried sample, grinding was impossible without loss of fat; therefore, the whole sample was extracted. All extractions were made with ethyl ether on the Bailey-Walker apparatus.

RESULTS AND DISCUSSION

Nitrogen balances

The data obtained for animals in series A to D are shown in table 1. Statistical significance of the differences between

[2] We wish to express our appreciation to Miss Elizabeth Hewston for advice in connection with methods used in the liver analyses.

TABLE 1

Average weights and daily nitrogen balance data for undepleted (series A and B) and protein-depleted (series C and D) adult male rats receiving diets containing low levels of amino acids with and without supplementation with glutamic acid and with either sucrose or corn dextrin as the source of carbohydrate

SERIES NO.	DIET NO.	DESCRIPTION OF DIET	NO. ANIMALS/ GROUP	INITIAL WEIGHT	WT. AT END OF ADJUSTMENT PERIOD	FINAL WEIGHT	FOOD INTAKE	CALORIE INTAKE	NITROGEN INTAKE		NITROGEN EXCRETION		NITROGEN BALANCE AND STANDARD ERROR [2]
									Total	EAAN [1]	Feces	Urine	
				gm	*gm*	*gm*	*gm*		*mg*	*mg*	*mg*	*mg*	*mg*
A	2	0.1% EAA, 0.1% NEAA [3]	6	299	280	272	12.0	54	37.1	14.8	13.0	48.0	— 23.8 ± 0.9
(undepleted,	3	0.1% EAA, 0.1% NEAA + 1.65% GA [4]	6	298	282	276	12.0	54	55.0	14.7	12.2	59.9	— 17.1 ± 1.0
sucrose)	4	0.1% EAA, 0.1% NEAA + 6.45% GA	6	299	281	276	12.0	54	108.0	14.8	13.0	116.7	— 21.7 ± 1.7
	5	0.2% EAA, 0.1% NEAA	6	301	285	290	12.0	54	55.2	29.5	11.6	41.6	+ 2.0 ± 1.0
B	2	0.1% EAA, 0.1% NEAA	6	297	278	276	11.8	54	40.1	14.5	15.6	40.1	— 15.5 ± 1.8
(undepleted,	3	0.1% EAA, 0.1% NEAA + 1.65% GA	6	295	275	276	12.0	55	58.6	14.7	15.3	56.9	— 13.5 ± 1.1
corn dextrin) [5]	4	0.1% EAA, 0.1% NEAA + 6.45% GA	6	297	275	271	11.9	54	110.7	14.6	16.2	106.1	— 11.6 ± 1.3
	5	0.2% EAA, 0.1% NEAA	6	293	275	276	12.0	55	58.7	29.4	17.7	40.8	+ 0.1 ± 0.9
C	1	Nitrogen-free	15	328	...	275	9.4	42	1.9	...	10.2	49.5	— 57.9 ± 1.4
(protein-	2	0.1% EAA, 0.1% NEAA	15	275	277	273	11.1	50	34.9	13.6	10.5	37.1	— 12.7 ± 0.7
depleted,	3	0.1% EAA, 0.1% NEAA + 1.65% GA	5	269	270	267	10.4	47	49.0	12.8	9.6	47.5	— 8.1 ± 1.3
sucrose)	4	0.1% EAA, 0.1% NEAA + 6.45% GA	5	267	265	265	11.2	50	102.7	13.7	10.8	98.9	— 7.0 ± 1.0
	5	0.2% EAA, 0.1% NEAA	6	267	266	269	10.8	48	50.8	26.6	9.8	33.6	+ 7.5 ± 0.6
D	1	Nitrogen-free	15	322	...	285	12.9	58	7.7	...	16.6	44.1	— 53.0 ± 1.2
(protein-	2	0.1% EAA, 0.1% NEAA	6	277	280	270	11.6	52	40.1	14.2	14.7	29.4	— 4.0 ± 0.9
depleted,	3	0.1% EAA, 0.1% NEAA + 1.65% GA	6	277	281	277	11.5	52	57.5	14.1	15.4	46.6	— 4.4 ± 1.4
corn dextrin)	4	0.1% EAA, 0.1% NEAA + 6.45% GA	5	280	286	283	11.7	53	110.2	14.4	16.4	97.6	— 3.8 ± 1.9
	5	0.2% EAA, 0.1% NEAA	6	278	288	290	11.7	53	57.4	28.8	15.7	34.9	+ 6.7 ± 0.6

[1] Essential amino acid nitrogen.

[2] $S_{\bar{x}} = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n(n-1)}}$.

[3] EAA, essential amino acids; NEAA, non-essential amino acids.

[4] Glutamic acid.

[5] Amidex, from Corn Poducts Refining Co.

the nitrogen balances was determined by the "*t*" test.[3] The value of "*t*" required for significance at the 1% level of probability was 2.92.

In series A (undepleted rats fed sucrose-containing diets), increasing the total nitrogen intake by the addition of 1.65% glutamic acid (diet 3) caused a statistically significant improvement in the balances when compared with those of control animals receiving diet 2 ($t = 5.06$), but when 6.45% of glutamic acid was fed (diet 4), the balances were not significantly different from those of animals fed the diet containing only 0.1% of the amino acids. No explanation is readily apparent for the finding that the addition of 17.9 mg of nitrogen from glutamic acid (diet 3) improves the balances, while 70.9 mg nitrogen per day (diet 4) does not. In series B, when corn dextrin was used instead of sucrose in the diets, neither level of glutamic acid improved the balances significantly. When a comparison was made between the balances obtained in series A and B on each diet, the differences were significant for diets 2, 3 and 4, but not for diet 5 (diet 2, $t = 4.17$; diet 3, $t = 2.43$, significant at the 5% level only; and diet 4, $t = 9.48$). The smaller difference obtained with diet 3 (3.6 mg per day) in comparison with the differences obtained on diets 2 and 4 (8.3 and 10.1 mg) suggests that the improvement in the balances brought about by glutamic acid and by corn dextrin are not additive. The animals did not achieve nitrogen equilibrium, as did those of an earlier study when corn dextrin was substituted for sucrose following a 4-week regimen of sucrose-containing diets during which large negative balances were maintained (Womack et al., '53).

In series C, using sucrose-containing diets and protein-depleted animals, both levels of glutamic acid brought about improvement; the nitrogen balances of animals fed diets 3

[3] The *t* value of the statistically significant difference between the averages of two sets of values was determined by the formula:

$$t = \frac{M_1 - M_2}{\sqrt{(S_{\bar{x}_1})^2 + (S_{\bar{x}_2})^2}}.$$

and 4 were significantly less negative than those of animals fed diet 2 ($t = 3.09$ and 4.76). In series D, using corn dextrin-containing diets and protein-depleted rats, supplementation with glutamic acid brought about no significant changes in nitrogen balances (table 1, diets 2, 3 or 4). Comparison of the nitrogen balances of series C and D indicates that there was a significant difference for the two groups of animals fed diet 2 ($t = 8.06$) but no significant difference between the balances of animals fed either level of glutamic acid (diets 3 and 4). However, this evaluation is complicated by small differences in weight, food and essential amino acid intakes of the groups of animals. For the depleted animals, it appears that if an improvement is brought about by the addition of glutamic acid to the sucrose rations, no further significant change is brought about by corn dextrin.

In all series (A to D), doubling the intake of essential amino acids (diet 5), irrespective of carbohydrate source, caused a significant improvement in the balances and the animals were in nitrogen equilibrium or in positive balance. Protein-depleted animals were able to store nitrogen at levels of essential amino acid intake at which undepleted animals were in equilibrium. Differences between the groups receiving the carbohydrates were not significant. Therefore, when the essential amino acid intake was adequate, the substitution of corn dextrin for sucrose was of no benefit. These findings are in accord with results recently reported by Harper and Katayama ('53), that when growing rats received sucrose diets containing 9% casein supplemented with essential amino acids which were limiting in casein, the difference in growth produced by feeding cornstarch instead of sucrose was almost eliminated.

Animals protein-depleted by being fed the nitrogen-free ration (diet 1) with sucrose had nitrogen balances significantly different only at the 5% level ($t = 2.58$) from those of animals depleted by being fed diet 1 with corn dextrin. Nitrogen intake was 5.8 mg per day greater for animals receiving corn dextrin. The total nitrogen excretion of the

two groups was almost the same, but urinary excretion of nitrogen was lower for animals receiving corn dextrin and fecal nitrogen higher by 6.4 mg, approximately the difference in nitrogen intake.

Microbiological analysis [4] of the essential amino acid content (excluding tryptophan) of the corn dextrin used in these experiments showed the following amounts, in milligrams per 100 gm dextrin: arginine, 4.4; histidine, 1.4; isoleucine, 1.0; leucine, 3.1; lysine, 2.3; methionine, 0.4; phenylalanine, 1.2; threonine, 1.1; and valine, 2.3. The nitrogen of this mixture of essential amino acids accounted for 34.4% of the total. Whether or not this combination of essential amino acids, added as a supplement to the diets containing 0.1% essential amino acids with sucrose, could cause the improvement in nitrogen balances when corresponding diets containing corn dextrin are fed will be investigated.

Liver composition

In every case, whatever the total nitrogen intake and prior state of nutrition, rats receiving diets containing only 0.1% of essential amino acids had livers whose absolute weight and whose weight as per cent of final body weight were higher when the carbohydrate was sucrose than when it was corn dextrin (table 2). Undepleted rats (series B) fed diets 2, 3 and 4 with corn dextrin had a normal percentage of liver water, whereas the rats fed the corresponding sucrose-containing rations (series A) did not. Rats protein-depleted for 18 days (diet 1, series C and D) had a normal percentage of liver water regardless of carbohydrate. Liver weight, liver weight as per cent of final body weight and per cent water content of the livers of rats fed 0.2% of essential amino acids (diet 5) with sucrose or corn dextrin were within the normal range.

The per cent of water in the livers remained essentially normal, irrespective of dietary carbohydrate, when the fat

[4] We wish to thank Mr. Amos Blum for carrying out the microbiological analyses.

TABLE 2

Average liver composition of undepleted (series A and B) and protein-depleted (series C and D) adult male rats receiving diets containing low levels of amino acids with and without supplementation with glutamic acid and with either sucrose or corn dextrin as the source of carbohydrate

SERIES NO.	DIET NO.[1]	NO. ANIMALS/ GROUP	LIVER WEIGHT	LIVER WT. AS PER CENT OF FINAL BODY WT.	TOTAL MOISTURE AND STANDARD ERROR	PER CENT MOISTURE	TOTAL PROTEIN AND STANDARD ERROR	PER CENT PROTEIN (wet basis)	TOTAL FAT AND STANDARD ERROR	PER CENT FAT AND STANDARD ERROR (wet basis)
			gm		*gm*		*gm*		*gm*	
A	2	5[2]	12.05	4.35	7.69 ± 0.37	64.2	1.68 ± 0.05	14.1	1.64 ± 0.39	13.0 ± 2.6
(undepleted,	3	5	11.17	4.10	7.00 ± 0.36	63.3	1.61 ± 0.03	14.7	1.83 ± 0.40	15.6 ± 2.6
sucrose)	4	6	11.30	4.12	6.92 ± 0.27	62.0	1.70 ± 0.06	15.2	2.06 ± 0.54	17.3 ± 3.2
	5	6	9.28	3.20	6.47 ± 0.11	69.7	1.69 ± 0.04	18.3	0.55 ± 0.03	5.9 ± 0.4
B	2	6	9.33	3.38	6.57 ± 0.21	70.4	1.52 ± 0.04	16.4	0.41 ± 0.07	4.3 ± 0.6
(undepleted,	3	6	8.69	3.15	6.05 ± 0.18	69.6	1.48 ± 0.04	17.0	0.43 ± 0.03	5.0 ± 0.2
corn dextrin)	4	6	8.01	2.95	5.64 ± 0.13	70.5	1.41 ± 0.04	17.7	0.41 ± 0.08	5.0 ± 0.8
	5	6	8.40	3.04	5.90 ± 0.22	70.4	1.55 ± 0.06	18.5	0.39 ± 0.06	4.5 ± 0.6
C	1	8[2]	9.10	3.22	6.27 ± 0.25	69.2	1.33 ± 0.04	14.7	0.65 ± 0.12	6.8 ± 1.0
(protein-	2	7[2]	14.80	5.36	8.24 ± 0.50	56.9	1.72 ± 0.09	11.9	3.76 ± 0.79	23.7 ± 3.1
depleted,	3	6	12.22	4.59	7.38 ± 0.32	60.6	1.54 ± 0.05	12.7	2.23 ± 0.30	18.0 ± 2.0
sucrose)	4	6	11.41	4.30	7.10 ± 0.37	62.6	1.57 ± 0.04	14.0	1.87 ± 0.36	15.9 ± 1.9
	5	6	9.23	3.43	6.29 ± 0.14	68.2	1.46 ± 0.06	15.9	0.51 ± 0.06	5.5 ± 0.7
D	1	7[2]	8.59	2.99	6.03 ± 0.42	70.5	1.33 ± 0.06	15.8	0.56 ± 0.18	5.9 ± 1.2
(protein-	2	6	8.01	2.97	5.42 ± 0.13	67.9	1.23 ± 0.04	15.5	0.62 ± 0.10	7.7 ± 1.1
depleted,	3	6	8.84	3.18	5.87 ± 0.22	66.5	1.34 ± 0.06	15.2	0.98 ± 0.19	10.9 ± 1.9
corn dextrin)	4	6	9.34	3.36	6.10 ± 0.30	65.5	1.43 ± 0.04	15.4	1.12 ± 0.17	11.7 ± 1.3
	5	6	8.75	3.02	5.86 ± 0.10	67.0	1.31 ± 0.04	15.0	0.71 ± 0.08	8.0 ± 0.7
76 days old	Stock	8[3]	11.63	3.81	8.12 ± 0.46	69.8	2.39 ± 0.11	20.6	0.18 ± 0.01	1.5 ± 0.1
91 days old	Stock	8[4]	11.41	3.37	7.94 ± 0.44	69.7	2.40 ± 0.13	21.0	0.23 ± 0.05	1.9 ± 0.3
79–92 days old	Stock	7[5]	9.81	3.25	6.86 ...	69.9	2.16 ± 0.08	22.1	... ...	.. ..

[1] See table 1 for description of diets.
[2] Different animals from those for which nitrogen balances were reported in table 1.
[3] Average wt. = 304 gm.
[4] Average wt. = 337 gm.
[5] Average wt. after 4 hrs. fasting = 301 gm.

content was normal. There was an inverse relationship between per cent of water and per cent of liver fat. Total moisture was higher in the livers of the animals fed the rations containing sucrose. However, no final conclusions can be drawn regarding differences in water content for the two carbohydrates, since glycogen was not determined. It has been demonstrated (Fenn, '39) that glycogen is deposited in the liver of rats with such an amount of water that the per cent of water in the whole liver does not change. In the present study, it was found that the removal of food 4 hours prior to removing the livers of rats fed the stock diet caused an appreciable reduction in liver weight, 67% of which was accounted for by change in the amount of liver water. Most of the remainder of the weight change was probably due to loss of glycogen.

The per cent of protein ($N \times 6.25$) in the livers of rats of series A and C fed 0.1% of amino acids and sucrose was lower than that of animals of series B and D fed the corresponding diets and corn dextrin (diets 2, 3 and 4). When the larger amounts of amino acids were fed (diet 5), there was no difference in per cent of protein attributable to the carbohydrate. As expected, the per cent of protein in the livers of protein-depleted animals was lower than that in the undepleted groups, regardless of the carbohydrate. However, the total amount of liver protein of animals receiving sucrose and 0.1% amino acids was significantly higher than that of the corresponding groups of animals fed corn dextrin.

When the protein-depleted animals were compared, the livers of the animals of series C receiving diets 2, 3 and 4 with sucrose had protein values not only significantly higher than those of the animals receiving corn dextrin (series D), but also significantly higher than the value for animals fed the nitrogen-free ration for 18 days, which represents the condition of the livers of the animals at the time they were placed upon the amino acid diets. In contrast, rats fed diet 5 with double the amount of essential amino acids had no more protein in the liver than rats fed the nitrogen-free

diet for 18 days. The livers of the rats fed diets 2 to 5 with corn dextrin (series D) also contained no more protein than those of animals fed the nitrogen-free diet for 18 days (differences between these groups were not statistically significant).

The livers of animals ingesting the stock ration had 1.5 and 1.9% fat (wet basis) at 76 and 91 days of age. Since the fat content of the diet influences liver fat accumulation, values for liver fat of 4 to 6% were considered normal for the experimental diets, which contained 15% fat. Undepleted animals fed the rations containing sucrose with or without glutamic acid (diets 2, 3 and 4) had much higher percentages of liver fat than animals fed corn dextrin. These findings were confirmed in the protein-depleted animals, although in contrast to the undepleted animals, the per cent liver fat of the animals receiving corn dextrin was higher than normal. It should be pointed out that in animals protein-depleted for 18 days on rations containing either sucrose or corn dextrin, per cent liver fat was normal or only slightly elevated; the addition of the lower levels of amino acids (diets 2, 3 and 4) to the sucrose-containing rations brought about large increases in the fat content, and the higher levels of amino acids (diet 5) resulted in normal liver fat.

When grams of fat in the liver were compared, the rats of series A receiving diets 2 to 5 containing sucrose had significantly more liver fat than the rats of series B fed corresponding diets containing corn dextrin. Within series A and C, but not within series B and D, doubling the essential amino acid intake of the animals significantly decreased the amount of liver fat. A comparison between the protein-depleted groups (series C and D) shows significantly more fat in the livers of rats fed diets 2 and 3 containing sucrose than in those of animals fed corresponding diets containing corn dextrin. The amount of fat in the livers of animals fed the protein-free rations was not significantly different from that of animals fed 0.2% of the essential amino acids (diet 5) with either carbohydrate. The data suggest that corn dex-

TABLE 3

Average liver composition of undepleted (series A-1) or protein-depleted (series C-1) adult male rats receiving diet 2 in which the carbohydrate was sucrose for 6 weeks, or sucrose for three weeks and corn dextrin for three weeks

SERIES NO.	CARBOHYDRATE IN RATION	NO. ANIMALS/ GROUP	LIVER WEIGHT	LIVER WT. AS PER CENT OF FINAL BODY WT.	TOTAL MOISTURE AND STANDARD ERROR	PER CENT MOISTURE	TOTAL PROTEIN AND STANDARD ERROR	PER CENT PROTEIN (wet basis)	TOTAL FAT AND STANDARD ERROR	PER CENT FAT AND STANDARD ERROR (wet basis)
			gm		*gm*		*gm*		*gm*	
A-1	Sucrose, 6 weeks	3	12.92	4.84	7.63 ± 0.29	59.3	1.67 ± 0.08	12.9	2.54 ± 0.46	19.4 ± 2.5
	Sucrose, 3 weeks; Corn dextrin, 3 weeks	3	11.03	3.88	6.88 ± 0.70	62.1	1.57 ± 0.07	14.4	1.68 ± 0.12	15.3 ± 1.2
C-1	Sucrose, 6 weeks	8	12.46	4.63	7.82 ± 0.27	63.0	1.71 ± 0.05	13.8	2.06 ± 0.28	16.1 ± 1.6
	Sucrose, 3 weeks; Corn dextrin, 3 weeks	8	9.43	3.42	6.21 ± 0.26	66.1	1.38 ± 0.05	14.7	1.09 ± 0.17	11.3 ± 1.4

trin in the diet is associated in some fashion with the use of liver protein, or amino acids that would become liver protein, in retarding the accumulation of liver fat and perhaps in reducing urinary nitrogen losses.

The effects on liver composition of changing the carbohydrate in diet 2 fed both undepleted (series A-1) and protein-depleted (series C-1) animals are shown in table 3. Although the fat content was not reduced to normal when corn dextrin was substituted in the diet after three weeks of sucrose feeding, a reduction occurred in per cent of fat, with an even greater reduction in amount.

The lipotropic effect of protein and certain essential amino acids has been repeatedly demonstrated. The lipotropic activity of methionine is well-known. Recently, Singal and co-workers ('53) reported the lipotropic activity of threonine and lysine. Litwack, Hankes and Elvehjem ('52) found that livers of growing rats fed diets containing 9% casein with 0.2% L-cystine and corn dextrin as the carbohydrate had less fat than those of animals receiving identical diets except that sucrose replaced dextrin. Although the corn dextrin used in the present study contained small amounts of threonine, lysine and methionine, it seems unlikely that the amounts received in the corn dextrin, per se, were sufficient to exert any significant effect upon the accumulation of liver fat. The lipotropic activity of choline is well-established, but it does not seem possible that the small amount of choline known to be present in corn dextrin would have a marked influence upon liver fat, particularly since 12 gm of these diets contained 24 mg choline chloride. There are conflicting reports concerning the role of vitamin B_{12} in liver fat accumulation. However, in the present study, all animals consuming 12 gm of food per day received 0.36 μg of B_{12}.

Hundley ('49) showed that the niacin requirement of the growing rat fed rations containing 9% casein was dependent upon the carbohydrate fed. When sucrose or fructose was the carbohydrate, the requirement was higher than when diets containing starch, dextrin or glucose were used. Whether

or not the animals in the present study fed corn dextrin diets containing 0.1% amino acids had a lower niacin requirement than animals fed sucrose is not known, and the lipotropic effect of niacin under the conditions of this experiment has not been determined.

SUMMARY

Animals fed 0.1% of the essential amino acids with sucrose as the carbohydrate had more negative nitrogen balances and a greater accumulation of fat in the liver than animals fed the same quantities of amino acids with corn dextrin. When the amounts of the essential amino acids were doubled (0.2%), differences in nitrogen balances were eliminated for both undepleted and protein-depleted animals fed the two carbohydrates. Glutamic acid fed at a level of 1.65% significantly improved the nitrogen balances of animals fed sucrose, but was without effect on corn dextrin-fed rats; 6.45% of glutamic acid caused a significant improvement in the nitrogen balances of protein-depleted animals fed sucrose but had no effect on the nitrogen balances of animals fed corresponding corn dextrin diets.

The livers of animals fed sucrose, except for the protein-free diet, contained more protein than those of animals fed corresponding diets with corn dextrin; the differences in protein content were statistically significant when the nitrogen balances differed between the groups.

It is suggested that corn dextrin in the diet in some fashion facilitates the use of liver protein, or amino acids that would become liver protein, and results in reducing nitrogen output and perhaps in retarding the accumulation of liver fat.

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RHYTHMIC RESPONSES OF CHICKS TO INJECTED THIAMINE [1, 2, 3]

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THREE FIGURES

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In a previous study the effectiveness of dietary antibiotics in reducing the thiamine requirement of chicks was shown to depend upon the route by which the thiamine was administered (Waibel, Cravens and Baumann, '53); e.g., penicillin significantly increased the effectiveness of thiamine in the diet, while it was without effect when the limiting vitamin was given parenterally. The present report concerns the relationship between frequency of thiamine injection and efficiency of growth. The growth curves show a marked periodicity, dependent primarily on thiamine injection and secondarily on an underlying diurnal rhythm.

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EXPERIMENTAL

The chicks were the progeny of New Hampshire males and Single Comb White Leghorn females fed diet B-1 of Robblee et al. ('48). They were kept in standard electrically heated batteries and under continuous artificial illumination, although daylight also entered through a few small windows. The following diet was fed from one day of age: dextrin (autoclaved starch) 60, hot alcohol-extracted casein 18, gelatin 10, salts V (Briggs et al., '43) 6, soybean oil 4, glycerol 1, feeding oil (2,000 A–300 D) 0.5, DL-methionine 0.3, choline chloride 0.2, and inositol 0.1 gm per 100 gm; the following vitamins were added, in milligrams per kilogram: biotin 0.2, menadione 0.5, thiamine · HCl 3.0, α-tocopherol 3.0, pyridoxine · HCl 4.0, folic acid 4.0, riboflavin 6.0, calcium pantothenate 20, niacin 50, *p*-aminobenzoic acid 100, and vitamin B_{12} 0.030. At 7 days of age the thiamine-free diet was fed, and on the 9th and 10th days each chick received 9 μg of thiamine by injection, an amount selected to support about half-maximum growth. The chicks were weighed individually on the 10th day and those showing extremes in weight discarded. Final separation into uniform groups of 10 chicks each was made after a further weighing on the 11th day.

In a typical series, 6 groups received thiamine by subcutaneous injection for 16 days, while two groups received either 1.0 or 6.0 μg of thiamine per gram of diet. The injected groups all received the same total amount of thiamine. The injection schedule follows:

GROUP	TIME OF INJECTION	QUANTITY OF SOLUTION INJECTED	FREQUENCY OF INJECTION
		ml	*hr.*
1	8:00 A.M. and 8:00 P.M.	0.05	12
2	8:00 A.M. and 8:00 P.M.	0.20	12
3	8:00 A.M.	0.10	24
4	8:00 P.M.	0.10	24
5	8:00 A.M.	0.20	48
6	8:00 A.M.	0.40	96

The doses of injected thiamine were increased as the experiment progressed, the daily amounts administered being: one to 4 days, 12 μg; 5 to 8 days, 14 μg; 9 to 12 days, 16 μg; and 13 to 16 days, 17 μg. The injected route was subcutaneous,

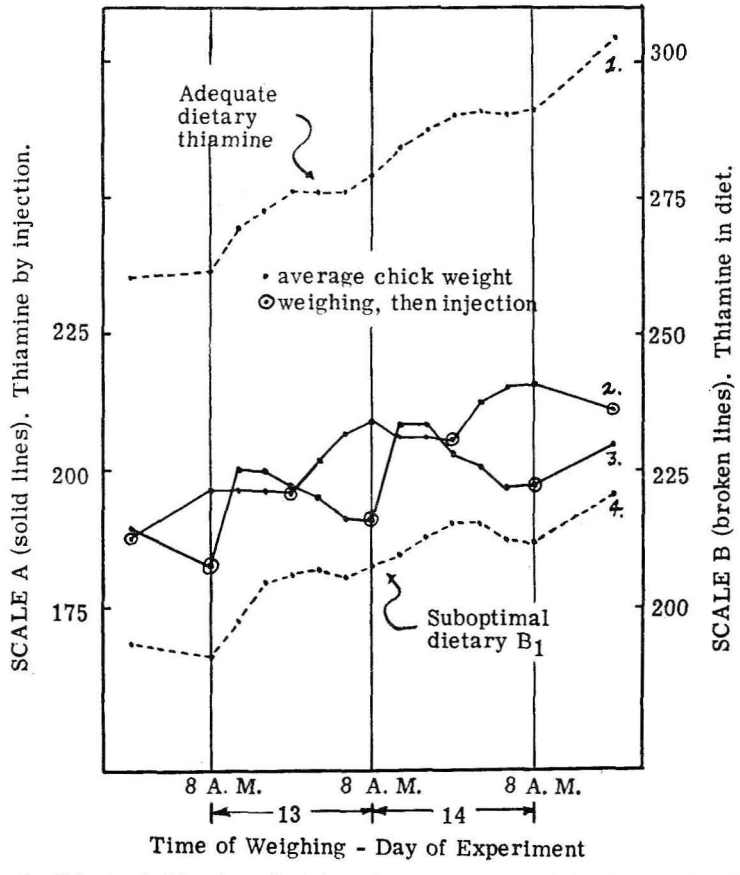

Fig. 1 Effect of thiamine administration on 4-hour weight changes in chicks (10 chicks per group).

over the breast muscle. Chicks receiving dietary thiamine also received 3 μg by injection at the beginning of the experiment to minimize the danger that certain birds might not eat. Weighings were made by groups at 8:00 A.M. and 8:00 P.M. each day, except at the beginning and end of the experiment, when individual weighings were made. On certain days

weighings were made at 4-hour intervals. During the first 8 days of the experiment the chicks were fed at 8:00 A.M. daily, and thereafter they were fed both in the morning and in the evening; in all cases feed was supplied ad libitum. The drinking water was renewed each morning.

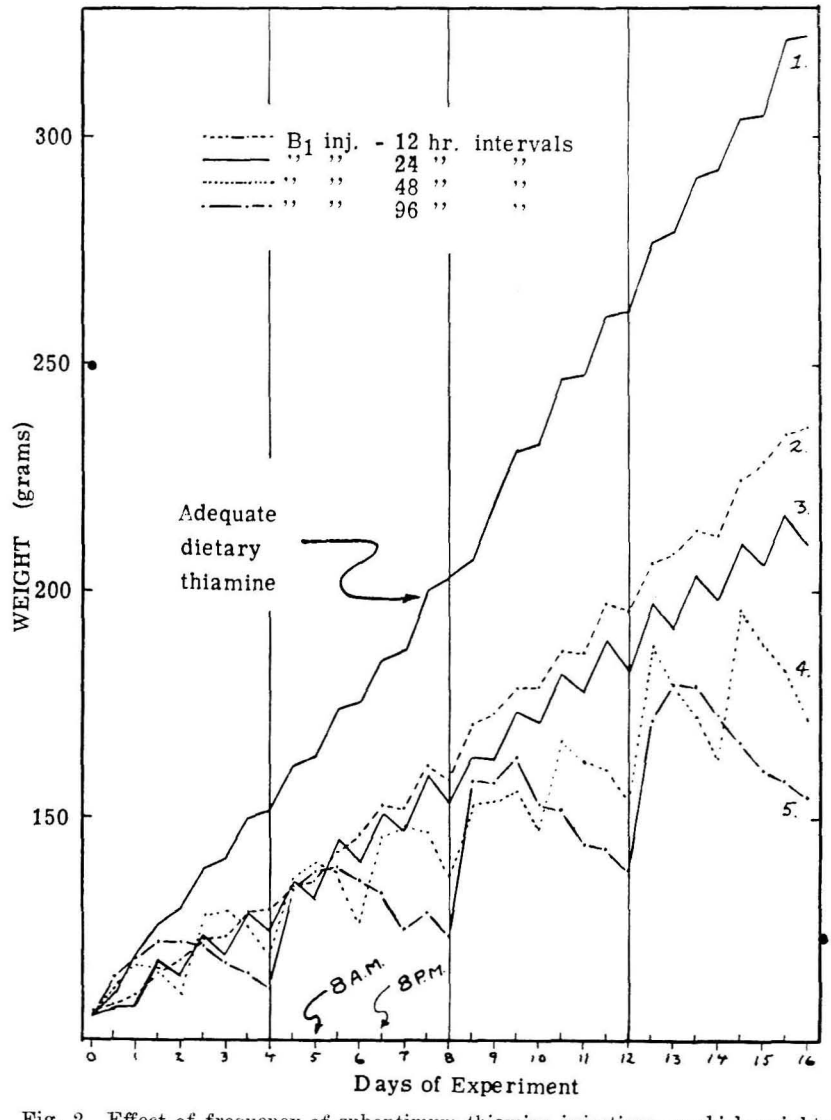

Fig. 2 Effect of frequency of suboptimum thiamine injections on chick weights (10 chicks per group).

RESULTS

With thiamine in the diet (fig. 1, lines 1 and 4; fig. 2, line 1) a diurnal growth rhythm occurred even though the chicks were subjected to continuous artificial illumination and had

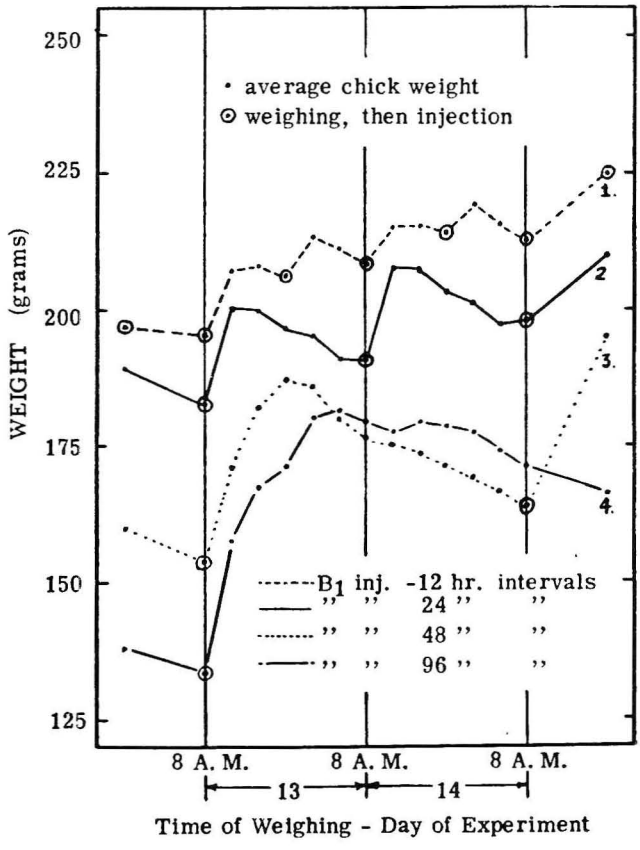

Fig. 3 Effect of frequency of thiamine injection on 4-hour weight changes in chicks (10 chicks per group).

feed available constantly. Birds receiving adequate dietary thiamine gained a small amount of weight during the night, while birds receiving the suboptimum level usually lost a little at that time. This diurnal rhythm was intensified in the groups receiving suboptimum amounts of thiamine par-

enterally at 8:00 A.M. (fig. 1, line 3; fig. 2, line 3), the sharp increase in weight by 6 hours after injection being followed by a decided loss in weight. The normal diurnal rhythm could be reversed by injecting the vitamin at 8:00 P.M. (fig. 1, line 2). In this case moderate increases in weight continued for 12 hours after injection (night) and the decline in weight during the day was relatively slight.

Very marked rhythmic changes in weight resulted when thiamine was injected at less frequent intervals, such as every second or every 4th day (figs. 2 and 3), and the terminal weights varied directly with the frequency of injection, although all injected groups received the same amount of thiamine. In this experiment the highest final weight and least intense fluctuation were in a group injected twice daily (fig. 3, line 1), 236 ± 18 gm; those injected daily (morning) weighed 210 ± 18 gm, while those injected once in 4 days weighed only 155 ± 11 gm. Thus, the wastefulness of semi-weekly injections of thiamine is clearly evident.

The increased efficiency of more frequent injection may be seen from a calculation of the micrograms of thiamine required per gram of gain in weight: injection twice daily, 1.82 and 1.78 for 0.05 and 0.20 ml of injected solution, respectively; injection once daily, 2.22; injection every other day, 3.53; and injection every 4th day, 4.82. The chicks receiving sub-optimum dietary thiamine (no antibiotic) required 2.15 μg of thiamine per gram of gain. The volume of injection solution did not appear to affect the growth response; birds injected twice daily with 0.05 ml of solution weighed 236 ± 18 gm after 16 days, while others receiving the same amount of thiamine in 0.20 ml weighed 239 ± 18 gm.

DISCUSSION

The periodicity in the growth responses observed in the present experiment parallels diurnal changes in other biological phenomena (Brody, '45). These include diurnal blood

sugar changes in the white rat (Pitts, '43), blood lactate levels of dogs (Swan, '43), blood and urine glucose levels in diabetics (Izzo, '49), feeding activity of salmon and trout (Haar, '42), and changes in the excretion of chromic oxide and lignin in bovines (Kane et al., '52). In chickens the maximum energy metabolism occurs at about 8:00 A.M. and the minimum at about 8:00 P.M. (Barott et al., '38). According to the data of these workers, oxygen consumption for chicks the size and age used in our experiments should be about 22% greater in the morning than in the evening. Since increased activity causes greater oxygen consumption (Hart, '52), the greater weight increment obtained in this study during the day is in harmony with diurnal variations in metabolism.

The marked increases in weight observed in the present study after thiamine injection were probably not due to any fundamental periodicity but rather were the result of a sudden increase in the intake of food and water. In fact, the normal periodicity (gain in weight during the day, loss at night) could be reversed by injecting thiamine only in the evening. Significantly, however, the magnitude of the subsequent gains in weight and the later losses was not nearly so great as when the vitamin was injected in the morning. Both curves can therefore be considered as the resultants of two phenomena, the broader "fundamental rhythm" plus the effects of a thiamine-induced intake of food and water. When the vitamin was injected in the morning, the two phenomena reinforced one another and the diurnal fluctuations became very great. However, when the vitamin was injected in the evening, the thiamine-induced intake of food took place when the more fundamental rhythm tended toward a decline in weight, and the net result was a relatively smooth curve, with the effect of the injection predominating somewhat.

Others have also succeeded in overcoming the normal metabolic rhythm, e.g., by exposing rats to continuous light and supplying one-eighth of the daily diet at three-hour intervals (Herring and Brody, '38). An intensification of the normal

rhythm in metabolism resulted when rats were fed relatively large amounts of food at 4:00 P.M. (Brody, '45, p. 240). These effects were designated "an algebraic summation of the effects of feed-intake and diurnal rhythm."

In a previous study the amounts of thiamine necessary for a unit increase in weight were usually less when the vitamin was injected than when it was incorporated into the diet (Waibel et al., '53), although the efficiency of dietary thiamine could be increased by stabilizing the vitamin against loss *in vitro* by glycerol (Kandutsch and Baumann, '53) and against the effect of microorganisms by penicillin (Lih and Baumann, '51). The present experiments show that parenteral administration does not necessarily result in a maximum utilization of the limiting vitamin, unless the injections are given at very frequent intervals, and that the process of injecting vitamins at intervals of several days may be very wasteful indeed.

SUMMARY

1. The growth of chicks fed diets suboptimum or adequate in thiamine took place mainly during the day, even though the laboratory was illuminated continuously.

2. When suboptimum amounts of thiamine were injected, chick weight increased rapidly for a few hours, followed by a decline in weight. The duration and intensity of the "growth" phase varied with dosage, and was sufficient to overcome and to reverse the normal diurnal rhythm.

3. Total growth varied with the frequency of injection; thiamine injected every other day was utilized less effectively than that injected daily or twice a day. Large injections were relatively wasteful.

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THE CORRELATION BETWEEN FEED CONSUMPTION AND FECAL FLORA IN CHICKS[1]

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FOUR FIGURES

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Scott and Glista ('50) reported that the inclusion of aureomycin in a chick diet gave a slight but insignificant growth response with ad libitum feeding and no growth response when feed intake was equated. Brown et al. ('52), working with pigs, observed a marked growth response to aureomycin on an unlimited feeding regime but no significant difference when the pigs were given an equalized feed intake. The latter workers concluded that aureomycin stimulates the rate of gain in the healthy pig by some mechanism or mechanisms operating almost entirely by way of an elevated daily feed consumption.

Antibiotics have not been found to stimulate the growth of germ-free chicks (Luckey, '52). Furthermore, little or no antibiotic growth response has been obtained in chicks maintained in quarters previously unused for rearing poultry (Coates et al., '51, '52; Hill et al., '53; Lillie et al., '53). These findings, together with the fact that the chemotherapeutic activity of these drugs is carried on through their effect on microorganisms, suggest that the intestinal flora is in some way involved in the growth response to antibiotics.

[1] This work was supported in part by a grant from Merck and Co., Inc., Rahway, N. J.

From a consideration of the above evidence it appeared to us that the effect of antibiotics on the intestinal flora may well be the primary mechanism which in turn influences feed consumption and thereby stimulates growth. An experiment was therefore carried out in an attempt to correlate daily feed consumption and fecal bacterial counts of chicks fed diets with and without an antibiotic, both on a free-choice and an equalized feed intake basis.

MATERIALS AND METHODS

Day-old New Hampshire ♂ ♂ × Columbian Rock ♀ ♀ male chicks were weighed individually and distributed into experimental groups on the basis of weight. Each group was comprised of 18 chicks. The birds were reared in electrically heated battery brooders with raised wire floors.

The basal diet had the following percentage composition: wheat 50, corn 10, soybean oil meal (44% protein) 28, soybean oil 1, dehydrated cereal grass 2.5, meat meal 1, fish meal 3, dried buttermilk 2, ground limestone 0.75, steamed bone meal 1.25, iodized salt 0.25, fish oil (300 I.C.U., D, and 1,500 I.U., A, per gram) 0.25. In addition, to each 100 lb. of diet the following were added: manganese sulfate 7.12 gm, riboflavin 150 mg, DL-methionine 11.4 gm and vitamin B_{12} supplement (3 mg B_{12}/pound) 45.4 gm. Various groups were fed this basal diet or the same diet plus 15 mg per kilogram of procaine penicillin G, both on the basis of free choice and with controlled feed intake. Both groups of birds on the controlled feed intake regime were fed the same amount of feed daily as was consumed by the group on the free choice regime which consumed the least on the previous day. The group fed the basal diet controlled feed consumption, with the exception of a single day, for the 28-day period.

Determinations were made daily for a 28-day period of body weight, feed consumption and fecal bacterial counts. From 29 days through 7 weeks of age those groups which had been on the controlled feed intake regime were given feed ad libi-

tum, while the remaining groups, as before, continued to receive an unlimited supply of feed. Weight and feed consumption data were kept on a weekly basis for this latter period and fecal floral analyses were not made.

Bacteriological examinations of the pooled, fresh feces (collected on waxed paper) of each of the 4 groups of birds were made as follows: a 50-gm sample of the thoroughly mixed feces was ground with 450 ml of sterile distilled water in a Waring Blendor for 5 minutes. From this initial dilution, serial dilutions of 10^{-2} to 10^{-10} were made and 1-ml aliquots of each were transferred, in duplicate, to the following media: (a) Levine EMB (Difco) plates for the enumeration of the coliform group of bacteria; (b) tomato juice agar (Difco) plates for the cultivation of the lactobacilli; (c) plates of tryptone glucose extract agar (Difco) with 2% yeast extract (Difco) and 2% skim milk added for the isolation of aerobes; (d) Linden thioglycollate medium (Difco) in tubes, with 2% agar added to each tube to form a plug, for the enumeration of anaerobes by the MPN method; (e) "S.F." broth of Hajna and Perry ('43) in tubes for estimation of numbers of enterococci, employing the MPN method.

The above media were incubated at 37°C. and readings were made at the end of three days, with the exception of the "S.F." broth tubes, which were read after 5 days' incubation at 37°C.

A 1-gm sample of the thoroughly mixed feces was taken each day, from each group, and dried to constant weight at 100°C. Bacterial counts were calculated on the basis of the dry weight of the feces.

RESULTS

The weight and feed efficiency data are presented in table 1. Statistical examination of the weight data by the "t" test (Snedecor, '46) indicated that penicillin resulted in a highly significant weight increase at both 4 and 7 weeks of age in the case of birds having free access to feed. In the groups where restricted feeding was practiced for 4 weeks, followed

by free choice feeding for three weeks, while there was no response to penicillin at the end of the initial period, it is evident that the antibiotic tended to result in some growth stimulation during the period from 4 through 7 weeks. The difference at 7 weeks of age did not prove to be statistically significant. Penicillin did not materially improve feed efficiency with either method of feeding.

Values for daily feed consumption (in grams) per chick and per unit body weight are plotted for the groups fed by free choice in figure 1. These data indicate that penicillin resulted in greater daily feed consumption per chick almost

TABLE 1

Influence of penicillin and method of feeding on weight and feed efficiency of chicks

GROUP NO.	DIET	FEEDING METHOD	RESULTS AT 4 WEEKS OF AGE		RESULTS AT 7 WEEKS OF AGE	
			Ave. wt.	Feed/ gain	Ave. wt.	Feed/ gain
			gm		*gm*	
1	Basal	Free choice for	394	1.85	939	2.21
2	Basal + penic.	7 weeks	431	1.83	1,036	2.17
3	Basal	Controlled for 4 wk.,	376	1.90	933	2.23
4	Basal + penic.	free choice for 3 wk.	377	1.89	959	2.23

without exception throughout the 28-day period. On the other hand, feed consumption per unit of body weight was increased by the antibiotic only to about 16 days of age, after which the group receiving penicillin consumed slightly less feed than the control group. Penicillin continued to result in greater feed consumption per chick during the period from 4 to 7 weeks of age but caused a slight decrease in feed consumption per unit body weight.

Since feed consumption per unit body weight was increased by the antibiotic for only the first 16 days, it was of interest to determine the influence of penicillin on the rate of growth during this time as compared with the effect during the 16- to 28- and 28- to 49-day periods. Rate of growth was calcu-

lated using the logarithmic method [2] (see Brody, '27). R values of 9.73 and 10.29%, 6.88 and 6.95% and 4.14 and 4.18% were obtained for the group fed the basal diet and that fed the basal plus penicillin for the one- to 16-, 16- to 28- and 28- to 49-day periods, respectively. It is apparent from these values that the difference in the rate of growth due to penicillin was greatest during the time feed consumption was being increased on a unit weight basis, and that there was a relatively small difference in the later periods.

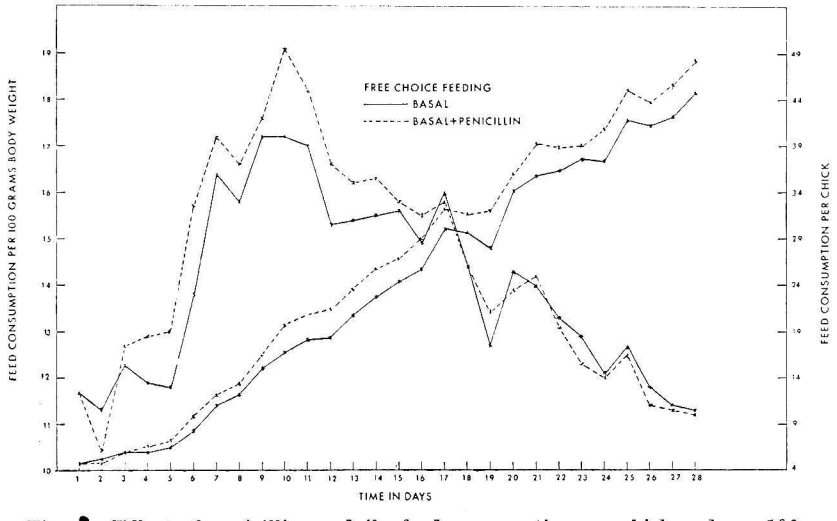

Fig. 1 Effect of penicillin on daily feed consumption per chick and per 100 gm of body weight.

Bacteriological findings

Daily coliform counts for the groups on the free choice feeding regime are plotted in figure 2. It may be noted that the coliform counts were very variable for about the first 7 days, with neither group showing consistently higher or lower counts than the other. From 7 to 28 days of age, however, the group receiving penicillin showed consistently higher

[2] $R = \frac{\log W_2 - \log W_1}{t_2 - t_1} \times 2.303 \times 100$, where W_1 and W_2 are the first and final weights at times t_1 and t_2.

coliform counts than the control group, and the numbers of coliforms tended to increase gradually in both groups during this period. Because of space limitation, the coliform counts for the groups on restricted feed intake will not be presented in detail. In this case the results were more variable than with the groups fed ad libitum. Here again, however, the counts tended to increase from about 7 to 28 days of age in both groups, and the birds receiving penicillin showed somewhat higher coliform numbers than the control birds.

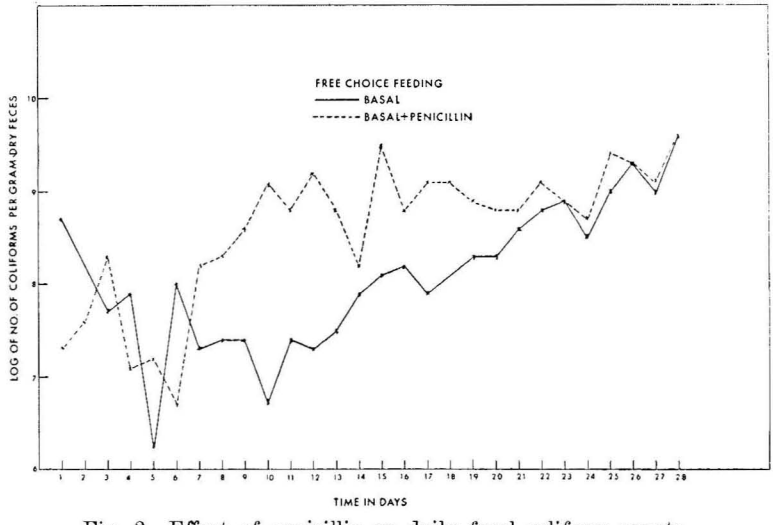

Fig. 2 Effect of penicillin on daily fecal coliform counts.

Enterococci counts are plotted in figures 3 and 4 for the period from 5 to 28 days of age. Exact enterococci counts were not obtained for the first 4 days, since the dilutions used proved to be too low. The enterococci counts for all groups were greater than 10^7 for each of the first 4 days. Enterococci counts tended to decrease with advancing age in the case of all groups. While the numbers of these organisms were extremely variable throughout, it is apparent that the groups fed penicillin showed higher enterococci counts, starting at about 15 days of age with ad libitum feeding and

at 18 days of age when feed intake was equated; the counts continued at the higher levels from then until the end of the 28-day period, with the single exception of the 20th day with the group fed by free choice.

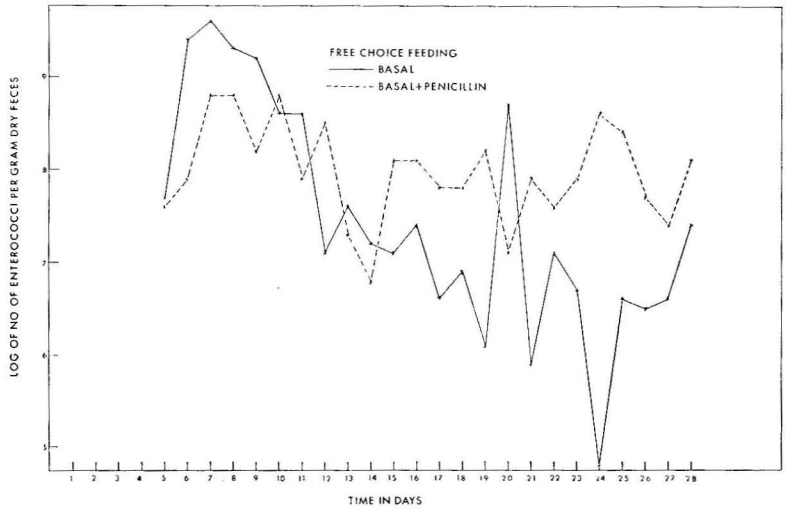

Fig. 3 Effect of penicillin on daily fecal enterococci counts.

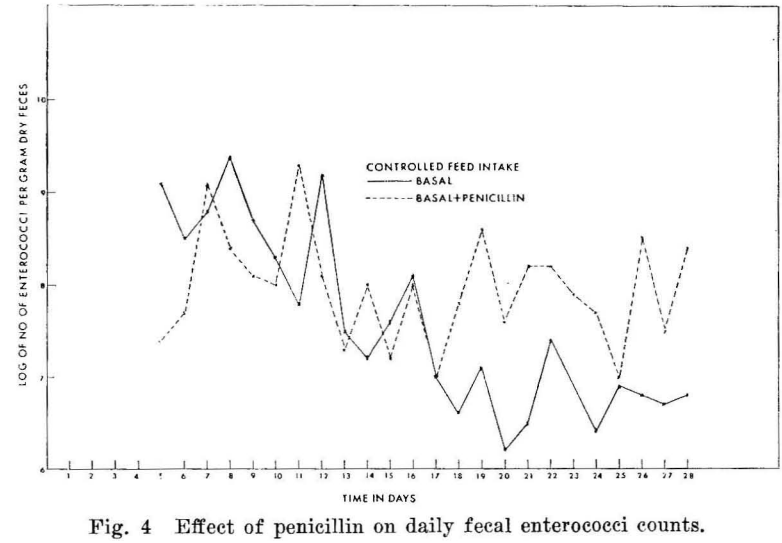

Fig. 4 Effect of penicillin on daily fecal enterococci counts.

The daily counts of lactobacilli are presented in table 2. It may be noted that penicillin tended to reduce the numbers in this group of microorganisms for about the first 9 days and to increase the counts from then until 28 days of age in birds fed by free choice. On the other hand, when feed intake was equated, there were no consistent differences in counts due to the antibiotic. The results of the aerobic and anaerobic counts are too voluminous to be presented in detail. It should be pointed out, however, that the influence of penicillin on the

TABLE 2

Effect of penicillin and method of feeding on fecal lactobacilli counts

AGE IN DAYS	FREE CHOICE FEEDING		CONTROLLED FEEDING	
	No penic.	+ Penic.	No penic.	+ Penic.
2	9.5 [1]	9.5	9.6	9.6
3	9.4	9.3	9.6	9.3
4	9.2	8.9	9.5	9.5
5	9.0	8.2	8.5	8.4
6	9.3	8.0	9.5	8.4
7	9.3	8.9	9.2	9.4
8	9.5	9.0	9.2	9.4
9	9.0	9.1	9.0	9.2
10	9.0	9.3	7.7	9.3
11	8.4	9.3	8.8	9.1
12	8.5	9.1	9.6	9.5
13	9.0	9.0	9.5	9.3
14	9.0	8.8	9.5	10.0
15	8.7	9.6	9.4	9.5
16	8.2	8.9	9.6	9.5
17	9.1	9.6	9.1	9.1
18	8.8	9.5	9.4	9.5
19	8.5	9.1	9.3	9.1
20	9.0	9.1	8.9	9.1
21	8.9	9.2	9.7	9.6
22	9.0	9.3	9.8	9.8
23	9.4	9.4	9.7	9.7
24	8.7	9.2	9.4	9.1
25	9.4	9.5	9.7	9.7
26	9.5	9.4	9.5	10.0
27	9.0	9.5	9.4	9.6
28	9.7	9.6	9.7	9.9

[1] Log. of number of bacteria per gram of dry feces.

counts of these groups of microorganisms was very similar to its effect on the lactobacilli group. There appeared to be no trend toward either an increase or a decrease in the numbers of lactobacilli, anaerobes or aerobes, with advancing age of the birds.

Correlation between feed consumption and fecal flora

The correlation coefficients between the daily counts for the various microorganisms and daily feed consumption per chick were calculated for the period from 7 through 28 days of age. The erratic nature of the counts for about the first 6 days suggested that this was a period of adjustment for the microorganisms. For this reason the early values were not included in the calculations. Highly significant positive correlations of 0.950 and 0.636 were found between feed consumption per chick and coliform counts for the groups fed the basal diet and this diet supplemented with penicillin, respectively, on a free choice basis. Significant positive correlations of 0.614 and 0.586 were obtained between feed consumption per chick and coliform counts for the groups fed the basal diet and this diet plus penicillin, respectively, on an equalized feed intake basis. The correlation coefficients between feed consumption per chick and enterococci counts for the groups fed the basal diet were — 0.694 and — 0.847, respectively, for the chicks on free choice and controlled feed intake regimes. These values proved to be highly significant. Negative but non-significant correlations also existed between feed consumption per chick and enterococci counts for the groups receiving penicillin. No significant correlation was found between feed consumption per chick and lactobacilli, aerobes or anaerobes.

Correlation coefficients between daily microorganism counts and feed consumption per 100 gm of body weight were calculated for the period from 6 through 29 days of age for the groups on ad libitum feed intake. Correlations of — 0.769 and — 0.335 were found between feed consumption per unit body weight and coliform counts for the groups fed the basal

diet and this diet plus penicillin, respectively. The former value proved to be highly significant, while the latter was not significant. Correlations of 0.625 and 0.238 were obtained by comparing feed consumption per unit body weight and enterococci counts for the groups fed the basal diet and this diet plus the antibiotic, respectively. The former value was highly significant and the latter non-significant. No significant correlations were found to exist between feed consumption per unit body weight and the daily counts of lactobacilli, aerobes and anaerobes.

Since penicillin resulted in an increase in feed consumption out of proportion to body weight only to about 16 days of age, it was of interest to determine the correlation between this measurement and the counts of certain microorganisms for the period from 6 through 16 days of age. Again, as in the period from 6 through 28 days of age, there was a significant correlation (— 0.664) between feed consumption per unit body weight and coliform counts for the period from 6 through 16 days in the group fed the basal diet. On the other hand, with the group fed penicillin, the correlation between feed consumption and coliform counts, for this period, proved to be positive (0.305) but non-significant. Correlations of 0.666 and 0.274 were obtained between feed consumption per unit body weight and enterococci counts for the group fed the basal diet and that receiving penicillin, respectively, for this early period. Only the former value proved significant. Correlation coefficients were not calculated for other microorganisms during the period from 6 through 16 days of age.

DISCUSSION

The present results confirm the findings of Brown et al. ('52) with pigs and indicate that the growth response of chicks to penicillin is dependent upon an increase in feed consumption. In contrast to the findings of Scott and Glista ('50), a significant growth response was obtained with an ad libitum feeding regime. While penicillin improved feed efficiency only slightly in the present work, more response is

ordinarily obtained with birds fed by free choice. An improvement in feed efficiency in no way detracts from the belief that the growth response is dependent upon an increase in feed consumption, since with the faster rate of growth a lesser proportion of the feed would be needed for maintenance.

Since body weight is known to be an important factor influencing feed intake, it is interesting to consider the effect of penicillin on this measurement on a unit weight basis. The fact that penicillin resulted in an increase in feed intake per unit body weight only for about the first 16 days suggests that the main growth-stimulating effect occurs during this initial period and that from then on the birds consume more feed and therefore maintain their weight advantage largely because they were heavier at 16 days of age. This is borne out by the observation that the growth rates of the two groups were very similar after the initial 16-day period. The finding that no growth response was obtained in birds fed on a restricted program for the first 28 days of life, but that a response did occur when these same groups were allowed free access to feed through 7 weeks of age, indicates that the growth-stimulating mechanism may become operative at other times than during the initial period of life.

The observation that a positive significant correlation existed between feed consumption per chick and coliform counts would suggest that this group of microorganisms is perhaps a causal factor in increasing feed intake. Evidence that the increase in coliforms preceded the increased feed intake is to be found in the fact that penicillin tended to result in larger numbers of this group of microorganisms in the chicks whose feed intake was controlled. This was not so in the case of lactobacilli, aerobes and anaerobes, which groups of microorganisms tended to be increased by penicillin in birds fed ad libitum but not in birds on restricted feed intake. The increase in these latter groups of microorganisms would thus appear to be as a result of the increase in feed intake.

The observation that penicillin increased feed consumption out of proportion to body weight during the first 16 days

suggests that some extraneous or non-physiological mechanism may be influencing feed consumption during this time. That coliforms may be a factor in this connection is suggested by the fact that with birds on the free choice feeding regime, the greatest difference in coliform counts between the birds fed the basal diet and those receiving penicillin occurred during a period which is roughly parallel to the time when feed consumption was being increased out of proportion to body weight. This is supported by the finding that while the negative correlation between feed consumption per unit body weight and coliform counts was significant for the 6- through 28-day period in the case of the group fed the basal diet, it was much less and non-significant in the presence of penicillin. Further evidence in favor of this is that a positive correlation existed between feed consumption per unit body weight and coliform counts for the 6- to 16-day period in the group fed penicillin. In addition, representative organisms of this group were isolated from the feces of these chicks and, after being grown in pure culture, were fed to other chicks. The results of this work are too extensive to be presented at this time. However, it should be pointed out that feeding the viable coliform cells caused a growth response which was due to an increase in feed consumption. Romoser et al. ('53) and Anderson et al. ('53) had previously observed growth stimulation upon the oral administration of coliforms to chicks.

Assuming that the mechanism of antibiotic growth response is dependent upon an increase in feed intake, the observations of Luckey ('52), Coates et al. ('51, '52), Hill et al. ('53) and Lillie et al. ('53) suggest that certain intestinal microorganisms depress feed consumption. The finding of a significant negative correlation between enterococci counts and feed consumption per chick, in birds fed the basal diet, suggests that this decrease may be a causal factor in increasing feed consumption. The tendency for penicillin to reduce enterococci numbers during the early period of life in the group fed ad libitum, while not particularly consistent, may help explain why the antibiotic effected an increase in feed consumption

on a unit weight basis. Further evidence that the enterococci group may depress feed consumption is the finding that the counts of this group of microorganisms were higher in birds fed penicillin than in the control birds during a time which coincided fairly well with the period when the feeding of penicillin no longer continued to result in an increase in feed consumption on a unit weight basis. That the increase in enterococci preceded the decrease in feed intake is evidenced by the similar increase in birds whose feed intake was equated.

It may well be that the inhibition of lactobacilli, anaerobes and aerobes brought about by penicillin, early in the life of the chick, is also a factor in the mechanism whereby feed consumption is enhanced. On the basis of the feed consumption data in the present experiment, there seems little doubt that intestinal floral changes occurring before the age of three weeks are the important ones. Unfortunately, much of the work reported in the literature dealing with this subject has been done using older birds.

The ability of the penicillin-fed group to maintain a slight superiority in rate of growth over the control group from 16 days through 7 weeks of age is surprising in view of the fact the former consumed somewhat less feed per unit body weight during that time. This might be explained if, as suggested by Braude and Johnson ('53), antibiotics decrease metabolic activity. On the other hand, the antibiotic may continue to exert a slight modifying influence such as to render the intestinal flora less host-competitive.

SUMMARY

Penicillin caused a significant increase in weight with chicks having free access to feed but no increase when feed intake was equated. With chicks fed ad libitum, penicillin resulted in greater daily feed consumption per chick almost without exception for the first 28 days after hatching. On the other hand, feed consumption per unit of body weight was increased by the antibiotic only to about 16 days of age. These results

suggest that penicillin stimulates chick growth by causing an increase in feed consumption early in the life of the bird.

The real increase in feed consumption — that is, the increase per unit of body weight — caused by penicillin appeared to be associated with an increase in fecal coliforms or a decrease in fecal counts of lactobacilli, anaerobes, aerobes and enterococci groups of microorganisms, or both.

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DIETARY PROTEIN AND GLYCINE AS PRECURSORS OF PORPHYRINS IN THE RAT [1]

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Comparatively little work has been done on study of the dietary precursors of the porphyrins in the living animal, other than protoporphyrin. Orten and Keller ('46) studied the influence of dietary protein on protoporphyrin formation in the rat. A low-protein intake resulted in a decrease in the protoporphyrin synthesis, as manifested by a decrease in fecal protoporphyrin. Totter, Amos and Keith ('49) likewise found a decrease in porphyrin synthesis with a decrease in the protein content of the diet. These investigators also showed that the addition of 3% of sodium benzoate to a purified diet was followed by a reduction in porphyrin synthesis (fecal excretion) in young rats. Glycine at a 2% level not only prevented the reduction in protoporphyrin excretion caused by the sodium benzoate, but probably increased the porphyrin synthesis to a level somewhat above that of the control animals. In both of the foregoing studies, however, fecal protoporphyrin was determined by a solvent extraction procedure which is open to question on the basis of specificity. The recent extensive work of Shemin and his collaborators (Shemin and Wittenberg, '51) using labeled glycine and acetate has established these two substances as the precursors of protoporphyrin in the nucleated duck erythrocyte.

[1] The data in this paper have been taken from the dissertation submitted by John Lucas for the degree of Doctor of Philosophy, Wayne University. The study was aided by a grant from the Nutrition Foundation, Inc.

The present investigation was undertaken to study the effect of dietary protein and glycine and of added benzoate on the formation of proto-, copro-, and uroporphyrins in the rat. The fecal excretion of these three porphyrins has been used as the criterion of porphyrin synthesis, since in the rat fecal porphyrins, at least protoporphyrin, appear to be derived exclusively from a metabolic surplus excreted by the Harderian glands (Watson, '38; Jakob, '39; McCoy, '43). The presence of protoporphyrin in the feces of the rat has been verified by its isolation as the methyl ester (Schultze, '42). The chromatographic method using silica gel (Lucas and Orten, '51) was employed for the isolation and subsequent determination of the individual porphyrins.

PROCEDURE

The experimental animals used were male albino rats of the Sprague-Dawley strain, ranging from 180 to 200 gm in weight. A total of 42 rats was divided into 5 groups for two 4-week periods of study, as shown in table 1. The group (12 rats) fed the basal diet only for the first 4 weeks was changed to a low-protein diet during the second 4 weeks. The group (6 rats) given the basal diet plus 2% glycine during the first 4 weeks was changed to the basal diet only, during the second 4 weeks. The group (12 rats) maintained on the basal diet plus 3% sodium benzoate during the first 4 weeks was changed to the basal diet plus 10% sodium benzoate for the seconds 4 weeks. The other group (12 rats) was given the basal diet plus 3% sodium benzoate plus 2% glycine for the first 4 weeks and was changed to the basal diet plus 10% sodium benzoate plus 5% glycine for the second 4 weeks. The rats were kept in individual cages and were supplied food and water ad libitum.

The purified basal diet had the following percentage composition: casein 20; corn oil 23; standardized cod liver oil 2; "vitaminized" sucrose 10; white corn dextrin 41; and salt mixture (Wesson, '32) 4. The vitaminized sucrose contained the following per 100 gm: thiamine hydrochloride 20 mg; riboflavin 20 mg; pyridoxine hydrochloride 10 mg; niacinamide 20

mg; calcium pantothenate 40 mg; choline chloride 2.0 gm; inositol 2.0 gm; *p*-aminobenzoic acid 600 mg; 2-methyl-4-naphthoquinone 4 mg; folic acid 20 mg. The rats were weighed twice weekly and food consumption was recorded. Hemoglobin determinations were made by the acid hematin method every two weeks, in order to determine whether any correlation existed between porphyrin and hemoglobin synthesis in the various groups of rats.

The feces were collected twice weekly and were placed in a bottle containing methyl alcohol to arrest any bacterial action. The feces were dried by vacuum desiccation at 60° C. The dried feces were weighed, pooled for each week into groups representing 6 rats each, and ground in a mortar. Aliquot portions of the pooled powdered feces were used for determination of the fecal porphyrins.

Both the "total" and individual porphyrins were determined in the fecal samples. The total porphyrins were determined by the solvent extraction method used by Orten and Keller ('46). The individual porphyrins were determined, following chromatography on a hydrated silica gel column (Lucas and Orten, '51), as follows: 2.0-gm aliquots of powdered dry feces were treated in a 125-ml ground-glass stoppered Erlenmeyer flask with four 25-ml portions of ethyl acetate-glacial acetic acid (3:1). The mixture was homogenized and the supernatant was decanted into a filter. The extraction was repeated until a negative pink fluorescence in ultraviolet light was obtained from the supernatant solution. The filtered ethyl acetate extract containing the total porphyrins was transferred into a separatory funnel with distilled water to remove any excess acetic acid and some fecal pigment, and the porphyrins were extracted with six 5-ml portions of 10% hydrochloric acid (w/v). This solution was neutralized with saturated sodium acetate solution to Congo red and the free porphyrin was extracted with ether and washed with 10 ml of 10% sodium carbonate, followed by distilled water. The combined washings were checked for pink fluorescence and, if positive, were extracted with two 5-ml portions of ether. The ether extract was

finally treated with five 5-ml portions of 5% HCl (w/v) and made up to volume in a 25-ml volumetric flask. An aliquot was read in a Coleman spectrophotometer and the total porphyrin concentration was determined from a calibration curve prepared with pure protoporphyrin.

The individual porphyrins were determined chromatographically on the above 5% HCl extract. It was first neutralized with saturated sodium acetate solution and extracted with ether. The ether-insoluble layer was checked for fluorescence in ultraviolet light. If positive, the porphyrins (uroporphyrins) were extracted with ethyl acetate. This extract was combined with the ether extract. The combined extracts were evaporated to dryness in a current of warm air and the residue was methylated with five 10-ml portions of methyl alcohol saturated with hydrogen chloride gas. An equal volume of chloroform was added and the mixture was washed with water, dilute ammonia and saline, as previously described. The chloroform solution was then diluted with an equal volume of ligroin and chromatographed in a silica gel column (Lucas and Orten, '51). This was prepared by mixing 1 gm silica gel, 1 ml of distilled water and 25 ml of ligroin; for the development of the chromatogram, 20% chloroform in ligroin was used. Protoporphyrin was eluted with this concentration. At the top of the column there appeared a dark brown zone which would not move down the column. This was an unidentified fecal non-porphyrin pigment. In some of the groups — i.e., those receiving sodium benzoate supplements — an unidentified green zone was also observed in the upper third of the column.

The eluted porphyrins were collected in pyrex test tubes (18 × 150 mm) and the chloroform was evaporated on a steam bath. The almost dry porphyrin ester was treated with 10 ml of 5% HCl (w/v) and warmed 30 seconds to aid solution. This was then washed into a 25-ml or 50-ml volumetric flask, made up to volume with 5% HCl and read in a spectrophotometer, or in a photofluorometer if concentrations were small. The porphyrin concentration was determined from calibration

curves prepared from solutions of the pure methyl esters of the respective porphyrins.

RESULTS AND DISCUSSION

The body weights for the animals of the various groups are omitted in the interest of brevity. The group given only the basal diet grew at a satisfactory rate. The addition of glycine had no perceptible effect. The change to a low-protein diet, however, resulted in a slow but definite loss of body weight. This was to be expected, since tissue protein was undoubtedly called upon to meet the needs of the organism for maintenance purposes. The addition of 3% sodium benzoate to the basal diet produced a slight inhibition of growth, whereas a change to 10% sodium benzoate-supplemented diet resulted in a marked drop in body weight and death in 11 out of 12 animals by the end of the 4-week period. Supplementation of the two levels of sodium benzoate-containing basal diet with 2% and 5% glycine, respectively, resulted in an almost complete counteraction of the growth-inhibiting effect of the sodium benzoate. The data on food consumption and on hemoglobin levels are also omitted for the sake of brevity.

Table 1 shows the average total fecal porphyrin, expressed in micrograms per day per rat. An increase in the total porphyrin synthesis, as measured by porphyrin fecal excretion, was found to occur with an increase in age and weight of the rat. The addition of glycine to the basal diet increased porphyrin synthesis slightly. On the other hand, the total porphyrin excretion was markedly diminished in the rats given sodium benzoate, particularly at the high level (10%), and in the low-protein group. The inclusion of glycine in the diets containing sodium benzoate resulted in an increase in porphyrin excretion toward the normal control value. The foregoing results thus confirm those of Orten and Keller ('46) and of Totter, Amos and Keith ('49).

The average fecal protoporphyrin, in micrograms per day per rat, as determined following chromatography on silica gel, is also presented in table 1. No more than traces of copro-

TABLE 1

Group average values for fecal total porphyrins and protoporphyrin [1]

	WEEKS OF EXPERIMENT	GROUPS				
		Basal diet only	Basal + glycine	Basal + Na benzoate [2]	Basal + Na benzoate [2] + glycine [3]	Low protein
Number of rats		12	6	12	12	12
Total porphyrin	1	150	161	139	153	...
μg per rat	2	161	162	148	151	...
per day	4	168	179	122	135	168
	6	187	...	74	135	107
	7	188	...	57	148	108
	8	207	...	26	141	120
Protoporphyrin [1]	1	73	76	53	64	..
μg per rat	2	84	95	58	74	..
per day	4	82	134	48	64	82
	6	98	...	37	81	47
	7	97	...	25	70	56
	8	135	...	13	64	61
Protoporphyrin	1	30	30	23	25	..
μg per 100 gm	2	31	35	23	26	..
weight per day	4	28	44	17	20	28
	6	29	..	19	29	18
	7	26	..	15	24	21
	8	37	..	9	21	24
Protoporphyrin	1	33	30	22	23	..
μg per gm protein	2	27	35	22	27	..
per day	4	30	45	17	22	30
	6	34	..	41	39	114
	7	35	..	31	26	140
	8	45	..	22	22	153
Protoporphyrin	1	67	3	..	9	...
μg per 10 mg	2	56	4	..	10	..
available glycine	4	61	4	..	8	61
per day	6	68	..	..	..	224
	7	69	..	..	..	280
	8	70	.	..	..	307

[1] Only traces of coproporphyrin and no uroporphyrin were found in any of the samples of feces. These data are therefore omitted from the table.

[2] Three per cent Na benzoate for first 4 weeks; 10% for final 4 weeks.

[3] Two per cent glycine for first 4 weeks; 5% for final 4 weeks.

porphyrin or of uroporphyrin were found in the feces of the rats in the various groups; therefore, data for these porphyrins are omitted in the table. It is evident that protoporphyrin constituted only about half of the total fecal porphyrin. This indicates that some unidentified non-porphyrin pigments are undoubtedly measured as porphyrins in the solvent extraction, "total porphyrin" procedure. However, the values obtained for protoporphyrin show the same general trend as does that for the "total' porphyrins.

When the values for protoporphyrin are calculated per 100 gm of body weight, a similar trend was found, as is shown in table 1. In the group receiving the basal diet only, there was a rather constant formation (excretion) of approximately 30 μg of protoporphyrin per day per 100 gm of body weight. This was somewhat increased when glycine was added, as a supplement. The group given sodium benzoate showed a reduction in protoporphyrin formation, particularly during the last 4 weeks when the animals received 10% sodium benzoate. When the benzoate diets were supplemented with an equivalent amount of glycine, a reversal of the inhibiting effect resulted, protoporphyrin synthesis approaching that of the rats receiving the basal diet. When the protein intake was restricted, the average protoporphyrin formation was decreased considerably below that of the control group.

When the amount of protoporphyrin excreted per day is calculated per gram protein ingested, an interesting and marked difference appears in the low-protein-fed group. There was a rather constant level of about 35 μg of protoporphyrin excreted per gram of protein ingested, in the rats fed the basal diet. Supplementation with benzoate appeared to decrease this amount, presumably because of the removal of one of the precursors of protoporphyrin, glycine. However, in the group fed the low-protein diet there were marked increases in the micrograms of protoporphyrin excreted per gram of protein ingested. This may be interpreted as indicating that protoporphyrin synthesis has a high priority on the limited amount of

protein available in the diet, as has also been shown for hemoglobin (Orten and Orten, '46).

Protoporphyrin excretion, calculated per 10 mg of available glycine, varied from 56 to 70 μg in the control group. When additional glycine was ingested, the value decreased, presumably because the excess glycine may have been excreted in the urine or used for other metabolic purposes. In the low-protein group, in which the glycine intake was necessarily restricted, there was again an efficient utilization of the available glycine for the synthesis of protoporphyrin. Values for the groups receiving sodium benzoate were not estimated.

The absence of more than traces of copro- and uroporphyrins in the feces of the rat, in contrast with the presence of significant amounts of protoporphyrin, is of considerable interest. These two porphyrins appear to be intermediates in the biosynthesis of protoporphyrin (Larsen et al., '53) and apparently do not accumulate in sufficient concentrations in the body to be excreted in significant quantities in the feces. Other investigators (Rimington and Hemmings, '38; Schwartz, '52) have likewise found only small amounts of coproporphyrin and even less uroporphyrin in the feces of the rat.

SUMMARY

A chromatographic procedure employing silica gel for separating and quantitatively determining the total and the individual porphyrins in rat feces is described.

The procedure has been applied to groups of rats receiving either a purified basal diet, or one in which the glycine intake is restricted by the simultaneous feeding of sodium benzoate, or one low in protein.

The results demonstrate that no more than traces of porphyrins other than protoporphyrin appear in rat feces. The amount of protoporphyrin in the feces of the normal rat is approximately 30 μg per 100 gm body weight, and serves as an index of protoporphyrin synthesis.

Fecal protoporphyrin decreases if the available glycine is decreased by the administration of sodium benzoate or if the

protein intake is restricted. However, protoporphyrin formation, calculated per gram of protein or per 10 mg glycine available, is markedly increased.

These data demonstrate the indispensability of an adequate protein or glycine intake, or both, for porphyrin formation in the rat, and the high priority of protein and of glycine for porphyrin synthesis if the intake of either is limited.

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ABSENCE OF A TIME FACTOR IN THE RELATIONSHIP BETWEEN LEVEL OF ENERGY INTAKE AND PROTEIN METABOLISM

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TWO FIGURES

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Experiments on human subjects (Cuthbertson and Munro, '39) and on rats (Cuthbertson, McCutcheon and Munro, '40; Munro, '49; Geiger, Bancroft and Hagerty, '50) have demonstrated that N balance is adversely affected by eating the protein and carbohydrate of an adequate diet at different meals, without altering the total dietary constituents. Since separation of the time of eating fat from that of eating dietary protein does not affect the N balance of adult rats (Munro, '49; Geiger, '51), it has been concluded that carbohydrate plays a special role in protein metabolism which demands close proximity in time of the eating of carbohydrate and protein.

By contrast, it has been observed that addition of either carbohydrate or fat to an adequate diet causes N retention; moreover carbohydrate and fat appear to be equally effective on a caloric basis (see review by Munro, '51). The question arises whether, in order to fulfill this energy-yielding effect on protein metabolism, the extra carbohydrate or fat need be fed along with the dietary protein. The experiments of Larson and Chaikoff ('37) on dogs and of Lathe and Peters ('49) on rats suggest that extra carbohydrate is effective only when given along with dietary protein, but neither series of experi-

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ments can be considered as conclusive (Munro, '51). Moreover, they are not in agreement with a single surfeit feeding experiment carried out on a human subject by Cuthbertson and Munro ('39). It therefore seemed desirable to obtain further data, and we have accordingly studied the N metabolism of dogs, human subjects and rats receiving a carbohydrate supplement separate from dietary protein.

EXPERIMENTAL

Experiments on dogs

In Larson and Chaikoff's ('37) experiments, the dogs received an adequate diet fed as one meal daily and additional sugar was given at various time intervals before or after this meal. They observed that N output was affected only when the extra carbohydrate was administered within 4 hours of the protein-containing meal. They did not give extra carbohydrate separate from protein for more than one day at a time. Our object has been to reproduce as closely as possible the conditions provided by Larson and Chaikoff in order to study the effect of *prolonged* feeding of extra carbohydrate given separately from dietary protein.

Experimental. Two female mongrel dogs weighing 8.0 kg and 3.6 kg, respectively, were perineotomized to facilitate catheterization and after recovery were put in individual metabolism cages under thermostatic conditions (26 to 29°C.). Larson and Chaikoff gave a diet based on Cowgill's ('23) synthetic mixture, but fed at a level of 60 Cal. per kilogram body weight instead of 80 Cal. We therefore gave our dogs a diet consisting of 5.5 gm casein, 5 gm sucrose, 2 gm lard, 1 gm margarine, 0.4 gm agar and 0.3 gm salt mixture (Cowgill, '23) per kilogram of body weight. Vitamins were added in the doses recommended by Allison and Anderson ('45), together with para-aminobenzoic acid, inositol and biotin. The diet provided some 68 Cal. per kilogram and was adequate to maintain the body weight of both dogs. The food was fed daily as a single meal at 9:30 A.M. and was immediately consumed. When re-

quired, extra glucose (50 gm for the larger dog and 30 for the smaller animal in water) was given by stomach tube. The animals had previously been trained to swallow the tube.

Urine was collected daily, the 24-hour period being completed by catheterization at 9:30 A.M. and the cages washed down; the feces were discarded. The urine was analyzed for N by the micro Kjeldahl procedure of Ma and Zuazaga ('42).

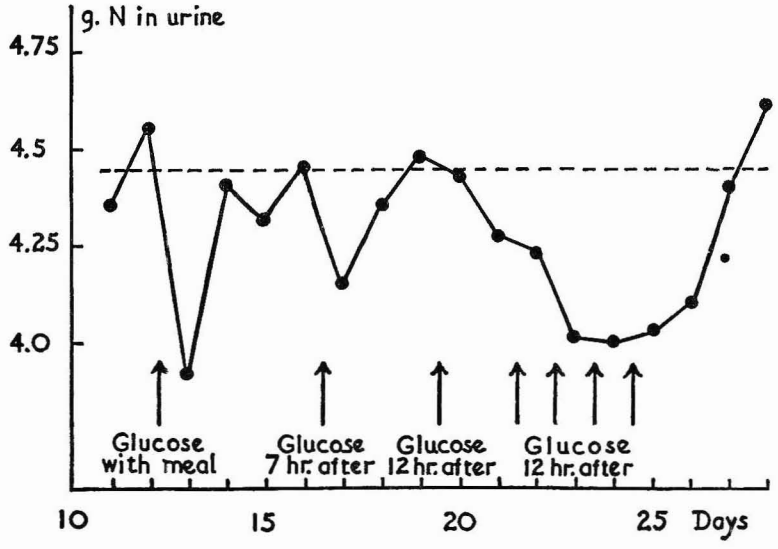

Fig. 1 Mean urinary N output of two dogs fed a constant basal diet and given extra glucose at different times in relation to the basal diet. The dotted line is the mean N output on the basal diet before addition of sugar.

With this method the recovery of N from urea solutions of known concentration was found to be satisfactory.

Results. Since both dogs showed essentially the same responses, the average urinary N output of the two animals is given in figure 1. After 10 days, N output had reached a steady level and then each dog received a single dose of extra glucose immediately after the protein-containing meal. This produced a considerable reduction in N output on the same day, followed by a return to the basal output on the following day. Four days later another single dose of glucose was given to

each dog, on this occasion 7 hours after the daily meal. This resulted in a smaller but still appreciable depression in N output. Three days later the glucose was given 12 hours after the daily meal and produced no reduction in N output on that day but a slight fall on the following day. These findings agree with those of Larson and Chaikoff in showing that a single dose of glucose spares protein more effectively when given along with the protein of the diet.

During the remainder of the experiment glucose was given 12 hours after the basal diet on 4 successive days. From the second day of administration onwards this resulted in a reduction in N output of the same magnitude as that obtained by feeding a single dose along with the basal diet meal. After the giving of extra glucose had been terminated, N output remained depressed for a further 24 hours and then rose to the basal output. These findings indicate that, under the conditions used by Larson and Chaikoff, extra carbohydrate given 12 hours apart from the protein of the diet does eventually lead to N retention.

Experiments on human subjects

Cuthbertson and Munro ('39) performed a single experiment in which a human subject on an adequate diet received repeated additions of extra sugar 4½ hours after the last meal of the day. This caused a definite N retention from the third day of administration onwards. The purpose of the present experiments was to confirm this observation on other subjects and to compare the extent of the N retention with that produced by feeding the additional carbohydrate along with the dietary protein.

Experimental. The subjects were 4 young adult males. They selected adequate diets from a limited number of standardized foodstuffs and this basal diet (table 1) was then kept constant throughout the experiment. Three meals were taken, between 8 : 30 and 9 : 00 A.M., 12 : 30 and 1 : 30 P.M. and 5 : 00 and 6 : 00 P.M. Fluid intake and energy expenditure were kept as constant as possible. From the second day onwards, 24-hour urine collec-

TABLE 1

The effect of superimposing 200 gm of sucrose on an adequate basal diet, at one time, along with the meals and at another time, apart from the meals

SUBJECT [1]	BODY WEIGHT	DAILY BASAL DIET [2]				CHANGES IN URINARY NITROGEN OUTPUT [3]									
						Extra carbohydrate with meals					Extra carbohydrate apart from meals [4]				
		Protein	Fat	Carbohydrate	Energy	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
	kg	*gm*	*gm*	*gm*	*Cal./kg*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
1 [5]	70.8	112	144	454	50	+ 0.73	+ 1.99	+ 0.87	+ 1.67	+ 1.44	...	...	...	...	...
2	77.9	102	115	392	39	+ 0.85	+ 2.72	+ 1.27	+ 2.30	+ 2.77	+ 0.03	+ 1.50	+ 1.09	+ 2.06	+ 2.72
3	67.7	102	141	394	48	+ 0.30	+ 0.84	+ 1.93	+ 1.96	...	— 0.24	+ 1.06	+ 1.54 [6]	+ 0.53	+ 0.56
4	65.7	79	104	398	45	+ 1.34	+ 2.16	+ 3.64	+ 3.09	...	— 0.09	+ 0.40	+ 1.54 [6]	+ 2.37	+ 1.65
Mean (subjects 2, 3 and 4)						+ 0.83 [7]	+ 1.91	+ 2.28	+ 2.45	...	— 0.10 [7]	+ 0.99	+ 1.39	+ 1.83	+ 1.64

[1] During the first surfeit period, extra sucrose taken with meals by subjects 1 and 2, apart from meals by subjects 3 and 4.

[2] Constituents calculated from tables of food composition (McCance and Widdowson, '46).

[3] The change in N output caused by the extra carbohydrate was measured from the mean N output of the subject during the first period on the basal diet. A + sign indicates N retention as a result of adding carbohydrate to the diet.

[4] Extra carbohydrate taken with water at 11: 30 P.M.; i.e. 5½ hours after the last meal of the basal diet. Subjects 3 and 4 also took the juice of a lemon at this time. During other surfeit period, the same amounts of water and lemon juice were taken at 11: 30 P.M.

[5] This subject developed a febrile illness during the second surfeit feeding period.

[6] The 24-hour specimens of these two subjects were accidentally mixed; the figure given is based on the average N content of the combined urines.

[7] The difference in response to extra carbohydrate fed with meals and apart from meals is statistically significant for the first day of surfeit feeding ("t" — 3.00; P = 0.05 — 0.02).

tions were made and analyzed for N as described in the dog experiments.

The subjects were first allowed several days on the basal diet until urinary N output had reached a fairly constant level. Then, for a period of 4 to 5 days, extra carbohydrate (200 gm of sucrose daily) was given to all subjects. Two of the subjects took the sucrose with the meals of the basal diet and the other two received it at 11:30 P.M. in water. After this period of surfeit feeding, the subjects ate the unsupplemented diet for a few days until N output had returned to the basal values obtained at the start of the experiment. Then a second period of surfeit feeding was begun, the first pair of subjects receiving the extra carbohydrate in the evening and the second pair taking it with the diet. In this way each subject received additional carbohydrate twice, on one occasion along with the dietary protein and on the other occasion some 5½ hours after the last meal of the day. Unfortunately, one subject developed a febrile illness during the second period of surfeit feeding and we have therefore comparative data for only three subjects who completed both periods of surfeit feeding.

Results. Assuming the fecal N output to be less than 1.5 gm per day, all 4 subjects were in positive N balance on the basal diet. Addition of carbohydrate to this diet caused a fall in N output, whether given along with the food or apart from it (table 1). When the extra carbohydrate was taken along with the basal diet, the protein-sparing effect was evident on the first day of surfeit feeding, whereas when the extra carbohydrate was given 5½ hours after the last meal a reduction in N output occurred only from the second day onwards; this difference in behavior on the first day of surfeit feeding is statistically significant (table 1).

In order to determine whether, apart from this delay in starting, the protein-sparing effects of extra carbohydrate given with and apart from dietary protein were similar, the daily N retentions (table 1) were transformed into cumulative curves (fig. 2) and the regression lines examined. After the initial delay in starting, the increments in N retention followed

a linear slope which, in the case of carbohydrate fed with protein, averaged 2.9 mg N per extra Calorie per day, and in the case of carbohydrate given separately from the dietary protein averaged 2.1 mg. The regression equations for individual subjects were then analyzed statistically. The slopes, which

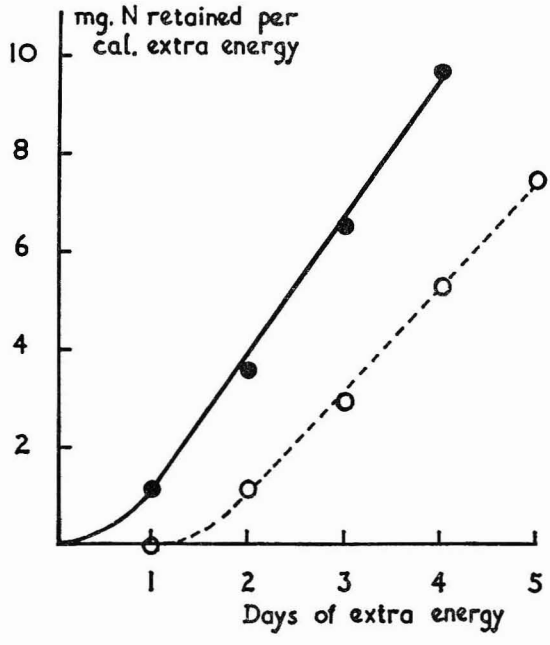

Fig. 2 Cumulative N retentions produced by adding sucrose to an adequate diet, either with the meals (●———●) or 5½ hours after the last meal (○---○). Each point is the mean observation on three subjects.

were approximately linear for all subjects, did not differ during the two surfeit feeding periods for subject 2, but in the case of subjects 3 and 4 the rate of increment was significantly less when the extra carbohydrate was taken apart from the dietary protein. Whether this is related to the order in which these experiments were performed cannot be decided from the present evidence, but too much weight should not be placed on the difference found with subjects 3 and 4 since, in the absence of fecal N determinations, we do not know that the digestibility

of the dietary protein was identical during the two periods of surfeit feeding.

Experiments on rats

While studying the protein metabolism of rats after they had been subjected to burns, Lathe and Peters ('49) made some observations on the effect of additional energy from carbohydrate on the metabolism of unburnt animals. In one experiment they compared urinary N output during a preliminary period on a fat-deficient basal diet with N output during a 6-day period in which extra carbohydrate was given several hours after the basal diet had been fed. Their results provide no evidence of a sparing effect. However, the basal diet alone was given for only two days before superimposing extra energy, and the rats may not have reached a steady N output during this two-day period. We have therefore carried out further studies on the influence of extra carbohydrate and fat on the protein metabolism of the rat, including an experiment in which the conditions used by Lathe and Peters were replicated.

Experimental. Three series of studies were carried out on young adult male albino rats weighing about 250 gm. In the first two experiments the rats were given a synthetic diet containing casein, glucose, margarine and a vitamin-mineral-roughage mixture (Munro, '49); this diet provided about 400 mg of N, 5.3 gm of carbohydrate, 0.8 gm of fat and 40 Cal. (1,200 Cal. per square meter of body surface area) per rat per day. The diet was checked for N content, using mercury as the digestion catalyst (Munro, '49). Before feeding, the food was moistened to prevent scattering. In the third experiment the rats received the fat-deficient diet used by Lathe and Peters ('49), prepared in the manner described by Lathe ('49). This provided 200 mg of N and 43 Cal. (1,300 Cal. per square meter) per rat per day.

The rats were housed in individual cages under thermostatic conditions. Separation of urine and feces was carried out by the procedure described by Cuthbertson, McGirr and Robertson ('39), iron oxide being used as a fecal marker to separate the

metabolic periods. Urine collections for 4-day periods were pooled and analyzed for N by the micro Kjeldahl procedure of Ma and Zuazaga ('42). The feces were digested with sulfuric acid and copper sulfate for two hours after clearing and aliquots of the digest were then submitted to the full micro Kjeldahl procedure.

In the first experiments 40 rats were distributed at random into 5 equal groups (table 2). They all received the same amount of the basal diet, fed twice daily. At 9:00 A.M. they were given 2 gm of the vitamin-mineral-roughage mixture and 2.5 gm of glucose; this was usually eaten quickly and at 12:00 noon the dishes were removed. At 5:00 P.M. they were fed a mixture containing 3 gm of casein, 2 gm of glucose and 0.5 gm of fat, which was always rapidly consumed.

After allowing one week for the animals to become adjusted to this regimen, the collection of excreta was started. During the first 4-day collection period all 5 groups of rats received only the basal diet. Thereafter each group was treated differently. One group received an extra 3 gm of glucose with the evening (protein-containing) meal. The second group received the same amount of glucose in the morning (protein-free) meal; three rats in this group did not completely finish the supplementary feed, but the amount uneaten was not sufficient to warrant rejection of the data obtained from these animals. Two other groups were given 1.34 ml (1.2 gm) of olive oil with the evening and morning feeds, respectively. Finally, a control group received no extra nutrient. The additional carbohydrate or fat was given daily for two successive 4-day periods (periods 2 and 3) and then during a final period all 5 groups of rats ate the basal diet only. Thus we can compare the effect of isodynamic amounts of extra carbohydrate or fat taken along with the dietary protein with their effect when eaten between 5 and 8 hours before the protein-containing feed.

In the second experiment 15 rats were distributed into three equal groups (table 3). The basal diet was similar to that used in the first experiment, but on this occasion the protein-

containing meal was given at 9:00 A.M. and the protein-free meal at 5:00 P.M. After a preliminary period of 7 days on this diet, excreta were collected for 4 days, during which all animals ate only the basal diet (period 1). Then during a second 4-day period one group received an extra 3 gm of glucose in the morning (protein-containing) meal, another group took it at

TABLE 2

The effect on the N balance of rats of adding carbohydrate (glucose) or fat (olive oil) to the diet along with or apart from the dietary protein

(The extra energy source was added during periods II and III. Each group consisted of 8 rats.)

ADDITIONS DURING PERIODS II AND III	MEAN INITIAL BODY WEIGHT	MEAN DAILY NITROGEN BALANCES [1]			
		Period I	Period II	Period III	Period IV
	gm.	*mg*	*mg*	*mg*	*mg*
1. Carbohydrate with protein	242	— 8.4	+ 34.3	+ 22.9	— 15.6
2. Carbohydrate apart from protein	254	— 0.4	+ 28.5	+ 18.4	— 8.8
3. Fat with protein	240	+ 19.9	+ 24.7	+ 21.1	+ 9.1
4. Fat apart from protein	245	+ 5.4	+ 27.8	+ 25.0	+ 0.2
5. No addition	247	— 1.7	— 8.7	+ 0.5	— 2.0

[1] There is a highly significant difference between the mean N balances of the 5 groups during periods II and III, after adjusting by covariance analysis for differences in N balance during period I. When the 5th group is excluded, the N balances during these two periods do not differ significantly. There are no significant differences during period IV.

9:00 P.M., and the third group continued on the unsupplemented basal diet as controls. This experiment provides a comparison between extra carbohydrate taken along with protein and extra carbohydrate taken about 12 hours after protein.

In the third experiment, 15 rats were divided into three equal groups (table 4). By stomach tube they received 7 ml of the fat-deficient diet described by Lathe and Peters ('49) at 9:30 A.M. and 8 ml at 3:30 P.M. After 5 days on this diet, one

group received in addition 4 ml of a 50% sucrose solution at 9:30 P.M.; the second group were given the same supplement along with the basal diet; and the third (control) group received 4 ml water at 9:30 P.M. This was continued for 4 days, during which urine and feces were collected.

Results. In the first experiment, the mean N balances in the 5 groups of rats (table 2) were essentially similar during period 1; i.e., when all animals were consuming the basal diet without supplements; the rather greater mean value for the

TABLE 3

The effect on N balance when carbohydrate (glucose) is superimposed on a diet adequate for rats, either along with or 12 hours apart from the dietary protein

(During period I, all animals received the unsupplemented basal diet; during period II, extra carbohydrate was given except to the control group.)

TREATMENT DURING PERIOD II	MEAN INITIAL BODY WEIGHT	MEAN DAILY NITROGEN BALANCES [1]	
		Period I	Period II
	gm	*mg*	*mg*
Extra glucose with protein	239	— 0.3	+ 36.4
Extra glucose apart from protein	249	— 0.8	+ 31.8
Control group	220	+ 3.9	— 15.3

[1] After adjustment of the values in period II by covariance analysis for differences in N balance during period I, the mean N balances during period II were shown to be highly significantly different ($P < 0.01$). This is entirely due to a difference between the control group and the other two groups; the groups receiving glucose gave statistically similar results.

group which were later to get extra fat with the protein-containing meal was chiefly caused by an abnormally high result obtained with one animal and was probably due to some technical error. During the time when the extra carbohydrate or fat was given (periods 2 and 3) there was a marked storage of N by all but the control group. Statistical analysis showed that this effect of the extra carbohydrate and fat was significant; the degree of N retention did not differ significantly among the 4 groups receiving supplements of carbohydrate or fat. During the 4th period, when all animals were again receiving the basal diet alone, statistical analysis did not

indicate a greater loss of N by the rats that had received supplements than by the animals of the control group. This experiment therefore demonstrates that surfeit carbohydrate and surfeit fat are effective in causing N retention, whether given with protein or some 5 or more hours beforehand.

The second experiment with rats confirms that surfeit carbohydrate is effective when administered apart from protein (table 3). On this occasion the time interval between the eating of the dietary protein and the extra carbohydrate was some 12 hours. The improvement in N balance caused by the extra

TABLE 4

The effect on N balance when sucrose is superimposed on a fat-deficient basal diet fed by stomach tube

(The extra sucrose was given either along with or 6 hours after the basal diet. Each group consisted of 5 rats. They received the unsupplemented basal diet for 5 days before excreta were collected.)

TREATMENT	MEAN INITIAL BODY WEIGHT	URINARY N	FECAL N	BALANCE DAILY N [1]
	gm	*mg*	*mg*	*mg*
None	237	205	21	— 26
Sucrose with meals	234	187	20	— 7
Sucrose apart from meals	236	182	21	— 3

[1] Analysis of variance shows that both sucrose supplements produced a significant reduction in N output ($P < 0.05$); the fall in N output was statistically similar in the two groups.

carbohydrate was highly significant by comparison with the controls, but the giving of the carbohydrate with or apart from protein did not significantly alter the degree of N retention.

The third experiment reproduced the essential features of Lathe and Peters' study, insofar as the same basal fat-deficient diet was given by stomach tube and extra sucrose was administered 6 hours after the basal diet. Table 4 shows that, although only one dose of additional sucrose was given, in contrast to the two doses used by Lathe and Peters, there was nevertheless a reduction in N output similar in magnitude to that produced by feeding the same amount of sucrose along with the basal diet. A second experiment carried out under

similar conditions confirmed this finding. In neither experiment was a positive N balance attained with the diet.

DISCUSSION

Previous N balance experiments (Cuthbertson and Munro, '39; Cuthbertson, McCutcheon and Munro, '40; Munro, '49; Geiger, Bancroft and Hagerty, '50) have shown that there is an interaction between the protein and carbohydrate of ordinary mixed diets which depends on these dietary constituents being eaten in close proximity to one another. On the other hand, variations in the time relationship of fat intake to protein intake do not affect the N balance of adult rats (Munro, '49; Geiger, '51). These findings indicate that carbohydrate plays a special part in protein metabolism which cannot be taken by fat.

Our present experiments show that addition of carbohydrate to the diet causes a reduction in N output even when it is taken separately from the protein of the diet. In the case of the human experiments, the maximum period of separation which was practicable was 5½ hours, but in the cases of the rat and the dog the sparing effect was demonstrated for a 12-hour interval between the giving of the two nutrients. Although there was a tendency in the human experiments for the N retention to be greater when the extra carbohydrate was fed with protein than when they were fed separately, the difference was not large and is of doubtful significance. The rat experiments also provide no definite evidence of such a difference in action. It may be concluded that the protein-sparing action of the extra carbohydrate is essentially independent of the time of its administration and thus does not operate through the mechanism referred to in the preceding paragraph, which demands close proximity in the time of eating protein and carbohydrate. Presumably the protein-sparing action of the additional carbohydrate is caloric in nature, since calculations based on these and other experiments recorded in the literature (see review by Munro, '51) indicate that added fat has a

protein-sparing action comparable on an energy-yielding basis to that of carbohydrate.

We may accordingly conclude that carbohydrate affects protein metabolism in two ways. First, protein utilization is favorably influenced by the presence of carbohydrate in the protein-containing meal; this effect is specific to carbohydrate. Secondly, carbohydrate acts like fat as an energy source which has a sparing action on protein metabolism irrespective of the time at which it is fed. It may be thought that this lack of a time factor indicates that the effect of energy level is on "endogenous" protein metabolism and not on dietary protein. However, studies on rats receiving protein-free and protein-containing diets show that the effect of energy level is on the dietary protein, even when the latter is fed separately from the variable energy source (Munro and Naismith, '53).

SUMMARY

1. Experiments are described in which additional carbohydrate (glucose) was given to dogs already receiving adequate diets. This caused a reduction in urinary N output, even when the extra carbohydrate was taken 12 hours apart from the rest of the diet.

2. Similar experiments on human subjects, in which extra carbohydrate was taken either with the diet or 5½ hours after the last meal of the day, also demonstrated a fall in N output.

3. In similar experiments on adult rats it was observed that extra carbohydrate given 5 hours or 12 hours apart from the dietary protein was just as effective as extra carbohydrate taken with the dietary protein. N retentions of similar magnitude were also observed when an isodynamic amount of fat was superimposed on the basal diet.

4. From a consideration of these and previously published experiments it has been concluded that protein utilization is affected in two ways by the other energy-yielding nutrients in the diet. First, protein utilization is favorably influenced by the presence of some carbohydrate in the protein-containing meals. Close proximity in the time of eating carbohydrate and

protein is necsssary in order that this interaction may take place, and fat cannot be used in place of carbohydrate. Secondly, carbohydrate and fat act interchangeably as energy sources in sparing protein; in order to exert this sparing action they do not need to be taken along with dietary protein.

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STUDIES ON THE BIOLOGICAL VALUE OF INORGANIC PHOSPHATES [1]

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ONE FIGURE

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Limited comparisons of the utilization of different phosphates by chicks have been published (Bird et al., '45; Gillis, Norris and Heuser, '48, '49; Sieburth et al., '52; Miller and Joukovsky, '53; Grau and Zweigart, '53), but the work to date has been mostly qualitative in nature, and precise comparisons of different phosphorus supplements have not been reported.

Chemical solubility tests analogous to those used in estimating the availability of nutrients to plants have been proposed as a measure of the availability of phosphorus for animals. However, efforts on our part to correlate the results of such tests with nutritional performance in animals have failed (Gillis et al., '48). In this paper a procedure is described for making more exact comparisons of the availability of phosphorus from different sources, and the comparative values of some of the phosphates studied are presented.

EXPERIMENTAL

Studies with a purified type of diet

The purified type of diet previously used by Gillis, Norris and Heuser ('48), slightly modified, has been very satisfactory

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for studies of this nature. The percentage composition of the diet used in the present work was as follows: dried blood fibrin [3] 20, gelatin 4, hydrogenated vegetable fat [4] 3, ground cellophane 3, liver fraction "L" 1, feeding oil (3,000 A, 400 D) 0.25; plus the following amounts of vitamins and minerals per pound; thiamine 6 mg, riboflavin 6 mg, Ca pantothenate 10 mg, pyridoxine 3 mg, niacin 12 mg, folic acid 2 mg, biotin 0.2 mg, vitamin B_{12} 0.01 mg, para-aminobenzoic acid 50 mg, vitamin K (menadione) 1 mg, inositol 500 mg, choline chloride 900 mg, alpha-tocopherol concentrate 320 mg, $MgSO_4$ 1.2 gm, $MnSO_4 \cdot 4H_2O$ 0.12 gm, iodized NaCl 2.27 gm, $FeSO_4 \cdot 7H_2O$ 50 mg, $CuSO_4 \cdot 5H_2O$ 5 mg, $ZnCl_2$ 5 mg, $CoCl_2 \cdot 6H_2O$ 5 mg, KCl 2 gm. After the addition of the desired amounts of phosphorus and calcium supplements, cornstarch was added to adjust the formula to 100%.

This diet, unsupplemented, contained about 0.03 to 0.05% phosphorus, an amount which will not support life in the young chick for more than a few days. When the phosphorus supplied in supplementary form was kept at appropriately low levels, the chicks' response was proportional to the amount of available phosphorus provided. When the amount of phosphorus added to the diet was optimum, growth and calcification were excellent, indicating that the diet was adequate in other respects for the chick.

The different phosphatic materials studied were each added to the P-deficient basal diet in graded increments, usually in amounts sufficient to provide 0.25, 0.30 and 0.35% dietary phosphorus. In those instances where the supplement did not provide sufficient calcium to establish a Ca:P ratio of 2:1 or greater, this ratio was established by adding $CaCO_3$.

Each level of the experimental phosphates in the test ration was fed to a lot of 10 or more White Leghorn male chicks. Feeding was begun when the chicks were approximately one day old. Wire-floored, electrically heated battery brooders were used and feed and water were allowed ad libitum. At

[3] Armour.
[4] Primex.

the end of 4 weeks the chicks were weighed and killed and the left tibiae were removed and cleaned. The bones were extracted for 24 hours with hot alcohol, followed by a 24-hour extraction with ether. The percentage of ash was then determined for the fat-free, dry bone.

The calcifying activity of pure beta-tricalcium phosphate was arbitrarily assigned a value of 100. It was used as a basis of reference in the same manner that well-characterized proteins such as casein or egg albumin have been used in studies on the nutritive value of proteins. In order to validate comparisons between experiments run at different times, beta-tricalcium phosphate was included in each study. Beta-tricalcium phosphate is generally regarded as the logical end product in the complete neutralization of phosphoric acid by lime. In practice, however, it is difficult to obtain as a pure compound. A sample of satisfactory purity was kindly prepared for use in these experiments by Mr. W. L. Hill of the U. S. Department of Agriculture.

Figure 1 shows the type of response curve which was obtained when the basal diet was supplemented with beta-tricalcium phosphate. The percentage of supplementary phosphorus was plotted against the percentage of ash in the tibiae of chicks. This figure also illustrates the response to another phosphate and the manner in which the availability was related to that of the reference material. The increase in bone calcification was sufficiently linear to permit valid comparisons between the curves.

A wide variety of inorganic phosphates were studied by the use of this assay procedure. Some of the results obtained are presented in table 1. These data indicate that most, but not all, of the phosphorus supplements commonly used in feeding poultry are satisfactory from the standpoint of availability. There is more variability between different samples of the same type of product than between the average values of the three most widely used classifications: dicalcium phosphate, defluorinated phosphate and bone meal.

The pure orthophosphates without exception showed a high degree of availability. The acid salts, monocalcium phosphate and potassium acid phosphate, were more completely utilized than tricalcium phosphate. This was not true in these experiments for sodium acid phosphate.

Most of the feed grade dicalcium phosphates exhibited a high degree of availability, comparable to that of reagent grade dicalcium phosphate. The data indicate, however, that

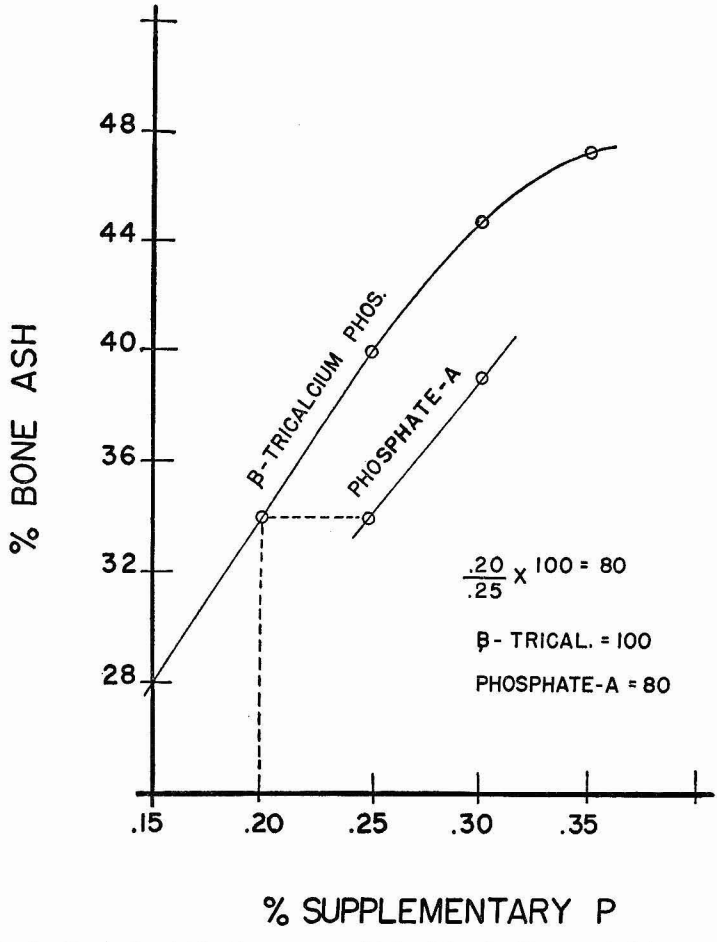

Fig. 1 Typical calcification curves obtained by feeding graded increments of beta-tricalcium phosphate and an experimental material. The method of relating the availability of phosphate A to that of beta-tricalcium phosphate is illustrated.

different methods for manufacturing this product result in some variation in quality.

The data for defluorinated phosphates presented in table 1 show that these supplements possess the same order of availability as feed grade or reagent grade dicalcium phosphate when prepared by calcination or precipitation. Material defluorinated by a precipitation process was slightly more satis-

TABLE 1

Comparative biological values of inorganic phosphates

(These values are based on ability to promote calcification in young chicks. All supplements are compared with beta-tricalcium phosphate, to which a biological value of 100 has been assigned arbitrarily.)

REAGENT GRADE ORTHOPHOSPHATES		FEED GRADE DICALCIUM PHOSPHATES	
Beta-tricalcium phosphate	100	Sample A	97
Dicalcium phosphate	98	Sample B	97
Monocalcium phosphate	113	Sample C	96
Potassium acid phosphate	109	Sample D	89
Sodium acid phosphate	101		
		DEFLUORINATED PHOSPHATES	
BONE PRODUCTS		Calcined	94
Steamed bone meal		Fused	82
Sample A	100	Precipitated	99
Sample B	100		
Sample C	100	RAW ROCK PHOSPHATES	
Sample D	95	Curacao Is. phosphate	87
Sample E	94	Fla. land pebble rock	50
Sample F	90	Tennessee brown rock	[1]
Sample G	70	Colloidal phosphate	[1]
Foreign bone meal	87		
Spent bone char	84	ACID PYROPHOSPHATES	
Bone ash	89	Calcium acid pyrophosphate A	67
		Calcium acid pyrophosphate B	50
PYROPHOSPHATES			
Alpha Ca pyrophosphate	0	METAPHOSPHATES	
Beta Ca pyrophosphate	0	Beta Ca metaphosphate	0
Gamma Ca pyrophosphate	0	Gamma Ca metaphosphate	0
Vitreous Ca pyrophosphate	[1]	Vitreous Ca metaphosphate	45
Na pyrophosphate, decahydrate	57 [2]	Sodium metaphosphate	[1]
		Potassium metaphosphate	0

[1] Availability too low to rate with purified diet. With practical type of diet the following values were obtained: vitreous Ca pyrophosphate 20, Tennessee brown rock 25, colloidal phosphate 25, sodium metaphosphate 28.

[2] Somewhat toxic.

factory than the calcined products. Although early samples of the fused product were credited with better availability than comparable material prepared by calcination, the one sample of fused material (T.V.A., experimental) included in these studies was not as well-utilized as the calcined products.

The various crystalline modifications of calcium pyrophosphate showed no detectable degree of availability to chicks in these studies. The amorphous or glassy form was very slightly available. Sodium pyrophosphate, decahydrate, showed an appreciable degree of availability but was sufficiently toxic to cause death in some chicks. Two samples of calcium acid pyrophosphate were respectively one-half and two-thirds as available under these conditions as tricalcium phosphate. Even this is considered too low for a satisfactory feeding supplement.

Among the metaphosphates studied, only the vitreous form of calcium metaphosphate showed any appreciable degree of availability. This material, however, is not a satisfactory source of phosphorus.

There were wide differences in the availability of phosphorus from the different untreated raw rock phosphates. Only Curacao Island phosphate showed a satisfactory degree of availability for the chick. The fluorine content of this material (0.5 to 1.0%) may preclude its safe use for other classes of domestic animals. Florida land pebble rock was appreciably more available than the brown Tennessee rock. The Florida rock has a significantly lower content of iron and aluminum than does rock from Tennessee. These results, which have been repeated in 5 different experiments, do not support the conclusion of Matterson et al. ('45) that "raw rock phosphate is as available if not more so than tricalcium phosphate."

The only domestic rock product widely used in feeds is the so-called "colloidal phosphate," or soft phosphate with colloidal clay. In the phosphate industry this product is generally known as waste pond phosphate, inasmuch as it is the waste product from washing or desliming phosphate

rock. It contains finely divided phosphate together with large amounts of clay and other impurities. The clay in these phosphates is the "colloidal" component (Sauchelli, '51). It has a slightly higher P : F ratio than high grade phosphate ore but contains a larger proportion of aluminum, iron and silica. When added to the low-P, purified basal diet, "colloidal" phosphate was so unavailable that most of the chicks died. Enough chicks did not survive the 4-week feeding period to permit an accurate measurement of availability. Five samples of "colloidal" phosphate from different sources were uniformly poor in availability. This confirms previous references to the poor utilization of these phosphates by chicks (Gillis, Norris and Heuser, '51; Miller and Joukovsky, '53; Grau and Zweigart, '53).

Studies with a practical type of diet

The purified type of diet described above is satisfactory when studying compounds having the same order of availability as pure orthophosphates. However, products of very poor availability such as "colloidal" phosphate cannot be adequately rated with this diet because of the high mortality which occurs. This difficulty can be overcome by using a diet containing enough phosphorus to prevent mortality but not an optimum amount. A diet containing a practical type of ingredients low in phosphorus is most convenient and economical for this purpose. An alternative is the addition of a limited amount of highly available phosphorus to the purified type of basal ration.

A practical type of basal diet which proved satisfactory had the following percentage composition: soybean meal 25, corn gluten meal 10, alfalfa leaf meal 3, iodized salt 0.5 and yellow corn meal to adjust the total to 100 after addition of calcium and phosphorus supplements. In addition, the following supplements were added per pound: vitamin B_{12} 4 µg, riboflavin 0.5 mg, choline chloride 450 mg, niacin 10 mg, $MnSO_4 \cdot 4H_2O$ 100 mg, vitamin A and D feeding oil, sufficient

to supply approximately 2,000 I.U. of A and 180 I.U. of D. The calcium and phosphorus contents of this diet varied with the source of ingredients but were always approximately 0.15% Ca and 0.35% P. A significant part of the latter element was present as phytin. The Ca : P ratio was maintained at 2 : 1 by adding limestone to the unsupplemented basal diet and to the basal diet containing the phosphorus carriers being studied. When this adjustment was made, the diet was adequate to keep the chicks alive for 4 weeks without additional phosphorus.

TABLE 2

Results of studies on availability of "colloidal" phosphates in a practical type of chick diet

PHOSPHORUS SUPPLEMENT	NO ADDED P		0.1% ADDED P		0.2% ADDED P		0.3% ADDED P	
	Wt. 4 wk.	Bone ash	Wt. 4 wk.	Bone ash	Wt. 4 wk.	Bone ash	Wt. 4 wk.	Bone ash
	gm	%	*gm*	%	*gm*	%	*gm*	%
None	209	28.0						
Beta-tricalcium phosphate			271	38.2	305	45.5	298	47.0
Colloidal phosphate A			190	30.1	217	31.9	244	37.9
Colloidal phosphate B			206	30.7	226	33.5	207	35.8

An example of the type of results which were obtained with the practical type of diet is presented in table 2. In this experiment two samples of "colloidal" phosphate were compared with beta-tricalcium phosphate. Growth and calcification in the chicks were readily improved by adding beta-tricalcium phosphate to the diet. However, the response of chicks to colloidal phosphate in the practical type of diet was much less satisfactory. There was no consistent or significant improvement in the growth of chicks fed colloidal phosphate. Of 6 lots which received this material, three weighed less than the basal lot and three weighed more after 4 weeks. Some improvement in bone ash was obtained by adding colloidal phosphate to the basal diet, although the increase was very much less than that caused by a comparable amount of phosphorus from beta-tricalcium phosphate.

By plotting the data on calcification obtained in this and other experiments employing the P-deficient, practical type of diet, it was determined that phosphorus from colloidal phosphate was approximately 25% as effective for increasing bone ash as that from beta-tricalcium phosphate. The reason for its failure to exert a comparable effect on growth is not clear but has been observed by other workers (Grau and Zweigart, '53).

DISCUSSION

The results obtained in these experiments show that equivalent amounts of phosphorus from different sources are not necessarily of equal nutritional value. The content of available rather than total phosphorus determines the usefulness of a supplement. Phosphates used as plant nutrients have long been sold on the basis of their availability as measured by certain legally defined tests. It would appear that information on availability as determined by some standard assay procedure would also be very valuable to the animal nutritionist in formulating rations.

The primary advantage of the biological assay for available phosphorus described in this paper is its reliability. Since the animals' response to graded increments of different phosphates is actually measured, there can be no question about applying the results in practice to the species of animal used in the assay. However, caution is required in applying the results to species other than the test animal. In experiments with rats, not reported, it has been found that qualitatively the same general relationships of phosphorus availability hold for this species as for the chick. The same numerical evaluation of phosphorus supplements does not apply for the two species, however.

The chief disadvantages of an assay of this type are time and expense. A three- or 4-week feeding period must be followed by a period of at least several days during which the bones are prepared, extracted, dried and ashed. It is usually at least 5 weeks from the initiation of the experiment

until results are obtained. A speedier reliable method for estimating availability would be very desirable. For some types of compounds the use of the radioisotope, P^{32}, offers some encouragement in this direction. Its use, however, is not likely to be more economical than the small animal assay. Possibilities exist that the length of the experimental feeding period can be shortened considerably. This problem is being investigated at the present time.

SUMMARY

A method for comparing the availability to chicks of phosphorus from different sources has been described. It involves feeding graded increments of the phosphates in a diet very deficient in phosphorus and determining the resulting increases in bone ash after 4 weeks. For compounds of moderate or high availability, a purified type of diet very low in phosphorus was used. For studying poorly available compounds, a diet with satisfactorily low phosphorus content was formulated from ingredients of a practical type.

Pure beta-tricalcium phosphate was used in all experiments as a standard of comparison and arbitrarily assigned a biological value of 100.

The chemically pure orthophosphates, mono-, di-, and tricalcium phosphate, sodium acid phosphate and potassium acid phosphate, were highly available. The acid salts, monocalcium phosphate and potassium acid phosphate, were better utilized than the other pure orthophosphates.

Feed grade materials of excellent availability were average samples of dicalcium phosphate, defluorinated phosphate and domestic steamed bone meal. Other bone products of slightly lower availability were spent bone char, bone ash and imported bone meal of unknown history.

None of the pyrophosphates or metaphosphates was satisfactory, although significant amounts of phosphorus were utilized from calcium acid pyrophosphate and vitreous metaphosphate.

Among the untreated rock products, only Curacao Island phosphate showed a satisfactory degree of availability. Florida land pebble phosphate was appreciably better utilized than brown phosphate rock from Tennessee.

Waste pond phosphate, the "colloidal" phosphate of commerce, was studied in the purified type of diet and also the practical type of basal ration. Excessive mortality occurred with the purified diet containing colloidal phosphate. In the practical diet, colloidal phosphate was approximately 25% as effective as an equivalent amount of phosphorus from beta-tricalcium phosphate in increasing the ash content of bones. However, it had no significant beneficial effect on growth.

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EFFECTS OF PHOSPHATE FERTILIZATION AND DIETARY MINERAL SUPPLEMENTS ON THE NUTRITIVE VALUE OF SOYBEAN FORAGE[1]

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The effects of fertilization on the nutritive value of food crops are not well understood and, in general, only hypotheses can be developed from the data available at the present time. A part of the problem is whether or not varying levels of soil fertility effect changes in the nutritive value of plants other than those measurable by the usual chemical methods. There is substantial evidence that differences do occur in the mineral content of plants grown on different soils (Beeson, '46), but there is a paucity of bioassay data concerning concomitant changes with respect to utilization of nutrients. One reason for the latter situation is the difficulty inherent in the evaluation of a complex involving the soil, the plant and the animal (Matrone et al., '49, '53). Another is the lack of bioassay techniques having sufficient specificity and sensitivity to reveal essential differences among experimental crops. The present report is a part of an investigation initiated in 1945 to study the effects of phosphate fertilization on the nutritive value of soybean plants (Matrone et al., '49, '53).

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In previous studies (Matrone et al., unpublished) no significant differences were found in the gains in weight of rabbits fed soybean hays grown on phosphated and non-phosphate-fertilized plots in 1948 and 1950, despite marked differences in the phosphorus content of the test hays. These results cast doubt on the suitability of either the bioassay technique employed or the rabbit as a test animal for these studies. A reappraisal of the bioassay technique led to the belief that the primarily roughage diets were suboptimum in protein, particularly in the early stages of the bioassay when the rabbits were younger. Accordingly, the test diets were supplemented with protein and certain procedures changed (see below). With these modifications, the 1948 and 1950 crops were reassayed, again using the rabbit, with the following objectives in mind: To evaluate (a) the phosphorus status and (b) the changes in growth rate arising from factors apart from phosphorus per se.

EXPERIMENTAL

The agronomic and chemical procedures, diet preparation and feeding techniques followed in these experiments were similar to those employed in earlier studies (Matrone et al., '53). The 1948 crop (Roanoke variety) was grown on 12 plots employed since 1945, whereas the 1950 crop (Ogden variety) was grown on 4 plots in an adjacent field. Lime and potash were added to all plots annually, but the phosphate level applied annually was added only to half of the plots in a randomized block design retained from year to year. Each crop contained less than 1% weeds and was harvested before the beans set in the pods. Both crops were barn-cured, but the 1950 crop was cured with the aid of artificial heat. The forages of each crop were separately composited into two lots; one was made up of forages from the control plots and the other of forages from the treated plots (hereafter designated by 48 — P, 48 + P, 50 — P, 50 + P, respectively).

For all studies, rabbits of the Dutch breed, weaned at 4 weeks of age, were placed on a preliminary diet (76% 48 — P hay, 10% cerelose,[2] 5% cottonseed oil[3] and 9% egg albumen[4]) for 11 to 12 days and then placed on the test diets. The experimental diets consisted of 76% of the forage to be tested (processed in a Wiley mill), 13% cerelose, 5% cottonseed oil and 6% egg albumen. The 1950 test hays were assayed with and without a supplement of dietary $CaHPO_4$ (designated as M hereafter) in a 2×2 factorial; thus, there were 6 experimental diets, two for the 1948 crop and 4 for the 1950 crop. The replications of the randomized block design employed were made on the basis of sex and initial weight; there were 7 rabbits receiving each diet. Each animal was given 20 μg of biotin subcutaneously biweekly and one drop of calciferol[5] orally weekly.

RESULTS AND DISCUSSION

The yield and composition data of the test forages are presented in table 1. There was a positive response to phosphate fertilization in terms of yield per acre and concentration of phosphorus and calcium in the plant, but an inverse response in terms of per cent leaf of the whole plant, for both crop years (table 1). It does not appear that the latter result is an effect of stage of maturity, since the small phosphorus-deficient plants reached the blossoming and pod-forming stage at approximately the same time as the treated plants. The protein concentration in the 1948 crop was significantly higher in the — P forage than in the + P forage. Similar differences were observed in the leaves and stems. Since different varieties of soybean were planted in 1948 and 1950, the variation in protein content between the test forages may have been

[2] Crystalline glucose, Corn Products Sales Company, Norfolk, Virginia.

[3] Wesson oil, distributed by Wesson Oil and Snowdrift Sales Company, New Orleans, Louisiana.

[4] Purified egg albumen, Nutritional Biochemical Corporation, Cleveland, Ohio.

[5] Drisdol, brand of crystalline vitamin D_2 (Calciferol) prepared by Winthrop-Sterns, Inc.: the commercial product was diluted with Wesson oil to contain approximately 62 I.U. per drop.

due to either year or variety, or to a year-by-variety interaction.

Rabbit experiment 1

In experiment 1 (table 2) the rabbits fed the — P forages unsupplemented with $CaHPO_4$ appeared to be deficient in phosphorus. After 11 weeks on experiment, measurements taken on the rabbits fed the 1948 test forages revealed that the 48 + P diet was superior to the 48 — P diet in terms of weight gains, inorganic phosphorus of blood serum and bone formation as indicated by breaking load and specific gravity

TABLE 1

Yield, leaf percentage and composition of experimental forages

(Moisture-free basis)

CHARACTERISTICS MEASURED	CROP YEAR AND FERTILIZATION [1]			
	1948 [2]		1950	
	- P	+ P	- P	+ P
Yield/acre, lb.	1,200	2,800	980	2,500
Leaf, %	46	41	49	43
Crude protein, %	19.21	14.34	15.56	16.86
Ether extract, %	1.94	1.67	2.23	2.71
Crude fiber, %	31.51	34.11	32.70	31.40
N. F. E., %	40.73	43.87	42.21	41.94
Ash, %	6.61	6.02	7.30	7.07
Phosphorus, %	0.106	0.144	0.098	0.164
Calcium, %	0.744	0.880	0.839	0.934

[1] — P = Grown on plots not receiving phosphate fertilizer.
+ P = Grown on plots receiving phosphate fertilizer.
[2] Roanoke variety grown in 1948 and Ogden in 1950.

of the femur. In addition, three out of the 7 animals on the 48 — P diet died between the 7th and 10th week of the 11-week experimental period. The cause of death was judged to be associated with the deficiency of the diet. Except for the fact that all animals survived, similar results were obtained with the groups of animals fed the 50 — P test diet and the 50 + P diet, after 7 weeks on experiment. Direct evidence indicating that the primary deficiency of the 50 — P diet was phosphorus was furnished by the response of rabbits receiving the 50

TABLE 2

Summary of gain, feed intake, blood and femur data of rabbits fed test diets in experiment 1
(Per rabbit basis)

ITEMS COMPARED	DIETS [1,2]								
	1	2	diff. (2–1)	3	4	diff. (4–3)	5	6	diff. (6–5)
Crop year and fertilizer [3] treatment	48 — P	48 + P		50 — P	50 + P		50 — P	50 + P	
Dietary $CaHPO_4$ supplement, %	0	0	0	0	0	0	0.75	0.75	
P content of diet	0.081	0.109		0.074	0.124		0.247	0.297	
Ca content of diet	0.560	0.672		0.635	0.707		0.859	0.931	
Duration of experimental period, wks.	11	11		7	7		7	7	
Gains, gm	607	771	+ 167** [4]	467	618	+ 151**	742	650	— 92
Intakes, gm	4,123	4,698	+ 575	2,576	3,010	+ 434	3,235	3,173	— 62
Hb, gm/100 ml of whole blood	11.09	10.81	— 0.28	11.38	13.14	+ 1.76	11.91	12.56	+ 0.65
Inorganic P of serum, mg/100 ml	4.85	6.05	+ 1.20**	6.36	7.39	+ 1.09**	7.17	7.14	— 0.03
Serum Ca, mg/100 ml	14.96	14.21	— 0.75	15.74	15.60	— 0.14	15.28	15.72	+ 0.44
Serum Mg, mg/100 ml	2.86	3.79	+ 0.93	3.38	4.89	+ 1.51	4.53	3.29	— 1.24
Breaking load of femur, gm	3,855	8,326	+ 4,471**	4,880	10,401	+ 5,521**	11,119	10,888	— 231
Sp. gravity of femur	0.8277	1.0057	+ 0.1780**	0.9267	1.0129	+ 0.1862**	1.0506	1.0585	+ 0.0079
Ash (moisture and fat free) of femur, %	49.6	56.6	+ 7.0	51.3	57.4	+ 6.1	59.6	60.1	— 0.5

[1] Basic formula of diets consisted of 76% test forage, 13% cerelose, 6% egg albumen and 5% Wesson oil.
[2] Seven animals per diet: three animals on diet 1 died before completion of experiment.
[3] + P and — P signify crops grown with and without phosphate fertilization, respectively.
[4] ** = highly significant (P = 0.01).

— PM diet, which was similar to the 50 — P diet in all respects except that it contained a supplement of $CaHPO_4$. Calcium per se can be ruled out as a factor, since all diets contained an adequate amount. The $CaHPO_4$ supplementation, moreover, eliminated the differences observed between the

TABLE 3

Summary of rabbit gains, feed intakes, phosphorus and calcium composition and digestion coefficients of diets in experiment 2 [1]

(Per rabbit basis)

ITEMS COMPARED	DIET 5 (50—PM)	DIET 6 (50+PM)	DIFF. 6 – 5
	Feeding trial 2 [2]		
P content of diet	0.548	0.572	
Ca content of diet	1.043	1.197	
First 5 weeks of experiment			
gains, gm	667	565	— 102**
feed intake, gm	2,508	2,336	— 172
Last 6 weeks of experiment only			
gains, gm	410	454	+ 44 n.s.
feed intake, gm	3,700	3,891	+ 191
	Digestion coefficients [3]		
Dry matter, %	64	63	— 1 n.s.
Crude protein, %	80	82	+ 2*
Ether extract, %	85	88	+ 3**
Crude fiber, %	37	33	— 4 n.s.
N. F. E., %	73	72	— 1 n.s.

[1] Eleven rabbits/diet.

[2] See table 2 for diet formulations; M = supplement of $CaHPO_4$.

[3] Digestion trial conducted at completion of feeding trial.

n.s. = not significant ($P \neq 0.05$).

* = significant ($P = 0.05$).

** = highly significant ($P = 0.01$).

non-supplemented — P and + P diets (table 2). In the comparison between the 50 + P and the 50 + PM diets, no significant improvement in any of the measurements taken was observed in the group of animals fed the 50 + PM diet, indicating that the phosphorus concentration in the diets formulated with the 1950 phosphate-fertilized forage was adequate.

The foregoing data indicate that the phosphorus concentration was a primary factor in the nutritional differences between the hays from phosphate- and non-phosphate-fertilized soils when the test diets were supplemented with protein.

Rabbit experiment 2

The difference in weight gain among the groups of rabbits on the 50 — PM, 50 — P, 50 + PM and 50 + P diets in experiment 1 suggested that after the phosphorus deficiency of the diet formulated from the — P forage was corrected, it was superior to the diets made up of the + P forage. To verify these results, a second experiment was conducted in which 11 rabbits were placed on each of the two diets, 50 — PM and 50 + PM. The experimental procedure employed was identical to that of experiment 1 except that the experimental period was extended from 7 to 11 weeks, and the $CaHPO_4$ supplements were increased from 0.75% to 1.6%.

The results of experiment 2 are presented in table 3. As in experiment 1, the animals on the 50 — PM diet gained more than those on the 50 + PM diet. The difference was greatest at the 5th week and then gradually became smaller. The accumulated gains for the first 5 weeks were significantly different, but the accumulated gains calculated for the last 6 weeks only were not significantly different (table 3). The results of a covariance analysis (Snedecor, '46) of these data, adjusting the gains to a mean food intake, indicated that the feed efficiency of the 50 — PM diet during the first 5 weeks was higher than that of the 50 + PM diet. Similar results were found for the diets of the first experiment. In experiment 2, the feed efficiency of the two diets under study was not significantly different after the first 5 weeks.

The next step undertaken was to determine, if possible, the factor causing the observed differences. Since the small phosphorus-deficient soybean plants had a higher percentage of leaves by weight than did the normal size plants grown on the phosphated soil (table 1), it was reasoned that the small

plants might be more digestible. Accordingly, after the feeding trial a digestion trial was conducted with the same experimental animals. During the digestion trial each animal was continued on the same diet it had received during the feeding trial. As is indicated in table 3, no significant differences were observed in digestibility of dry matter, crude fiber and nitrogen-free extract, but the crude protein and the ether extract of the 50 + PM diet were slightly more digestible than those of the 50 — PM diet. It appears from these data, therefore, that the digestibility of the diets did not differ sufficiently to account for the observed differences in weight gain. There remains the possibility, however, that if the digestion trial had been conducted with younger rabbits, differences in the digestibility of the diets might have been revealed. This possibility is strengthened by the feed efficiency results discussed above.

In view of the amount and quality of protein which was added to the test diets, and of the positive nitrogen balances of similar magnitude obtained in studies conducted on these test diets with other rabbits in a previous experiment, the probability of protein quality or quantity being a factor influencing the differences observed was discounted.

A third possibility considered was a difference in mineral elements other than calcium and phosphorus. Spectrographic analysis [6] of the test forages revealed that the 50 — P forage was lower in magnesium and higher in aluminum and silicon than the 50 + P forage. No significant differences between the test forages were found for sodium, potassium, boron, manganese or copper. It is unlikely that the higher concentration of the aluminum and silicon in the 50 — P forage had a beneficial effect on growth. Silicon has not been shown to be essential for animals, whereas Williams et al. ('38, '42) suggest that large amounts of aluminum in low phosphorus hays might decrease the availability of phosphorus by forming insoluble phosphates in the intestinal tract of the animal.

[6] Acknowledgment is made to Dr. W. L. Lott, Chemistry Department, North Carolina State College, for the spectrographic analyses.

Of the factors considered as possible explanations of the observed differences, the one relating to the difference in leafiness appears most promising. Whether it was due to a difference in the digestibility of the test forages by the young rabbits in the early part of the experiment, or to some other factor correlated with per cent leafiness, is a problem for further study.

SUMMARY

1. Data from two crops (1948 and 1950) showed that phosphate fertilization doubled the yield of soybean forage and produced a plant (+ P) having a higher concentration of calcium and phosphorus but a lower leaf percentage, by weight, than the plants grown on soil not fertilized with phosphate (— P).

2. A rabbit assay is described which was successfully employed in the study of the relative nutritive value of these crops. Rabbits fed diets containing the — P forage made smaller gains and had a lower level of inorganic phosphorus in the blood serum, and bones of greater fragility, than did those fed the diets containing the + P forage.

3. A supplement of $CaHPO_4$ (M) added to the test diets not only eliminated the differences observed between the non-supplemented diets, but in addition the rabbits on the — PM forage diet gained more than those on the + PM forage diet. Data on the digestible nutrients of these diets and spectrographic analysis for 8 other elements offered no explanation for the last-observed difference.

4. Phosphorus concentration and a factor not identified, but possibly associated with leafiness, were the principal differences found between soybean forages differentially fertilized with phosphate.

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