Effect of Protein Intake on Ribonucleic Acid Metabolism in Liver Cell Nuclei of the Rat [1]

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ABSTRACT Liver cell nuclei were isolated from 3 groups of rats, 1) animals that had been fed a protein-free diet, 2) animals in the post-absorptive state after receiving a diet of adequate protein content, and 3) animals actively absorbing amino acids from a recent meal of protein. The nuclei were fractionated by successive extraction with a phosphate buffer and M NaCl, leaving a "nucleolar" residue. Determinations of RNA in whole liver nuclei and their subfractions showed significant effects of protein intake. In the protein-depleted group, there was a reduction in the amount of RNA extractable with phosphate buffer and with M NaCl, and an increase in the RNA content of the nucleolar residue (group 1 vs. group 2). Two hours after feeding protein to fasting rats (group 3 vs. group 2), the total RNA content of the nucleus increased significantly. This was associated with a large increment in the amount of RNA in the nucleolar residue. However, the feeding of protein reduced the relative uptake of adenine-C^{14} into nucleolar RNA, indicating that the extra RNA in the nucleolar residue was of low metabolic activity. Protein feeding also caused a marked increase in adenine-C^{14} uptake by the RNA of the M NaCl extract. Attempts to identify dietary effects on the amounts of different molecular species of nuclear RNA by sedimentation analysis led to equivocal results.

When rats are fed a protein-free diet, the amount of RNA in the liver diminishes rapidly (1, 2). Munro and Clark (3) concluded that the RNA lost from the liver as a result of protein deficiency comes mainly from the endoplasmic reticulum of the cell cytoplasm. There is, however, histological evidence that protein depletion also affects the nucleus of the liver cell, particularly the nucleolus. Early studies of nucleolar size in liver cells produced conflicting evidence of the effects of protein deficiency (4, 5). However, Stenram has extensively re-investigated the effect of dietary protein intake on liver cell nuclei of rats (6–8) and mice (9) and has demonstrated considerable enlargement of the nucleolus after a protein-free diet has been fed for a few days. By measuring the dry matter of the nucleolus interferometrically before and after ribonuclease treatment, he was able to show that the total amount of RNA in the nucleolus increases during protein depletion (10, 11). It thus appears that a short period of protein deficiency causes changes in nucleolar RNA content that are opposite in direction to the changes taking place in the RNA content of the cytoplasm. It seemed desirable to apply other techniques to confirm and extend these histological findings on liver nuclei. We have accordingly isolated nuclei from the livers of rats receiving dietary protein at different levels and have measured the amounts of RNA in the nuclei and their subfractions. In addition, animals were injected with adenine-C^{14} and the effect of diet on the metabolic activity of nuclear RNA was examined. These studies show that the protein content of the diet affects the amount and metabolic activity of RNA in the liver cell nucleus.

EXPERIMENTAL

Animals and diets. Male albino rats of about 180 g were caged separately and fed isocaloric quantities of protein-free or protein-containing diets described previously (12). The total daily food intake per rat provided 8 g carbohydrate and 0.5 g fat for the protein-free diet and 5.6 g carbohydrate, 2.4 g protein (casein) and 0.5 g fat for the protein-containing diet. Both diets contained adequate quantities of vitamins and minerals. The diets were fed twice daily in fixed amounts. The morn-

Received for publication December 3, 1964.

[1] This study was supported by U.S. Public Health Service Research Grant no. CA 07087 from the National Cancer Institute.

ing meal contained carbohydrate, fat, vitamins and minerals and the evening meal carbohydrate, fat and all the protein of the diet; for animals fed the protein-free regimen, carbohydrate was substituted isocalorically for the protein of this meal. The rats soon learned to consume each meal promptly and completely. After the animals had been fed these diets for 5 days, they were killed under ether anesthesia in the morning in the fasting state, 18 hours after consuming the last meal on the previous evening, except for some animals that were given 2 g casein shortly before death. In this way, 3 dietary groups were obtained: 1) rats in a depleted condition as a result of eating a protein-free diet, 2) rats in the post-absorptive state after a diet of adequate protein content, and 3) rats actively absorbing amino acids after a recent meal of protein. For dietary experiments not involving the use of radioactive isotopes, the rats in this third category were killed 2 hours after receiving casein. For experiments in which the effect of diet on the uptake of labeled precursors by RNA was examined, adenine-8-C^{14}[2] (5 μc/rat) was injected intraperitoneally 1 hour after feeding casein and the rats were killed at various times thereafter. At all these time-intervals, the stomachs of the casein-fed animals contained food, indicating that continuous absorption of amino acids was proceeding throughout the period of isotopic labeling of liver RNA.

Isolation of liver-cell nuclei. After exploring a number of published methods for isolation of nuclei, a new procedure was devised. This was based on the principle used by Wilczok and Chorazy (13) of separating crude nuclei by centrifugation in a conventional homogenization medium and then purifying them by sedimentation through dense (2.2 M) sucrose. We have found that their experimental conditions gave a poor yield of liver nuclei, and the following modification was adopted. Rats were anesthetized with ether, their livers were perfused with a solution containing 0.25 M sucrose, 0.002 M $CaCl_2$ and 0.001 M $MgCl_2$, and were then disintegrated in a Potter-Elvehjem homogenizer with 10 volumes of the solution used for perfusion. This homogenization medium is similar to that originally used for isolation of nuclei by Allfrey et al. (14) with the addition of Mg^{++} to minimize clumping of nuclei (15). The homogenate was first spun at 100 × g for 5 minutes to remove unbroken cells. The supernatant fraction was layered onto a solution containing 0.34 M sucrose, 0.002 M $CaCl_2$ and 0.001 M $MgCl_2$, and then spun at 600 × g for 10 minutes. The crude nuclear pellet obtained from this spin was resuspended in a small volume of the 0.25 M sucrose medium and 19 volumes of a solution containing 2.31 M sucrose, 0.002 M $CaCl_2$ and 0.001 M $MgCl_2$ were added to give a final sucrose concentration of 2.20 M (density 1.273). On centrifugation at 30,000 × g for 1 hour, a precipitate of pure nuclei was obtained in good yield. After rinsing gently with ice-cold water, the nuclei were resuspended in 0.25 M sucrose containing 0.002 M $CaCl_2$ and 0.001 M $MgCl_2$.

Fractionation of nuclei. The nuclei were fractionated according to the scheme used by Allfrey et al. (16) for calf thymus nuclei, in which the nuclei are successively treated with phosphate buffer and M NaCl, leaving a nucleolar residue. Purified nuclei, prepared as described above, were first sedimented from the suspension by spinning at 1000 × g for 10 minutes. They were then extracted by stirring for 5 minutes with 1 ml ice-cold 0.1 M phosphate buffer (KH_2PO_4/K_2HPO_4 at pH 7.1) and recovered as a pellet by spinning at 1000 × g for 15 minutes. The extraction procedure was repeated twice, using 1 ml and 0.5 ml phosphate buffer, respectively. The 3 supernatant fractions were combined to give the "phosphate extract." The residue was then stirred for 10 minutes with 1 ml ice-cold M NaCl and spun at 2000 × g for 20 minutes. This extraction was repeated using 1 ml and 0.5 ml M NaCl, and the supernatant fractions were combined ("sodium chloride extract"). The precipitate spun down from the final NaCl extraction is the "nucleolar residue."

Estimations on nuclei and subfractions. These were based on the techniques described by Fleck and Munro (17). The nucleic acids and protein in the preparations of whole nuclei and their subfractions were precipitated by addition of

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$HClO_4$ to give a final concentration of 0.2 N. In the case of nuclear specimens containing adenine-C^{14}, non-radioactive adenine (3 mg/ml) was added to the $HClO_4$. The precipitate was then washed twice centrifugally with cold 0.2 N $HClO_4$ without added non-radioactive adenine and finally digested at 37° for 1 hour with 2 ml of 0.3 N KOH to release all the RNA in acid-soluble form (17). Thereafter protein and DNA were precipitated by adding 1 ml of 1.2 N $HClO_4$. The precipitate was spun down and washed twice centrifugally with 0.5 ml of 0.2 N $HClO_4$. The supernatant fractions from each treatment with $HClO_4$ were combined for measurement of RNA content by ultraviolet absorption at 260 mμ (17); examination of the RNA fractions obtained from whole nuclei and from each subfraction gave ultraviolet spectra typical of uncontaminated RNA. The precipitate containing DNA was taken up in 0.1 N KOH and its deoxyribose content was measured by the Ceriotti procedure (18). A few estimates of the phospholipid content of nuclei were also made; after precipitation of the nuclei with 0.2 N $HClO_4$, the precipitate was extracted with a series of organic solvents (17) and the phosphorus content of the combined solvents was estimated (19). Phospholipid content was obtained by multiplying phosphorus by 23 (20).

Measurement of adenine-C^{14} uptake by RNA. The RNA fraction obtained on acidification of the digest, as described above, was made alkaline with KOH and the $KClO_4$ precipitate was spun off. Samples of the supernatant fluid were then plated onto metal planchets, using lens paper to give uniform self-absorption (21). After drying, the samples were counted to 1000 counts in a Nuclear Chicago gas-flow counter.

Sedimentation analysis of nuclei and whole liver RNA. Purified RNA was prepared by a modification of the phenol procedure (22) from isolated nuclei and from whole liver samples. The nuclei were isolated from livers homogenized in sucrose medium to which was added a small amount of bentonite to inhibit ribonuclease (23). The final purified nuclear pellet was taken up in acetate buffer containing 0.05 M NaCl, 0.01 M sodium acetate, 0.001 M $MgCl_2$ and 0.5% naphthalene-1:5-disulphonate, with a small amount of bentonite added, and brought to pH 5.25 with acetic acid. In experiments with whole cell RNA, homogenization was performed directly in this buffer. Sodium lauryl sulphate was added to both preparations to give a final concentration of 1%. After shaking, an equal volume of 90% phenol containing 0.1% 8-hydroxyquinoline was added and the mixture shaken for 1 hour at 0°. Following centrifugation at 30,000 × g for 30 minutes to separate the phases and remove DNA and glycogen, the aqueous layer was recovered and bentonite was added. The phenol layer was washed with the acetate buffer (without naphthalene disulphonate) and the 2 aqueous supernatants were combined and extracted repeatedly with ether to remove traces of phenol. After precipitation of the RNA with ethanol, it was redissolved in acetate buffer at pH 6.8 and incubated for 20 minutes at 37° with 40 μg/ml deoxyribonuclease (DNase I). Chloroform was added to precipitate the DNase, the aqueous layer was separated and the RNA precipitated with ethanol. The RNA, suspended in acetate buffer, was finally dialyzed overnight against acetate buffer at pH 5.25 to remove ethanol and products of DNA digestion. The final product contained only traces of protein and DNA. The RNA sample was analyzed for its spectrum of molecular sizes in a Spinco Model E analytical ultracentrifuge, using both the Schlieren and ultraviolet optical systems. Sedimentation coefficients (S values) were computed (24) for the different peaks present and approximate estimates of the relative amounts in each peak were obtained by measuring the areas under them.

RESULTS

Properties of liver-cell nuclei isolated by our procedure. The method of nuclear isolation described above yielded about 50% of the total population of nuclei in the liver, as judged by recovery of DNA from liver samples of known DNA content. Examination by light microscopy and by electron microscopy showed no evidence of significant contamination with whole cells or cytoplasmic components. Chem-

ical analysis for RNA and phospholipid content also suggested that the nuclei were free from contaminants. Whole rat liver cells have an RNA-to-DNA ratio of 3.1 and a phospholipid-to-DNA ratio of 9.8 (25), whereas the best preparations of rat liver nuclei recorded in the literature have RNA-to-DNA ratios of 0.2 to 0.3 and phospholipid-to-DNA ratios of 0.1 to 0.3 (26). Consequently, measurement of these ratios provides a sensitive means of detecting contamination of nuclear preparations with cytoplasmic components and whole cells. Our nuclei had an RNA-to-DNA ratio of 0.25 (mean of 22 preparations); we also prepared rat liver nuclei by several reliable published procedures (13, 27, 28) and obtained RNA-to-DNA ratios around 0.25. The phospholipid-to-DNA ratio of our nuclear preparations was 0.25. Gurr and co-workers (29) report a ratio of 0.33 (ratio of lipid phosphorus to DNA phosphorus of 0.13) for liver nuclei with double membranes and one-half this figure for nuclei that had lost their outer membrane during preparation. The presence of double nuclear membranes in our preparation was confirmed by high resolution electron microscopy.[3]

Properties of nuclear subfractions. The nuclei were fractionated by the scheme devised by Allfrey et al. (16) for thymus nuclei. The first step in this procedure is extraction of the nuclei with phosphate buffer ("phosphate extract"), followed by extraction with M NaCl ("sodium chloride extract"), leaving an unextracted fraction ("nucleolar residue"). Extraction with M NaCl removes the DNA from thymus cell nuclei (16) but no DNA was extracted from our liver cell nuclei. This may be due to a tissue difference, to the method of nuclear preparation, or to the conditions of NaCl extraction.

About one-quarter of the total RNA of the nucleus was extracted by the phosphate buffer and one-quarter by M NaCl, leaving one-half of the original RNA in the nucleolar residue remaining after extraction. Maggio and associates (30) consider that the material extracted by the phosphate buffer represents cytoplasmic contamination and have devised a method of isolating liver nuclei which reduces the amount of RNA in the phosphate extract to a negligible quantity. However, the RNA in the phosphate fraction prepared by us always showed a higher uptake of adenine-C^{14} than did the RNA in the cytoplasm, suggesting that it is a separate species.

The nature of the nucleolar residue obtained by us was examined[4] using an antibody present in the sera of some patients with autoimmune diseases. This antibody reacts specifically with the nucleoli of mammalian cells (31). Nucleoli to which this antibody becomes attached can be identified by using a fluorescein-tagged rabbit antibody which reacts with human antibodies in general and thus with nucleoli containing the human antibody (32). The nucleoli observed microscopically in our isolated nuclei gave a strong positive reaction to the anti-nucleolar antibody; on the other hand, the "nucleolar residue" left after extraction gave no reaction or only a weakly positive reaction. The reactant was present in the M NaCl extract. The distribution of nucleolar antibody reactant among the nuclear subfractions was not influenced by diet.

Effect of protein intake on RNA content of liver nuclei. Table 1 shows the effect of diet on the amount of RNA in whole nuclei and in the nuclear subfractions. Since diet has no significant effect on the DNA content of liver cell nuclei (33), the results are expressed per 100 μg DNA in the whole nuclei. In testing the significance of differences between groups statistically, the phosphate and sodium chloride extracts have been combined, since the changes occurring in these subfractions parallel one another but are not independently significant. In addition, the amounts of RNA recovered in the different subfractions have been compared with the RNA content of the whole nuclei and the influence of diet on RNA recovery has been examined.

Statistical evaluation of the data was made by analysis of variance, from which fiducial intervals have been computed in order to show minimal significant differences between pairs of observations (table 1). This demonstrates that animals fasting overnight after receiving the protein-

[3] We are indebted to Dr. J. Swanson Beck for electron microscopy.

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TABLE 1
Effect of protein intake on the amount of RNA in rat liver whole nuclei and nuclear subfractions

Dietary group [1]	Whole nuclei	Nuclear subfractions				
		Phosphate extract	NaCl extract	Combined extracts	Nucleolar residue	Unre-covered RNA
		μg RNA/100 μg DNA			*μg RNA/100 μg DNA*	
Protein-depleted (fasting 18 hr)	25.4	5.2	5.3	10.5	13.2	1.7
Adequate protein (fasting 18 hr)	25.8	6.6	6.7	13.3	11.0	1.5
Adequate protein (fed protein 2 hr)	26.7	6.9	6.6	13.5	12.8	0.3
Statistical analysis: Significance level (*P*)	< 5%	—	—	< 0.1%	< 0.1%	< 0.1%
Standard deviation [2]	± 1.5	—	—	± 2.0	± 1.2	± 0.7
Minimum significant difference [2]	0.9	—	—	1.2	0.7	0.4

[1] The results are the mean data of 22 animals in each dietary group.
[2] Computed from the residual error variance obtained on analysis of variance of the results.

free diet (group 1) do not have significantly less RNA per liver nucleus than that in the nuclei of rats fasting after a diet containing protein (group 2). However, this obscures a redistribution of RNA between subfractions; there is less RNA in the 2 extracts, but more in the nucleolar residue, thus confirming the histological observations of Stowell (5) and Stenram (6–9) of nucleolar enlargement in protein-depleted animals. The amount of RNA unrecovered after fractionation was not affected by protein depletion.

When a meal of protein is fed 2 hours before killing (group 3) there is a small but significant increase in the amount of RNA per nucleus above the level observed in animals that received a similar diet but were kept fasting (group 2). This is due to a striking increase in the amount of RNA in the nucleolar residue in the protein-fed animals without any reduction in the RNA content of the phosphate and saline extracts. There is, however, a very significant reduction in the amount of RNA unrecovered after fractionation. It is possible that some rather labile nuclear RNA species may become stabilized to the extraction procedure when protein is fed; its inclusion in the nucleolar residue could be partly responsible for the large increase (16%) occurring in the RNA of this fraction 2 hours after the protein meal.

Effect of protein intake on adenine-C^{14} uptake by nuclear RNA. Uptake of adenine-C^{14} into the RNA of liver-cell cytoplasm, nucleus and subnuclear fractions was investigated in rats injected intraperitoneally with the isotope and killed at intervals from 15 minutes to 180 minutes thereafter (table 2). Throughout this period, the nuclear RNA was much more heavily labeled than that of the cytoplasm, in agreement with the observations of many other workers. Among the nuclear subfractions, the RNA of the nucleolar residue had the highest general activity and the RNA of the phosphate extract had the lowest activity, although it was still higher than that of cytoplasmic RNA. The sodium chloride extract showed an initial rapid uptake which fell quickly and was followed by a low level of incorporation similar to that of the phosphate extract; this suggests that the sodium chloride extract contains 2 species of RNA of quite different metabolic activities (see also fig. 1).

Metabolism of RNA in the different dietary groups cannot be directly compared, since changes in precursor pool activities may be responsible for differences in RNA labeling without necessarily implying any alteration in rate of RNA synthesis. However, there was a constantly higher labeling of RNA in all cell fractions obtained

TABLE 2

Effect of dietary protein intake on uptake of adenine-C¹⁴ in vivo by nuclear and cytoplasmic RNA

Dietary group [1]	Cell fraction	Time after C^{14} injection					
		15 min	30 min	45 min	60 min	120 min	180 min
		count/min/100 μg RNA			*count/min/100 μg RNA*		
Protein-depleted (fasting 18 hr)	Whole nuclei	201	425	523	608	725	801
	Nucleolar residue	194	426	714	846	1109	1559
	NaCl extract	87	294	472	112	132	111
	Phosphate extract	28	85	73	191	287	352
	Cytoplasm	16	29	61	78	109	205
Adequate protein (fasting 18 hr)	Whole nuclei	111	174	201	262	303	393
	Nucleolar residue	112	251	324	447	644	850
	NaCl extract	83	170	244	47	32	40
	Phosphate extract	21	20	37	54	69	154
	Cytoplasm	14	13	23	48	65	99
Adequate protein (fed protein)	Whole nuclei	112	130	180	227	408	456
	Nucleolar residue	81	105	176	297	600	759
	NaCl extract	206	239	242	51	75	34
	Phosphate extract	18	27	39	52	102	192
	Cytoplasm	5	13	31	46	86	119

[1] The results are the mean data from 2, 2, 2, 8, 4 and 4 animals, respectively, per group at the different time intervals.

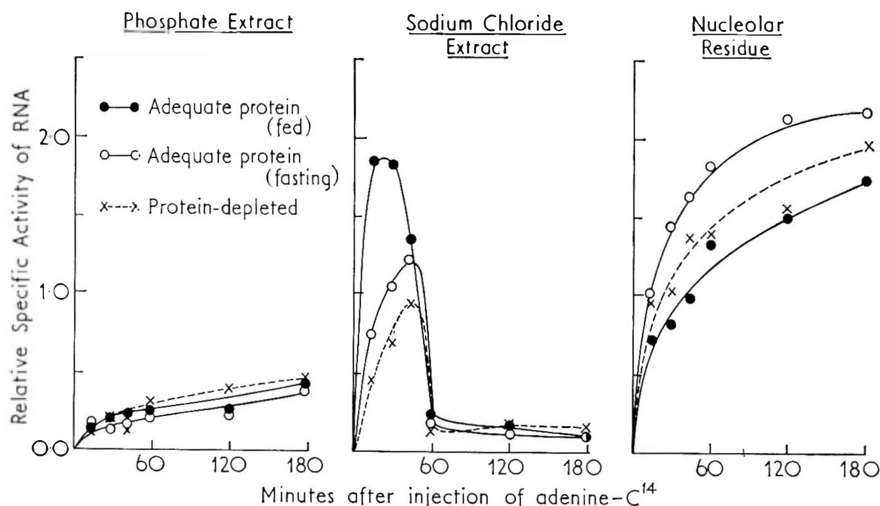

Fig. 1 The effect of diet on adenine-C^{14} incorporation by RNA in nuclear subfractions at different times after C^{14} injection. The relative specific activity of the RNA in each nuclear subfraction was calculated from the data given in table 2 by relating the activity of the RNA in the fraction to the activity of whole nuclear RNA.

from animals fed the protein-free diet; this agrees with other observations made by biochemical means (3) and by autoradiography (34) showing that precursors are incorporated more rapidly into the liver RNA of protein-depleted animals.

It is, nevertheless, possible to evaluate the action of diet on the RNA of different nuclear fractions by comparing their activities with that of whole nuclear RNA. In this way, relative changes in rate of synthesis of the RNA in different nuclear fractions can be detected (fig. 1). Expressed in this way, adenine-C^{14} uptake of the RNA of the phosphate fraction remained low throughout the labeling period

and was not affected in a consistent way by diet. There were, however, quite definite changes in relative C^{14} uptake into the RNA of the other 2 nuclear subfractions when the intake of protein was altered (fig. 1). The relative uptake into the rapidly turning over RNA of the sodium chloride extract was greatly enhanced in the group of rats fed casein before killing. On the other hand, the relative uptake of C^{14} into the RNA of the nucleolar residue was highest in animals fasting after having received an adequate protein intake (fig. 1). This group had the lowest amount of RNA in the nucleolar residue (table 1), which implies that the additional RNA observed in the nucleolar residues of the other 2 dietary groups must be of a low metabolic activity.

Sedimentation patterns of RNA extracted from liver nuclei of rats fed different diets. The Schlieren optical system of the Spinco analytical ultracentrifuge was used to determine the number of components and approximate sedimentation coefficients of RNA samples extracted from whole liver cells and cell nuclei after feeding the different diets. At the concentrations of RNA used (about 15 mg/ml), the rates of sedimentation are retarded and consequently the sedimentation coefficients obtained are too low. These were assigned a more accurate coefficient by checking the components with ultraviolet optics at a lower concentration of RNA. The amount of each component observed represents the area of each peak as noted by the Schlieren system; these are expressed in table 3 as percentages of the total RNA present in the sample (the sum of all the peaks).

Table 3 shows that one-half of the RNA from our nuclear preparations is of low molecular weight (4–7S). Sporn and Dingman (35) also observed this material in liver nuclei, but in their samples it amounted to only 31% of the total nuclear RNA. They reported an 18–20S component in nuclear RNA from rat liver (35) which corresponds to the 18S peak in our samples. The component sedimenting at approximately 28S in our samples is probably similar to their 33S peak. There did not appear to be any real difference due to diet in the proportions of RNA sedimenting at 4, 18 and 28S (table 3).

In addition to these major components, there was a small amount of RNA sedimenting at 35S or more; this has been reported previously in rat liver nuclei by Sporn and Dingman (35) who noted 19% of the nuclear RNA to be of this type. In our studies, the amount of this material was least abundant in rats receiving protein in the diet, but was somewhat higher (3%) for rats fed the protein-free diet (table 3).

The whole cell RNA showed 4 peaks with sedimentation values similar to those obtained with nuclear RNA. The amount of RNA heavier than 35S was again greatest in the group given the protein-free diet, the increase being more marked than that observed with nuclear RNA (table 3). This action of diet was confirmed by sucrose density gradient analysis (not reported here). Since the contribution of nuclear RNA to whole cell RNA is small, the increase in the amount of this very

TABLE 3

Effect of dietary protein intake on the relative amounts of nuclear and whole liver cell RNA of different sedimentation classes

Dietary group [1]	Nuclear RNA				Whole cell RNA			
	4–8S	18S	28S	35+S	4–8S	18S	28S	35+S
	%	%	%	%	%	%	%	%
Protein depleted (fasting 18 hr)	58	10	29	3	25	14	54	6
Adequate protein (fasting 18 hr)	49	21	30	1	39	15	47	0
Adequate protein (fed protein 2 hr)	56	21	30	1	53	10	41	1

[1] The RNA examined came from the livers of 4 rats in each dietary group.

heavy RNA must have been located principally in the cytoplasm.

DISCUSSION

Both published histological studies and the present investigations show that the nucleus of the liver cell is sensitive to the amount of protein in the diet. Using histological and electron microscopic techniques, 4 groups of authors have identified changes in nucleolar size arising from variations in protein intake, although only one of the authors, Stenram, established that the RNA content of the nucleoli changed in parallel with size. Stowell (5), Stenram (6–9) and Svoboda and Higginson (36) observed larger nucleoli in the livers of animals fed protein-deficient diets than in animals fed diets containing adequate amounts of protein. Lagerstedt (4) performed 2 series of studies which add some detail to the picture. In the first series, he observed a sharp reduction in nucleolar size in rats fasted for 24 hours, and a slower shrinking of nucleoli in the liver cells of a small number of animals fed a low protein diet for 18 days. In the second series of experiments, rats were fasted for 5 days and then given either a protein-rich diet or a protein-deficient diet. Within 3 hours of giving the protein-rich diet, the nucleolar mass was twice that of the fasting animals. After feeding the low protein diet, no change occurred in nucleolar size until some 36 hours had elapsed, when the nucleolar mass was observed to be 50% above that of the starving animals. Stenram (7) has confirmed this difference in the response of liver nucleoli of fasting animals a few hours after feeding a protein-rich or a protein-free diet. However, continued administration of the protein-free diet resulted in nucleoli that eventually became much larger than those of rats fed protein.

It thus appears established that enlargement of liver nucleoli can be observed when animals fed a protein-free diet for a sufficiently long period are compared with fasting animals or with those fed a diet adequate in protein content. Our own studies confirm the observations of Lagerstedt and Stenram insofar as they show an increase in the RNA content of the nucleolar residue in rats fed a protein-free diet for 5 days, as compared with rats fasted overnight after having received an adequate protein intake (table 1). The changes in nucleolar RNA content are not as striking as those observed histologically in nucleolar mass by Lagerstedt and by Stenram. This may be because our "nucleolar residue" probably contains RNA from other nuclear structures, such as nuclear membrane and chromosomes, as well as from the nucleoli proper, and these other sources of RNA may not be affected by diet although contributing to the total RNA content of the fraction. No useful comment can be made as to why the nucleoli enlarge when a protein-free diet is fed. Some years ago we established (2) that rate of RNA synthesis in the liver is determined by energy intake and is not affected by removal of protein from the diet. Thus in starving animals fed the protein-free diet the rate of RNA synthesis would be expected to increase and this might well lead to its slow accumulation in the nucleus.

The marked and rapid increase in nucleolar mass observed by Lagerstedt (4) 3 hours after feeding a protein-rich diet to fasting rats is also confirmed by our studies of RNA in the "nucleolar residue" (table 1). This is not due to deposition of metabolically highly active RNA, since the specific activity of nucleolar RNA relative to that of whole nuclear RNA was reduced in the group fed protein (fig. 1). It is therefore of interest that there is a significant improvement in RNA recovery during fractionation of the nuclei of protein-fed animals (table 1). This suggests that some rather labile form of nucleolar RNA may become stabilized during absorption of amino acids after a meal of protein. This thesis would be compatible with our observation of more RNA in the nucleolar residue without an increase in adenine-C^{14} uptake.

The effect of a protein meal on adenine-C^{14} uptake by RNA extractable into M NaCl was the opposite of that observed with the RNA of the nucleolar residue. Instead of a reduction in C^{14} uptake, the RNA of the M NaCl extract showed a much increased incorporation (fig. 1). The intranuclear origin of this RNA is obscure. We have noted that an antibody which is specific

for nucleoli in intact nuclei no longer reacts with the nucleolar residue, the reactant being extracted into M NaCl. Although this nucleolar antigen is not RNA (31), its presence in the M NaCl extract demonstrates that this treatment can remove components of liver cell nucleoli. The RNA in the M NaCl extract that shows metabolic stimulation after a protein meal may thus originate from the nucleoli.

There is now a considerable body of evidence to show that RNA metabolism in the liver undergoes extensive changes within 2 to 3 hours after feeding protein to fasting animals. There is an immediate and marked increase in the uptake of precursors into whole cell RNA, the proportion of glycine-C^{14} taken up by adenine and guanine alters, allantoin formation diminishes (37) and the RNA content of the post-microsomal fraction of liver (breakdown fragments from ribosomes?) diminishes (38). These changes have been interpreted as representing increased stability in ribosomal RNA associated with the influx of amino acids from the protein meal (3). The present study shows that equally rapid changes take place in the metabolism of RNA in liver nuclei after a meal of protein. These nuclear and cytoplasmic events undoubtedly form part of an integrated mechanism by which the liver cell rapidly changes its protein content in response to variations in the quantity and quality of protein in the diet.

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Effect of Simple and Complex Carbohydrates upon Total Lipids, Nonphospholipids, and Different Fractions of Phospholipids of Serum in Young Men and Women [1]

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ABSTRACT Eight young, healthy persons, 4 men and 4 women, were fed experimental diets for 4 dietary periods of 4 weeks each. Except for the type of carbohydrate and total protein in the second period, diets were constant in composition. Total fats, proteins, and carbohydrates constituted 40, 16 and 44% of total calories of the basic diet, respectively. The ratio of complex to simple carbohydrates was 1:4 in periods 1 and 3 and this was reversed in periods 2 and 4. Serum total lipids, nonphospholipids, and the different fractions of phospholipids (ethanolamine phosphatides, inositides, lecithins, lysolecithins, and sphingomyelins) were determined using silicic acid column chromatography. Serum total lipids, phospholipids, and nonphospholipids were found to be significantly reduced with the high cereal diet and increased with the high sugar diet when the total calories and fats were held constant for both men and women. The percentage of the ethanolamine fraction to the total phospholipids increased with the high sugar diet and decreased with the high cereal diet. The proportion of lecithins to total phospholipids showed the opposite trend. The other phospholipids showed minimal changes with dietary change. The possible importance of the changes in the phosphatides in relation to the coagulation of the blood was pointed out. An hypothesis was suggested for the mechanism for the lipid-lowering effect of complex carbohydrates in contrast with the lipemic effect of high sucrose diet.

During the past half century, most of the dietary studies concerning atherosclerosis have emphasized the role of fats in the diet. Unfortunately little work has been carried out with dietary carbohydrates, ignoring the fact that they are readily converted into fats in the body and may contribute to serum lipids.

In a previous report (1), it was pointed out that the increase in the incidence of coronary heart disease over the last 60 years in the United States cannot be attributed to a relative deficiency of the polyunsaturated fatty acids in the diet since the polyunsaturated-to-saturated fatty acid ratio of the food supply in 1961 has increased about 30% over the value in 1909. The ratio of complex to simple carbohydrates, on the other hand, had decreased in 1961 to about 70% of the value in 1889. A possible role of lower molecular weight dietary carbohydrates (sucrose), in the development of coronary heart disease has been suggested (1–3).

Experimental work in animals has shown that starch is associated with lower serum cholesterol than is sucrose (4, 5). In rabbits the amount of lipid in the liver is greater when sucrose is given than when starch is fed, with glucose occupying an intermediate place (6).

Few studies of the influence of dietary carbohydrate upon serum lipids in man have been reported. Keys et al. (7) observed that diets containing sucrose and milk carbohydrate to provide 17% of the calories, produced higher serum cholesterol levels in their schizophrenic subjects than did diets in which an equivalent amount of carbohydrate was provided by a mixture of fruit, vegetables, and legumes, Quite recently, Macdonald and Braithwaite (8) have shown that sucrose resulted in an increase in serum lipids in

Received for publication November 9, 1964.
[1] Work supported in part by grants from the College of Medicine Trust Fund.
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man, mainly in the glyceride fraction, whereas the starch diet produced a decrease in serum lipids, mainly evident in the sterol ester and the phospholipids. Irwin et al. (9), however, observed that there was no significant difference in serum cholesterol and phospholipids in man when they changed only 40% of dietary carbohydrate from rice to sucrose.

There is now much evidence that changes in coagulability of the blood may play a major role in development of coronary heart disease (10, 11) and that one or more phosphatides play an essential role in the coagulation process (12). The nature of the phosphatide, however, is still a controversial matter.

This study was a part of a larger project [4] designed to test the effect of dietary regimens providing carbohydrates primarily as starches or simple sugars. Since pre-menopausal women are relatively free from coronary heart disease in contrast with men of the same age group, it seemed pertinent to study the total serum lipids, nonphospholipids, and the different fractions of phospholipids of the blood of both young men and women.

MATERIALS AND METHODS

Eight healthy persons, 4 men and 4 women whose ages ranged from 20 to 25 years, participated in the study as outpatients. Three spaced meals were eaten daily in the metabolic ward under the supervision of a dietitian. The dietitian provided a bedtime snack which the subject ate during the evening. Each subject was given a complete physical examination after a careful medical history was taken. After the subjects had been adjusted to an ordinary American diet for a few days, they were given a weighed diet divided into 4 dietary periods, each of which was 4 weeks. Table 1 gives the basic dietary plan for 2500 kcal/day. The diet was adjusted for each person by a percentage increase or decrease of all nutrients to maintain constant weight throughout the study. By this procedure the relation of nutritents ingested was the same for all subjects although absolute intakes differed.

Periods 1 and 3 were alike in every respect, and total carbohydrates were held constant throughout the 4 periods. The main variable was the source of carbohydrates. The intake of simple sugars (table 1) in periods 1 and 3 was 80%. In periods 2 and 4, 80% of carbohydrate was ingested as starch. The intake of total protein was the same except in period 2 when it was increased by 20 g/day by the protein content of the vegetable and cereal foods which replaced sugar. Dietary fat was the same in all periods and fats used were

[4] Hodges, R. E., and M. A. Ohlson 1963 Total serum lipids, cholesterol and triglycerides following ingestion of diets with 2 sources of carbohydrates. Federation Proc., 22 (2): 209 (abstract).

TABLE 1

Dietary plan for 2500 kcal

	Period 1	Period 2	Period 3	Period 4
	High simple [1] CHO	High complex CHO	High simple CHO	High complex CHO [2]
Total CHO, g	272	273	272	273
Simple CHO, g	220	56	220	56
Complex CHO, g	52	217	52	217
Total proteins, g	100	120	100	100
Animal proteins, g	92	90	92	70
Vegetable proteins, g	8	30	8	30
Total fat, g	111	111	111	111
Saturated fatty acids, g	54.9	55.2	54.9	54.8
Polyunsaturated fatty acids,[3] g	7.7	7.7	7.7	7.4
Cholesterol, mg	829	834	829	779

[1] In this report "simple carbohydrates" consisted mainly of the mono- and the disaccharides, and included the carbohydrates of the refined sugars, sirups, milk, and those of fruits. The "complex carbohydrates" included those of cereal products, potatoes, legumes and vegetables.
[2] Protein was equalized (see text).
[3] As linoleic acid.

those characteristic of the diet of this area. The ratio of polyunsaturated to saturated fatty acids was calculated to be 0.25.

Blood samples were obtained under basal conditions from an antecubital vein with a siliconized needle and syringe before the study and at the end of each dietary period.

Serum total lipids were extracted by a chloroform-methanol mixture (2:1, v/v) using 19 parts solvent and 1 part serum, purified by washing with 0.2 volume saline 3 times according to Folch et al. (13), and finally dried under vacuum to a constant weight. Phospholipid and nonphospholipid [5] portions were separated by silicic acid column chromatography by the method of Borgstrom (14). They were further fractionated by chromatography using activated silicic acid columns according to the method of Hanahan et al. (15). The eluting mixtures of chloroform-methanol were 4:1, 3:2, and 1:4 (v/v). Separation of the fractions was made by phosphorus determination according to Bartlett (16). Each sample was evaporated under vacuum, diluted to volume, and phosphorus determination was made for an aliquot. The mean recovery of phosphorus in the different fractions of phospholipids was 91.5 ± 1.75[6]% as compared with the phosphorus observed in the total phospholipids. These results compare with the recoveries reported by Hanahan et al. (15) which were 88 to 95%.

Two of the four women investigated became pregnant during the third dietary period and their data are reported separately.

Analysis of variance, according to Snedecor (17), was applied to test significance of differences. The results were considered significantly different at $P < 0.01$).

RESULTS

Total serum lipids, nonphospholipids and phospholipids are reported in table 2. The same data are expressed as percentage change in total serum lipids, phospholipids, and neutral lipids during different dietary periods considering the values at the end of the first high sugar period as 100 (table 3). All of these components of serum showed the same trend; namely, an increase after the feeding of high sugar diets and a decrease with the complex carbohydrate diets. The changes occurred in men and women but were of lesser degree in the women. The

[5] Includes total cholesterol, glycerides, and nonesterified fatty acids.
[6] SD.

TABLE 2

Serum lipids with substitution of starch for sugar in a controlled diet

Subjects	Pre-experimental period	Period 1	Period 2	Period 3	Period 4 [1]
		Simple CHO	Complex CHO	Simple CHO	Complex CHO
		Total lipids, mg/100 ml			
Men (4) [2]	563(504–644) [3]	616(542–672)	545(450–704)	691(558–866)	470(423–513)
Women (2)	467(424–510)	545(492–598)	478(414–542)	589(512–666)	446(407–484)
Women (2) [4]	563(425–701)	532(526–537)	450(444–456)	512(458–566)	431(362–500)
		Nonphospholipids (neutral), mg/100 ml			
Men (4)	364(291–450)	393(329–398)	350(263–464)	454(386–587)	292(238–344)
Women (2)	336(263–408)	345(320–370)	301(245–357)	364(324–405)	266(233–299)
Women (2) [4]	347(246–448)	346(341–351)	275(273–277)	319(273–365)	260(216–304)
		Phospholipids, mg/100 ml			
Men (4) [2]	196(181–217) [3]	212(188–238)	192(171–237)	226(162–273)	173(151–210)
Women (2)	156(one case)	189(163–215)	174(160–189)	209(173–245)	170(164–176)
Women (2) [4]	200(166–234)	179(175–183)	171(163–179)	185(183–187)	174(148–199)
		Phospholipids, % of total lipids			
Men (4)	35.4(29.0–41.2)	35.1(33.5–36.4)	35.7(33.8–39.4)	33.2(29.6–36.0)	37.4(30.4–42.2)
Women (2)	37.1(one case)	35.3(33.8–36.8)	37.4(34.6–40.3)	36.3(34.8–37.8)	39.2(37.0–41.3)
Women (2)	37.3(34.3–40.3)	34.0(33.8–34.3)	38.4(37.4–39.3)	37.0(34.0–40.1)	40.1(39.5–40.7)

[1] Protein intake was equalized (see text).
[2] Number of subjects.
[3] Range of values.
[4] Pregnancy occurred during period 3.

TABLE 3

Percentage change in total lipids, nonphospholipids and phospholipids during dietary periods 2, 3, 4 (expressed as % of values observed in dietary period 1)

	Pre-experimental period	Period 1	Period 2	Period 3	Period 4
		Total lipids			
Men	91.4	100.0	88.5	112.2	76.3
Women	85.7	100.0	87.7	108.1	81.8
Pregnant women [1]	105.8	100.0	84.6	96.2	81.0
		Nonphospholipids			
Men	92.6	100.0	89.1	115.5	74.3
Women	97.4	100.0	87.2	105.5	77.1
Pregnant women [1]	100.3	100.0	77.2	92.2	75.1
		Phospholipids			
Men	92.5	100.0	90.6	106.6	81.6
Women	—	100.0	92.1	110.6	89.9
Pregnant women [1]	111.7	100.0	95.5	103.4	97.2

[1] Pregnancy occurred at the midpoint of period 3.

rate of increase after sugar feeding was greater in the nonphospholipid fraction than in the phospholipids and the change in total lipids was intermediate between these 2 fractions. It also was noted that the decrease in serum lipid concentrations in pregnant women fed the complex carbohydrate diet was less pronounced than in the case of 2 women in the nonpregnant state.

Tables 4 and 5 show the mean and range of cephalins, (phosphatidyl ethanolamines) lecithins, inositides, lysolecthins, and sphingomylins, as percentages of total serum phospholipids.

The cephalin fraction showed a characteristic trend, namely, an increase when the subjects were fed a high sugar diet (periods 1 and 3) and a decrease in the case of the diet rich in complex carbohydrates (periods 2 and 4). This trend occurred in both the men and women except when pregnancy occurred, at which point the cephalin fraction increased.

The lecithins have shown essentially the opposite trend to that of the cephalins in

TABLE 4

Mean concentration of the phosphatidyl ethanolamines and lecithins (% of total serum phospholipids)

Subjects	Pre-experimental period	Period 1	Period 2	Period 3	Period 4
		Phosphatidyl ethanolamines			
Men (4) [1]	6.3 (5.6–6.7) [2]	7.3 (6.8–7.9)	6.3 (6.0–6.9)	7.3 (6.8–7.7)	5.8 (5.3–6.6)
Women (2)	5.8 (5.6–6.0)	6.2 (6.1–6.2)	5.2 (5.2–5.3)	6.2 (5.9–6.4)	4.8 (4.7–4.8)
Women (2) [3]	5.8 (5.6–6.0)	6.5 (6.4–6.6)	5.4 (5.4–5.5)	6.2 (5.9–6.4)	7.0 (6.9–7.1)
		Lecithins			
Men (4) [1]	69.0 (68.3–69.9) [2]	67.4 (66.4–68.5)	71.3 (70.0–73.2)	68.9 (68.1–70.7)	72.4 (70.6–75.2)
Women (2)	72.0 (71.4–72.5)	70.6 (70.2–70.9)	73.1 (72.8–73.4)	70.4 (70.2–70.5)	73.6 (73.2–74.1)
Women (2) [3]	73.4 (73.1–73.8)	71.0 (70.8–71.3)	73.6 (73.1–74.1)	70.9 (70.2–70.6)	70.1 (69.5–70.7)

[1] Figures in parentheses indicate the number of subjects.
[2] Range.
[3] Pregnancy occurred in the third period.

TABLE 5

Mean concentration of inositides, lysolecithins, and sphingomyelins (% of total phospholipids)

Subjects	Pre-experimental period	Period 1	Period 2	Period 3	Period 4
			Inositides		
Men (4) [1]	0.4 (0–0.8) [2]	0.8 (0.4–1.0)	0.8 (0.5–1.1)	0.7 (0.5–0.9)	0.7 (0.6–0.8)
Women (2)	0.4 (0.2–0.5)	0.4 (0.4–0.5)	0.6 (0.4–0.7)	0.8 (0.5–1.1)	0.8 (0.6–1.0)
Women (2) [3]	0.6 (0.4–0.7)	0.6 (0.6–0.7)	0.8 (0.7–0.8)	1.0 (0.9–1.0)	0.8 (0.8–0.9)
			Lysolecithins		
Men (4) [1]	4.7 (4.1–5.7) [2]	5.8 (4.1–7.8)	4.8 (3.8–5.3)	5.3 (4.7–6.5)	4.7 (4.2–5.1)
Women (2)	5.6 (5.2–5.9)	5.2 (4.8–5.7)	5.2 (4.9–5.6)	5.2 (4.5–6.2)	4.4 (4.2–4.5)
Women (2) [3]	4.2 (4.2–4.3)	5.0 (5.2–5.9)	4.4 (4.2–4.7)	4.2 (4.2–4.3)	5.0 (5.0–5.1)
			Sphingomyelins		
Men (4) [1]	19.6 (18.9–20.5) [2]	18.7 (16.2–20.4)	16.8 (16.4–17.4)	17.7 (16.7–18.5)	16.4 (14.2–18.0)
Women (2)	16.4 (15.4–17.4)	17.6 (17.4–17.8)	15.8 (15.7–16.0)	17.4 (16.4–18.3)	16.3 (16.0–16.6)
Women (2) [3]	16.0 (15.7–16.2)	16.8 (16.3–17.2)	15.8 (15.4–16.1)	17.8 (16.8–18.7)	17.0 (16.3–17.7)

[1] Figures after men or women indicate the number of subjects.
[2] Range.
[3] Pregnancy occurred in the third period.

TABLE 6

Summary of analysis of variance for the indicated variables for the 4 dietary periods [1]

	Between subjects	Between sexes	Between diets
Degrees of freedom	5	1	1
Required F value [2]	4.56	8.68	8.68
	Observed F value		
Total lipids	10.09	9.30	42.28
Nonphospholipids	11.32	11.93	40.51
Phospholipids	4.97	2.48	13.82
Phospholipids, % of total lipids	2.01	1.94	4.35
Ethanolamine phosphatides	12.58	55.58	71.0
Lecithins	13.00	32.52	118.50
Inositides	0.16	0.01	0
Lysolecithins	0.68	0.08	4.24
Sphingomyelins	5.15	3.42	22.50

[1] Based on 24 observations.
[2] Probability, 0.01.

all subjects. The change in inositides was small and inconsistent. The changes in the concentration of lysolecithins also were inconsistent. The sphingomyelin fraction was higher in periods 1 and 3 in comparison with periods 2 and 4. This trend was similar to that of the cephalin fraction except that when pregnancy occurred the percentage of the sphingomyelins did not increase as did that of the ethanolamine fractions.

Table 6 presents the analysis of variance for the different parameters measured. The effect of the 2 different diets upon the

total lipids, phospholipids, and nonphospholipids of the serum in milligrams per 100 ml was highly significant. The differences in concentrations of cephalins, lecithins and sphingomyelins in percentage of total serum phospholipids were also significant. On the other hand those of inositides and lysolecithins were not significant.

DISCUSSION

The effect of the 2 diets upon the total lipids, phospholipids, and the nonphospholipids of the serum was highly significant (table 6). There was a greater reduction in serum lipids when the total protein was held constant in period 4 than in the case of period 2. The differences between periods 2 and 4, however, were not significant.

The lipid-lowering effect of complex carbohydrates in the diet and the lipemic effect of the simple sugars reported in this study are in agreement with the data of Keys et al. (7) which has been confirmed recently by Macdonald and Braithwaite (8) in 7 male subjects. Irwin et al. (9), however, did not find any significant difference in serum cholesterol and phospholipids when only 40% of the dietary carbohydrate was given as rice rather than sucrose. About 42% of the carbohydrate in their diets was derived from wheat, oats, potatoes, beans, and celery and 18% from fruits and other vegetables. Both the total amount of carbohydrate and the percentage of complex-to-simple carbohydrates may be critical to demonstrate a change in metabolic pathways.

The values reported here for the individual phosphatides agree with those published by Gjone et al. (18), Phillips (19), Marinetti et al. (20), and Nye et al. (21). Many workers (20, 21) have failed to isolate phosphatidyl serine from human plasma although other investigators (18) have reported traces of this constituent. Therefore, it can be assumed that the cephalin fraction reported here is essentially ethanolamine phosphatides. The percentage of phosphatidyl ethanolamines increased after maintaining the subjects with a high sugar diet and decreased after a high cereal diet. This trend occurred in both men and women except when pregnancy occurred. On the other hand, lecithins have shown the opposite trend. In another study [7] evidence has been presented that the concentrations of both ethanolamine phosphatides and lecthins may be important factors in the coagulation process. The former is thought to stimulate coagulation and the latter to inhibit or possibly have a neutral effect (12).[8]

It is recognized that complex carbohydrates were given as food rather than as pure starch in this experiment, thus increasing somewhat the intake of fiber and sitosterol, both of which could influence the serum cholesterol (22–24). The amounts added could not have been large, as white bread, white rice, macaroni, spaghetti, refined breakfast cereals and potatoes were substituted for the sugars of candies, syrups and jellies. The amount of fruit, vegetables and milk used was kept constant throuhgout the experiment by the use of a 7-day menu plan, this portion of which was replicated during the entire 4 months of study.

TABLE 7

Mean fecal nitrogen and fat during diets varied in carbohydrate source

	Diet no.			
	1	2	3	4
	g/day	*g/day*	*g/day*	*g/day*
Nitrogen	1.29	1.41	1.26	1.18
Fat	0.81	0.80	0.95	0.70

Changes in intestinal flora which would affect the serum lipids could have occurred (4). These must have been minimal as there was no change in the character or frequency of stools. Mean values for fecal fat and fecal nitrogen are shown in table 7 and are within the ranges expected for healthy young adults; no differences were observed that could be related to diet.

An important fact is that the lipids of the blood reflect not only dietary fats, but also the carbohydrates and proteins ingested in excess of immediate tissue needs.

[7] Antar, M. A., M. A. Ohlson and R. E. Hodges 1964 The effects of simple and complex carbohydrates in the diets upon serum phospholipids and the relation to *in vitro* thrombus formation. Federation Proc., 23 (1): 300 (abstract).

[8] See footnote 7.

A diet of concentrated sugars has been shown to result in rapid lipogenesis and in storage of hard fats in the body composed largely of palmitic, stearic, palmitoleic and oleic acids (25). These fats are released as needed for energy purposes. Thus feeding large amounts of sugars at spaced intervals may result in the same serum lipid values as those after feeding highly saturated dietary fats. For example, Anderson et al. (26) observed that isocaloric substitution of sucrose for corn oil in the diet resulted in an increase of serum cholesterol, phospholipids, and triglycerides in man.

Differences in rate of absorption of the different kinds of carbohydrates could lead to change in certain metabolic pathways. Hill et al. (27) have shown that substitution of a diet containing 60% of glucose for a stock diet containing no free hexoses resulted in an eight- to tenfold increase in the capacity of rat liver to convert glucose carbon to fatty acids and in a twofold increase in its capacity to incorporate acetate carbon into fatty acids. Fitch et al. (28) reported that the activities of both 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (2 enzymes of the hexosemonophosphate shunt) are greatly increased by glucose or fructose feeding instead of ground whole wheat. It also has been shown (28) that α-glycerophosphate dehydrogenase, which is responsible for the glycerol moiety of neutral fats, was elevated by the feedings of the hexose containing diets. The hexose monophosphate pathway generates TPNH, considered necessary for lipogenesis and cholesterologenesis (29). Furthermore, the rate of cholesterol synthesis in rats has been shown to increase with a high sucrose diet in contrast with the normal chow diet (30).

The experiments of Cohn and his associates (31) and Hashim et al. (32) indicate that increased lipogenesis and higher concentrations of serum lipids accompany the meal-eating habit, as compared with the nibbling habit.

Unpublished data from this laboratory indicate that ingestion of simple sugar leads to a more rapid and higher glycemia than the ingestion of bread providing the same amount of total carbohydrate.[9] Similar data have been reported by Orent-Keiles (33). Thus, feeding with starches may simulate the effect of nibbling and the active body in caloric equilibrium may metabolize the absorbed glucose from starch without resort to the same degree of lipogenesis and storage as required following large doses of sucrose.

Another factor which may account, at least in part, for such a lipemic effect of high sugar diets is that starch is hydrolyzed in the intestine into gluose only whereas sucrose hydrolyzes into glucose and fructose. Hill et al. (27, 34, 35) reported that normal rats and dogs fed a diet containing 58% fructose as the sole source of carbohydrate developed an impaired capacity for utilizing glucose by the liver. Impaired glucose utilization has also been observed in the livers of human subjects fed fructose (36). Kritchevsky et al. (5) reported that the serum cholesterol in chickens fed high sucrose diets was considerably higher than in chickens fed high glucose diets.

Fitch and Chaikoff (26, 28, 29) have shown that substitution of fructose for glucose in the diet resulted in a significant increase of the activity of the hexose monophosphate shunt. There was also an increase in the activity of α-glycerophosphate dehydrogenase. These changes, as pointed out, will increase the rate of lipogenesis.

It seems probable that the marked changes observed during high sugar intake could be explained on the basis of increased lipogenesis due to difference in absorption rate, the presence of fructose in sucrose, or both. If this explanation proves to be correct, there is no conflict between these observations and those reported after ingestion of large amounts of saturated fatty acids (37) or after nibbling (31).

ACKNOWLEDGMENTS

Dr. Genevieve Stearns kindly provided use of certain equipment and Dr. R. E. Hodges allowed the additional amount of blood needed for these studies to be drawn from the experimental subjects.

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Influence of Caloric Restriction and of Reduced Feeding Time on Experimental Dental Caries in the Rat [1,2]

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ABSTRACT A series of experiments was conducted on caries-susceptible rats to determine the influence of feeding habits on the occurrence of dental caries and to study further the reason for the effectiveness of caloric restriction on reduced incidence of caries. In these studies, caloric restriction caused major reductions in the occurrence of experimental dental caries. When the same caries-producing diet was fed for limited periods, comparable reductions in dental caries were observed. Length of food availability per day was more closely related to occurrence of dental caries than gain in body weight. The data are strongly suggestive that caloric restriction operated through the shorter period that food was available and thereby the shorter oral exposure than through caloric insufficiency per se.

Caloric restriction in cotton rats and in white rats has been shown to cause highly significant decreases in the incidence of experimental dental caries (1–3). The reduction may not be attributable entirely to starvation per se, but may be due at least in part to food consumption by the restricted subjects in a short interval with a resultant brief exposure of the oral cavity to the dietary components. The present series of experiments was conducted to attempt to define in greater detail why caloric restriction was effective and also to determine the influence of the feeding habits of caries-susceptible rats upon the occurrence of dental caries.

EXPERIMENTAL

Three experiments were conducted with the Harvard caries-susceptible strain of the common laboratory rat. Each experiment began when the rats were weaned at 21 days of age and was continued for 60 days. The rats were weighed weekly throughout. At the end of this period the rats were killed under excessive ether anesthesia and the heads preserved in 95% alcohol in preparation for skinning and scoring by the process of Shaw et al. (4). Water was provided ad libitum from an automatic watering fount in each wire-bottom cage. Diet was provided in the individual control and experimental groups as noted in tables 1, 2 and 3.

The first experiment consisted of 5 groups as described in the second column of table 1. The rats in the first group served as controls and were provided diet 2700 (5) ad libitum throughout the 24 hours of each day. Food consumption was determined each morning and used as the basis for calculation of the weights of food to be provided to littermates of the same sex in groups 2, 3 and 4. The individual rats in these 3 groups received 80, 65 and 50%, respectively, of the calories consumed by its mate in group 1. These 4 groups were comparable to those in previous experiments (2, 3) but the experiments were repeated to provide information on our more clearly defined strain of caries-susceptible rats.

The rats in group 5 were provided with an unlimited amount of diet for 2 hours each morning but were not allowed access to food for the remaining 22 hours. This period was arbitrarily selected on the basis that rats in groups 3 and 4 had routinely consumed their food allowances in less than this amount of time. Our desire was for the rats in group 5 to train

Received for publication November 6, 1964.

[1] This investigation was supported in part by Public Health Service Research Grant no. DE-00204 and Career Award, 1-K6-DE-22,634-01 from the National Institute of Dental Research, National Institues of Health.

[2] We are indebted to Dr. Lyon P. Strean of Merck, Sharp and Dohme Research Laboratories, West Point, Pennsylvania, for the generous supplies of B-complex vitamins used in the diets in these studies.

TABLE 1

Influence of caloric restriction on body weight gain and on occurrence of experimental dental caries [1]

Group no.	Diet 2700 provided	No. of rats		Wt gain				No. of caries-free rats	No. of carious molars		No. of carious lesions		Extent of carious lesions	
				Males		Females								
		Males	Females	Avg	P [2]	Avg	P		Avg	P	Avg	P	Avg [3]	P
				g		g								
1	Ad libitum	8	8	247(5)	—	157(4)	—	0	6.6(0.4)	—	13.1(1.1)	—	48.9+(5.2+)	—
2	80% of calories	8	10	201(8)	< 0.001	129(5)	< 0.001	0	4.7(0.4)	< 0.01	7.9(1.1)	< 0.01	26.4+(4.4+)	< 0.01
3	65% of calories	9	8	152(3)	< 0.001	102(4)	< 0.001	7	2.2(0.6)	< 0.001	3.0(0.9)	< 0.001	9.5+(3.2+)	< 0.001
4	50% of calories	9	8	108(2)	< 0.001	74(5)	< 0.001	10	1.5(0.5)	< 0.001	1.8(0.7)	< 0.001	4.9+(2.0+)	< 0.001
5	2 Hours/day	9	7	157(8)	< 0.001	94(6)	< 0.001	3	2.1(0.5)	< 0.001	3.5(0.9)	< 0.001	7.8+(2.1+)	< 0.001

[1] Values in parentheses are standard errors of means.
[2] Probability that the difference between experimental and control values occurred by chance.
[3] According to the "plus scoring" method of Shaw et al. (4).

TABLE 2

Influence of feeding interval on gain in body weight and on occurrence of experimental dental caries [1]

Group	Period diet 2700 provided	No. of rats		Wt gain				No. of caries-free rats	No. of carious molars		No. of carious lesions		Extent of carious lesions	
				Males		Females								
		Males	Females	Avg	P [2]	Avg	P		Avg	P	Avg	P	Avg [3]	P
				g		*g*								
1	Without interruption	11	8	226(13)	—	125(5)	—	0	6.6(0.5)	—	13.4(1.1)	—	47.7+(5.4+)	—
2	Days only	9	10	164(11)	< 0.01	111(5)	< 0.05	7	2.4(0.5)	< 0.001	3.7(1.0)	< 0.001	9.6+(2.7+)	< 0.001
3	Nights only	10	10	218(14)	> 0.05	133(9)	> 0.05	1	5.7(0.5)	> 0.05	10.1(1.3)	> 0.05	33.7+(4.6+)	< 0.05
4	Four days out of five	10	10	191(11)	< 0.05	137(6)	> 0.05	0	6.0(0.5)	> 0.05	10.9(1.3)	> 0.05	36.9+(5.1+)	> 0.05
5	Three days out of four	11	10	188(7)	< 0.01	126(5)	> 0.05	0	5.9(0.4)	> 0.05	10.6(1.0)	> 0.05	35.1+(3.7+)	> 0.05
6	Two days out of three	10	10	138(7)	< 0.001	110(5)	< 0.05	1	4.9(0.5)	< 0.02	8.4(1.1)	< 0.001	26.6+(3.4+)	< 0.01

[1] Values in parentheses are standard errors of means.

[2] Probability.

[3] According to the "plus scoring" method of Shaw et al. (4).

TABLE 3

Average day and night food consumption during fifth experimental week of rats in experiment 3

Group no.	Diet		Day		Night		Total	
	Day	Night	Males	Females	Males	Females	Males	Females
			g	g	g	g	g	g
1	No. 2700	no. 2700	2.2	1.8	12.2	9.5	14.4	11.3
2	No. 2700 + 2% NaH_2PO_4	no. 2700 + 2% NaH_2PO_4	2.8	2.2	12.3	10.9	15.1	13.1
3	Laboratory meal	laboratory meal	2.9	1.8	14.2	11.5	17.1	13.3
4	Laboratory meal	sucrose	6.2	4.5	6.5	7.2	12.7	11.7
5	Laboratory meal	sucrose + 3% NaH_2PO_4	6.6	5.0	7.5	6.7	14.1	11.7
6	Sucrose	laboratory meal	3.6	3.5	13.0	9.5	16.6	13.0
7	Sucrose + 3% NaH_2PO_4	laboratory meal	3.4	2.3	13.0	9.5	16.4	11.8

themselves to eat a normal amount of food for a day in 2 hours. No records were kept of their food consumption.

The second experiment was composed of 6 groups of rats. The first group served as controls and received diet 2700 throughout the entire 60-day period. From 9:00 AM to 5:00 PM on Monday through Friday and from 9:00 AM to 12:00 noon on Saturday (our normal working hours) the littermates in group 2 were provided with filled ration cups containing diet 2700. No diet was provided from late afternoon until early morning on weekdays nor from noon Saturday to Monday morning. Thus diet 2700 was provided ad libitum for 43 hours per week but was not available for the remaining 125 hours. The rats in the third group were fed on the reverse pattern, with starvation during the working days and ad libitum feeding of diet 2700 at night and on Sunday. The rats in group 4 were provided unlimited amounts of diet 2700 for 4 consecutive 24-hour periods followed by 24 hours of starvation. Comparably, the rats in groups 5 and 6 were fed for either 3 or 2 consecutive 24-hour periods followed in each case by a 24-hour starvation period. The importance of weighing food consumption in this type of experiment did not become fully apparent until after its termination and appraisal.

The third experiment provided the opportunity to evaluate the day-night alternating feeding of diets of varying caries-producing properties. The design is shown in table 3. Control group 1 received diet 2700 continuously throughout the 60-day experimental period. The rats in group 2 received the same diet supplemented with 2% monosodium orthophosphate continuously. The rats in group 3 received laboratory meal [3] continuously. The rats in groups 4 and 5 received laboratory meal during the same weekday working hours as in group 2 of experiment 2. At night the rats in group 4 were given access to unlimited amounts of sucrose, whereas the rats in group 5 were provided ad libitum with sucrose that had been supplemented with 3% monosodium orthophosphate. The rats in groups 6 and 7 received the reverse alternation of diets to those in groups 4 and 5: laboratory meal at night, with sucrose or sucrose plus 3% monosodium orthophosphate during the daytime to groups 6 and 7, respectively. In this experiment, intake of all diets during the day and night feeding periods was determined.

RESULTS

The results of experiment 1 are presented in table 1. The rats in control group 1 grew normally and were maintained in good health by the ad libitum feeding of diet 2700. Progressively higher caloric restriction in groups 2, 3 and 4 resulted in very closely parallel reductions

[3] Purina Laboratory Meal, Ralston Purina Company, St. Louis.

in weight gain. For example, male rats in group 3, which received only 65% of the calories consumed by the control group, grew at 61.5% the rate for control males, whereas females in group 3 grew 65.0% as rapidly as control females. The rats in group 5 that were permitted to eat ad libitum for 2 hours per day grew approximately as well as the rats provided with the 65% allocation of calories in group 3.

The occurrence of tooth decay in the control group was typical for representatives of the Harvard caries-susceptible strain maintained under these experimental circumstances. In group 2, reduction to 80% of the calories consumed by their control littermates of the same sex resulted in a highly significant reduction in tooth decay. Further caloric restriction to levels of 65 or 50% in groups 3 and 4 resulted in still greater reductions. The 90% reduction in extent of carious lesions observed in group 4 was markedly greater than either their 50% caloric restriction or their 43.7% decrease in growth. The dental caries incidence in group 5 provided with food ad libitum for 2 hours per day was closely similar to the incidence of caries for the rats provided with 65% of the normal caloric consumption. No food consumption records were kept for the animals in group 5. However, since the weight gains in groups 3 and 5 were very similar, presumably food consumption in the 2 groups was similar. The 84.0% reduction in extent of caries for group 5 appears more likely to be related to the absence of food for 91.6% of the day than to a 35% reduction in calorie consumption.

The results of experiment 2 are presented in table 2. The weight gains for the control males and the females were normal. When the diet was provided only during the 43 daytime working hours, a highly significant reduction in weight gain was observed among the males; among the females a reduction of lower statistical significance was observed. When diet 2700 was fed for the 125 hours outside of normal working hours (nights and week ends) no significant penalty was imposed upon the weight gain in either male or female. In groups 4, 5 and 6 where the diets were fed for four, three or two 24-hour days, respectively, followed by a single day of starvation, the weight gains of the males were progressively decreased. Among the females in these 3 groups, no adverse influence upon weight gain was observed in groups 4 and 5, but in group 6 a significant reduction in weight gain was observed. Since the food intake was not recorded in this experiment, no values are available to indicate the relative amounts of food consumed.

The occurrence of dental caries among the controls was typical of this strain. In group 2 where diet 2700 was provided only during our working hours, a highly significant reduction in dental caries occurred. This level of reduction was very comparable to that observed in the previous experiment in groups 3 and 5: on the one hand where only 65% of the normal calories was fed and on the other where the food was allowed only 2 hours per day. In group 3 where the diet was available outside of our working hours, the incidence of dental caries was slightly reduced. Only the reduction in the average extent of carious lesions was statistically significant (5% level). When the rats in groups 4 and 5 were fed diet 2700 for 4 consecutive 24-hour days and starved on the fifth, no significant reductions in dental caries were observed, although slight trends in this direction were noted. In group 6 where the rats were starved one day in three, a statistically significant reduction was observed. This latter result deserves special comment: the penalty with respect to weight gain was slightly greater for males in group 6 than in group 2 of this experiment and for both males and females in groups 3 and 5 of experiment 1. Yet the reduction in dental caries for both males and females in group 6 was small compared with that in these other groups. Thus the reduction in dental caries incidence in group 6 appears to be clearly proportional to the longer hours that food was available rather than to the gain in body weight, which was presumably proportional to the food consumed.

The relation of duration of food availability to extent of carious lesions is shown in figure 1. This straight-line rela-

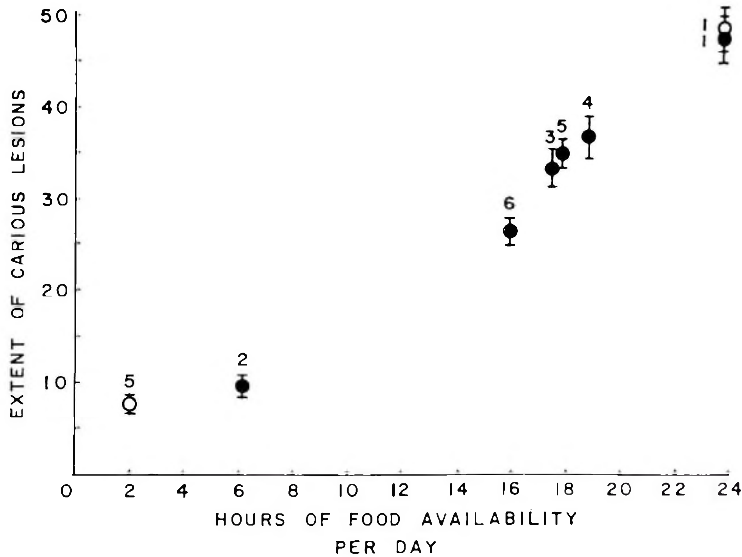

Fig. 1 The relationship between hours of food availability and extent of carious lesions in experiments 1 and 2. Data for experiment 1 are presented by open circles and for experiment 2 by closed circles. The lines above and below represent the limits of the standard errors of each mean. The number beside or above each datum is the group designation within each experiment.

tionship can be more clearly seen from graphic presentation of the results than from the absolute values in table 2. No comparable relationship was seen to exist between extent of carious lesions and gain in body weight, which is presumably closely indicative of caloric intake. These data strongly suggest that the length of time that food was available for the rat to consume was more important than caloric insufficiency with respect to the occurrence of dental caries.

The results on food consumption for experiment 3 are presented (table 3) for the fifth experimental week, which was typical of the entire experiment. In groups 1, 2 and 3 where diets 2700, 2700 plus 2% monosodium orthophosphate, or laboratory meal, respectively, were fed continuously throughout the experiment, food consumption for males and females during the 16 night hours was 5 to 6 times as great as food consumption during the 8 daytime working hours. The relatively comparable total consumption of the 3 diets was consistent with their comparably high nutritional values and the relatively comparable caloric value per gram of diet. In groups 4 and 5 where laboratory meal was fed during the day, food consumption more than doubled for males and females, whereas the weight of sucrose or of sucrose plus 3% monosodium orthophosphate eaten during the night hours was approximately one-half to two-thirds the weights of the 3 previous diets consumed at night by the rats in groups 1, 2 and 3. Total food consumption by the rats in groups 4 and 5 was appreciably less than the total food consumption in groups 1, 2 and 3. Increased consumption of laboratory meal during the days was insufficient to compensate for decreased night consumption. In groups 6 and 7, the intake of sucrose or of sucrose plus 3% monosodium orthophosphate during the days was higher in 3 or 4 comparisons than with the 3 diets consumed by groups 1, 2 and 3 during the daytime hours. Food consumption of laboratory meal at night in groups 6 and 7 was approximately as high as night consumption in groups 1, 2 and 3.

The influences of the experimental procedures in experiment 3 on gain in body weight and dental caries are shown in table 4. The dietary modifications in groups 4 and 5 caused the only highly

TABLE 4

Influence of alternation of diets and phosphate supplementation on body weight gain and on occurrence of dental caries [1]

Group no.	No. of rats		Wt gain				No. of caries-free rats	No. of carious molars		No. of carious lesions		Extent of carious lesions	
			Males		Females								
	Males	Females	Avg	P [2]	Avg	P		Avg	P	Avg	P	Avg [3]	P
			g		g								
1	11	13	217(14)	—	144(7)	—	0	4.6(0.4)	—	7.9(1.0)	—	27.8+(3.7+)	—
2	9	8	235(9)	> 0.05	138(9)	> 0.05	0	3.1(0.3)	< 0.01	3.8(0.5)	< 0.001	13.1+(1.8+)	< 0.001
3	9	8	215(16)	> 0.05	134(8)	> 0.05	12	0.9(0.4)	< 0.001	1.2(0.5)	< 0.001	4.4+(1.9+)	< 0.001
4	10	9	138(10)	< 0.001	110(8)	< 0.01	0	7.2(0.4)	< 0.01	11.4(0.8)	< 0.01	42.3+(3.1+)	< 0.01
5	9	10	150(7)	< 0.001	107(9)	< 0.01	0	5.7(0.4)	< 0.05	7.6(0.7)	> 0.05	28.4+(2.9+)	> 0.05
6	10	9	204(11)	> 0.05	134(6)	> 0.05	5	2.6(0.4)	< 0.01	3.6(0.7)	< 0.01	13.9+(2.6+)	< 0.01
7	9	10	189(10)	> 0.05	119(8)	< 0.02	7	1.5(0.3)	< 0.001	1.7(0.4)	< 0.001	6.5+(1.4+)	< 0.001

[1] Values in parentheses are standard errors of means.
[2] Probability.
[3] According to the "plus scoring" method of Shaw et al. (4).

significant reductions in weight gain. In group 7 where sucrose plus 3% monosodium orthophosphate was fed during the day and laboratory meal at night, a lower weight gain for females was observed that was significant at the 2% level.

The occurrence of dental caries in the control group of this experiment was somewhat lower than for experiments 1 and 2. In group 2 where diet 2700 was supplemented with 2% monosodium orthophosphate, a highly significant reduction in experimental dental caries was observed. This influence was typical of many experiments where phosphate supplementation of caries-producing diets had been evaluated previously (6). When laboratory meal was fed throughout the entire experiment, a greater reduction in experimental dental caries was observed. The low caries-producing ability of laboratory meal when fed as the only diet has also been reported (7). In group 4 where laboratory meal was fed during the working hours and sucrose at night, a striking increase in dental caries was observed over the control group. In group 5 where the sucrose was supplemented with 3% monosodium orthophosphate, the dental caries rate was the same as in the control group, but was appreciably lower than was observed in group 4 where the sucrose was not supplemented. In group 6 where sucrose was fed during the working hours and laboratory meal at night, the incidence of experimental dental caries was significantly less than for the control group, but was more than double the level for group 3. Evidently this brief exposure to sucrose during our working hours was adequate to adversely affect the caries process. When the sucrose in group 7 was supplemented with monosodium orthophosphate, again a reduction in experimental dental caries was observed from that noted in otherwise comparable animals in group 6. Thus in both groups 5 and 7 monosodium orthophosphate acted as an effective anti-caries agent when fed with sucrose only for a part of the day.

A comparison may serve to highlight the relationship between amount of cariogenic agent consumed and the length of time that the agent was available. In group 6 the 8-hour consumption of 3.6 g of sucrose by male rats or 3.5 g by female rats during the day caused an increase in extent of caries from the average score[4] of 4.4 + in group 3 to an average of 13.9 +. In contrast, in group 4 the 16-hour consumption of roughly twice as much sucrose, 6.5 g for males and 7.2 g for females, caused an increase in caries score from 4.4 + to 42.3 +. A similar comparison may be made for groups 5 and 7 where the sucrose was supplemented with 3% NaH_2PO_4.

DISCUSSION

The striking influence of caloric restriction on the occurrence of dental caries has raised questions of interpretation in many experiments where reductions in dental caries incidence were accompanied by decreased rates of growth and altered levels of food consumption. If the reductions in growth rate in an experimental population could be traced to decreased food consumption rather than inefficient food utilization, was the reduction in dental caries attributable to the reduced caloric intake rather than to the experimental variable? Larson and associates (8) were concerned about this difficulty in interpretation of their studies on the influence of ethylenediaminetetraacetic acid injections on dental caries incidence. From their series of experiments in which 3 caries-producing diets were fed ad libitum for 8, 16 or 24 hours per day, they concluded that caries activity was directly related to the length of time food was available and not to the amount of food consumed.

In the current experiments conducted under quite different, more varied and possibly more rigorous feeding circumstances than those of Larson et al., the direct relationship between caries activity and the length of time that food was available was re-affirmed. In experiments 1 and 2, the relationship observed was less between caries activity and amount of food consumed than between caries activity and length of time food was available. Probably additional support for the latter relationship could have been obtained if

[4] According to the "plus scoring" method of Shaw et al. (4).

we had had the foresight to weigh food consumption in group 5 of experiment 1 and for all groups in experiment 2.

In our experiment 3, which was an attempt to determine the relative impact of feeding diets of varying caries-producing properties alternating day and night, the length of time that the cariogenic agent was available was again demonstrated to be a more important factor in the causation of dental caries than the amount consumed. When a caries-inhibiting agent was tested under these circumstances, its usefulness was observable when fed only with the cariogenic substance rather than throughout the entire 24 hours.

For an adequate interpretation of the relationship to dental caries of an experimental variable that caused both a caries reduction and a reduced gain in weight, an understanding of the influence upon food habits such as frequency of eating would probably be more important than a knowledge of the actual weight of food consumed. This evaluation would require a knowledge not only of how frequently eating had occurred but also how much had been consumed at each meal. Actually, neither the experiments of Larson et al. nor our experiments 1 and 2 have explored the frequency of eating in terms of the actual eating pattern of the rat with its frequent nibbling, especially during the night hours. More important than the provision of food for 6 or 8 consecutive hours per day followed by prolonged starvation in contrast with 16 or 24 hours per day would be the provision of food 15 or 20 minutes per hour throughout the day and night or for these brief periods for 4 hours followed by 2 hours of starvation. Such experiments would be difficult or unreasonable without some adequate form of mechanization to replace technical personnel.

On the surface, dilution of a cariogenic diet with a nutritionally inert substance such as cellulose [5] might be suggested as a procedure to require rats to consume more diet and possibly to eat more frequently in order to obtain adequate calories. Conclusions from an earlier study are relevant here (9): ". . . incorporation of varying amounts of cellu flour from 10 to 50% into the caries-producing diet did not cause any significant change in the incidence of dental caries in the white rat. The average rates of growth and average increases in weight for the several groups were practically identical. Again the quantities of ration consumed increased in proportion to the amount of cellu flour." Another experiment in the same study was conducted with cotton rats where the cariogenic diet had been diluted with as much as an equal weight of the cellulose without influencing the caries incidence. The answer to the question posed in this present study is not obtained from the above results. Whereas the feeding time and frequency probably increased appreciably with the increased food required, the cariogenicity of the diet would be expected to decrease as a result of the 25 or 50% decrease in absolute concentration of sucrose in the diets with high amounts of cellulose.

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[5] Cellu Flour, The Chicago Dietetic Supply House Inc., Chicago.

Comparisons of Casein and Soy Proteins upon Mineral Balance and Vitamin D_2 Requirement of the Baby Pig [1]

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ABSTRACT Comparisons were made of the effects of dietary protein source (casein versus isolated soy protein) upon growth, serum mineral level, skeletal development, mineral balance and vitamin D_2 requirement of the baby pig. Purified casein diets containing zero, 50 or 100 IU of vitamin D_2/kg or soy protein diets containing 50, 100, 250 or 500 IU of vitamin D_2/kg were utilized. The casein diet containing 100 IU of vitamin D_2/kg gave optimal values for all criteria. Using these values as standards, 50 IU of vitamin D_2/kg in a casein diet resulted only in substandard serum and bone Mg concentration. Levels of vitamin D_2 of 50 or 100 IU/kg in soy protein diets gave suboptimal values for many of the criteria. Only soy protein diets containing 500 IU of vitamin D_2/kg produced optimal growth rate. Soy protein diets containing 250 or 500 IU of vitamin D_2/kg gave optimal concentrations of serum Ca, P, Mg and alkaline phosphatase activity and bone ash. Pigs receiving soy protein diets excreted excessive amounts of fecal Ca, P and Mg resulting in decreased mineral retention and this decrease was not overcome by increasing dietary vitamin D_2 levels in this study. The data indicate that the vitamin D_2 requirement of the baby pig receiving purified diets containing isolated soybean protein is greater than the 100 IU/kg requirement level of casein diets and may be several times this level depending upon the criteria selected, the level of isolated soybean protein in the diet and the method of isolation of the soybean protein.

Comparisons of the proteins of milk and soybeans as sources of dietary protein for baby pigs have generally shown lower rates of bodyweight gain and reduced digestibilities of protein and total dry matter in the diet by those animals receiving soybean protein (1–9). Digestibility of protein and energy of diets containing soybean protein improves in pigs as their age increases from birth to 5 to 7 weeks of age (3, 5, 7, 9–11). Soybean meal (solvent 44 or 50% crude protein) has produced better growth rate in young pigs than isolated soybean protein in semi-purified diets (5). Supplementation of either soybean meal or isolated soybean protein in semi-purified diets with DL-methionine has improved growth rate in young pigs (2, 4). Isolated soybean protein has rachitogenic activity for the chick (12, 13) which may be overcome by other fractions of soybean meal or by increased dietary vitamin D.

The present study was undertaken to compare the effect of purified casein and isolated soybean protein in the diets of baby pigs upon calcium, phosphorus and magnesium balance and upon the dietary vitamin D_2 requirement of the baby pig. The criteria used were similar to those of earlier studies (14, 15) which indicated that the dietary vitamin D_2 requirement of baby pigs receiving casein diets is not greater than 100 IU/kg.

MATERIALS AND METHODS

Two trials were conducted using 44 Yorkshire-Hampshire crossbred pigs of either sex. Pigs were taken from their dam at one to three days of age and reared in metal cages with wire mesh bottoms. Room temperature was maintained at 20° and infrared heat lamps were utilized to maintain a cage temperature of 30° during the first two or three weeks of either trial. All windows in the room were covered with heavy opaque paper to eliminate the entrance of sunlight and there was no measurable intensity of ultraviolet energy at any point in the room as determined by a thermopile with a microvolt-

Received for publication November 13, 1964.

[1] Published with the approval of the Director of the Michigan Agricultural Experiment Station as journal article no. 3505.

TABLE 1
Composition of purified diets

	Casein [1]	Soy [2]
	%	%
Purified protein	30(20) [3]	29.7(19.7) [3]
DL-Methionine	—	0.3
Lard	5	5
α-Cellulose [4]	5	5
Glucose [5]	53(63) [3]	53 (63) [3]
Mineral mixture	6	6
Corn oil [6]	1	1
Vitamin mixture [7]	+	+

Mineral mixture

	%
KCl	10.0
KI	0.002
$FeSO_4 \cdot 2H_2O$	0.7
$CuSO_4$	0.1
$CoCO_3$	0.1
$MnSO_4 \cdot H_2O$	0.1
$ZnSO_4 \cdot H_2O$	0.4
$MgCO_3$	25.0
$CaHPO_4 \cdot 2H_2O$	36.0(42.4) [3]
$CaCO_3$	12.5(8.8) [3]
Glucose [5]	13.1(10.4) [3]

[1] Vitamin-Free Casein, Nutritional Biochemicals Corporation, Cleveland.
[2] ADM C-1 Assay protein, Archer-Daniels-Midland Company, Minneapolis.
[3] Changed to this value during final 2 weeks of either trial.
[4] Solka Floc, Brown Company, Chicago.
[5] Cerelose, Corn Products Company, Argo, Illinois.
[6] Mazola, Corn Products Company, Argo, Illinois.
[7] See Miller et al. (14).

indicating amplifier.[2] The purified diet and the methods of adjustment to the diet were similar to those described in an earlier study (14). By one week of age the animals had become well adjusted to both the diet and the environment and were assigned to experimental diets on the basis of size, sex and litter. The experimental diets (table 1) contained either purified casein [3] or isolated soy [4] proteins. Soy protein diets were complemented by the inclusion of 0.3% DL-methionine. Irradiated ergosterol [5] was diluted in the corn oil and supplied vitamin D_2 levels of 50 or 100 IU/kg to casein and soy protein diets in the first trial and levels of 100, 250 or 500 IU/kg of soy protein diets in the second trial. In addition, casein diets not supplemented with vitamin D were utilized in either trial to serve as negative control diets and a casein diet supplemented with 100 IU of vitamin D_2/kg was used again in the second trial to serve as a positive control since this diet had been proven to support optimal growth, skeletal development and mineral balance in the baby pig (14, 15). Constant dietary levels of calcium (0.8%) and phosphorus (0.6%) were maintained throughout the 5-week experimental period by alteration of the mineral mixture when protein level was changed. With the exception of the time during which mineral balance studies were conducted food and tap water were made available for individual ad libitum consumption.

Blood was withdrawn on 3 occasions (initial, 3 weeks and final) during either trial from the anterior vena cava for the determination of levels of serum calcium, inorganic phosphorus, magnesium and alkaline phosphatase activity by methods previously described (14, 16–18). Determinations of bone composition and strength were also by methods indicated in these studies.

Mineral balance studies were conducted during the final 2 weeks of the second trial by methods described in recent reports (15, 19, 20). Two 3-day adjustment periods followed by separate collections of urine and feces were carried out for each pig with constant daily food intake. Food, feces and urine were analyzed for Ca, P and Mg concentration (15, 20). Water intake was controlled during the balance studies amounting to twice the weight of food intake. The drinking water analyzed 25 ppm of magnesium and this was taken into account in the magnesium balance. The mean values from the magnesium analyses of casein and soy protein diets were 320 and 340 ppm, respectively.

Growth, serum and bone data from the 2 trials were combined and statistical analyses were carried out with application of Duncan's (21) multiple range test to compare treatment means.

[2] Eppley Thermopile no. 5232, Eppley Laboratories, Inc., Newport, R. I., with Microvolt Indicating Amplifier no. 9835-A, Leeds and Northrup Company, Philadelphia.
[3] Vitamin-Free Casein, Nutritional Biochemicals Corporation, Cleveland.
[4] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Minneapolis.
[5] Viosterol, Nutritional Biochemicals Corporation. Vitamin D_2 in corn oil, 400,000 USP units vitamin D/g. Potency was verified in our laboratory by rat line-test comparison with USP vitamin D_3 reference standard. USP Reference Standards, New York.

TABLE 2

Growth, serum analyses and skeletal development of baby pigs fed casein or soy protein with different levels of vitamin D_2

Type of protein	Soy	Soy	Soy	Soy	Casein	Casein	Casein
Dietary level of vitamin D_2, IU/kg	50	100	250	500	0	50	100
No. of pigs	5	9	4	4	8	5	9
Initial wt, kg	2.8 ± 0.6 [1]	2.6 ± 0.3	2.5 ± 0.2	2.5 ± 0.2	2.6 ± 0.2	2.8 ± 0.8	2.6 ± 0.3
Daily gain, kg	0.20 ± 0.02^{a}	0.22 ± 0.02^{aa}	0.22 ± 0.01^{aa}	0.25 ± 0.01^{aa,b}	0.15 ± 0.01	0.25 ± 0.03^{aa,b}	0.25 ± 0.01^{aa,b}
Daily food intake, kg	0.32 ± 0.02^{aa}	0.32 ± 0.02^{aa}	0.32 ± 0.01^{aa}	0.34 ± 0.01^{aa}	0.24 ± 0.02	0.34 ± 0.02^{aa}	0.34 ± 0.01^{aa}
Gain/food	0.63 ± 0.01	0.70 ± 0.01^{b}	0.68 ± 0.02	0.73 ± 0.03^{bb}	0.62 ± 0.03	0.74 ± 0.01^{bb}	0.75 ± 0.01^{bb,c}
Serum Ca, mg/100 ml							
Initial	11.2 ± 0.3	11.2 ± 0.2	11.1 ± 0.2	11.4 ± 0.2	11.2 ± 0.1	11.0 ± 0.2	10.7 ± 0.2
3 weeks	8.9 ± 0.5	10.6 ± 0.3	12.0 ± 0.1^{bb}	12.0 ± 0.4^{bb}	9.3 ± 1.4	11.1 ± 0.4^{b}	11.8 ± 0.2^{bb}
5 weeks	8.1 ± 0.3	9.0 ± 0.5^{aa}	11.2 ± 0.1^{cc}	11.2 ± 0.1^{cc}	7.4 ± 0.2	10.9 ± 0.4^{cc}	11.0 ± 0.2^{cc}
Serum Mg, mg/100 ml							
Initial	3.0 ± 0.1	3.4 ± 0.2	3.2 ± 0.1	3.1 ± 0.1	3.3 ± 0.1	3.1 ± 0.2	3.1 ± 0.1
3 weeks	2.0 ± 0.1	2.3 ± 0.2	3.2 ± 0.3^{bb,d}	3.0 ± 0.6^{aa,b}	1.7 ± 0.1	2.2 ± 0.2	2.9 ± 0.3^{aa,b}
5 weeks	2.0 ± 0.2	2.1 ± 0.2	2.6 ± 0.4^{a}	2.5 ± 0.3^{a}	1.7 ± 0.1	2.4 ± 0.1^{a}	2.4 ± 0.1^{a}
Serum alkaline phosphatase, Bessey-Lowry units							
Initial	19.2 ± 1.6	19.4 ± 2.7	19.4 ± 3.8	16.6 ± 1.4	17.2 ± 0.8	17.2 ± 2.4	20.1 ± 2.2
3 weeks	11.5 ± 1.4	11.8 ± 1.0	9.1 ± 1.5	11.2 ± 2.6	17.4 ± 2.4^{e}	10.8 ± 1.6	11.2 ± 1.1
5 weeks	13.0 ± 1.5	11.8 ± 1.4	7.0 ± 1.0	6.7 ± 1.4	20.6 ± 4.6^{dd}	7.0 ± 1.6	9.0 ± 0.8
Humeral analysis, dry, fat-free basis, %							
Ash	46.5 ± 1.0	47.9 ± 0.1^{aa}	49.0 ± 0.1^{aa,b}	47.5 ± 0.2^{aa}	44.5 ± 0.8	48.5 ± 1.0^{aa}	49.5 ± 0.5^{bb}
Ca	16.5 ± 0.3	17.2 ± 0.2^{aa}	17.5 ± 0.1^{aa}	16.9 ± 0.1^{a}	15.7 ± 0.3	17.9 ± 0.7^{bb}	17.7 ± 0.2^{bb}
P	8.6 ± 0.2	9.0 ± 0.1^{aa}	9.2 ± 0.1^{aa,b}	9.0 ± 0.1^{aa}	8.2 ± 0.1	9.0 ± 0.2^{aa}	9.3 ± 0.1^{aa,b}
Mg	0.42 ± 0.02	0.49 ± 0.02^{a}	0.52 ± 0.06^{b}	0.52 ± 0.01^{b}	0.33 ± 0.04	0.36 ± 0.04	0.51 ± 0.03^{b}

[1] Mean ± SE.

[a] Significantly greater than least value ($P < 0.05$); [aa] $P < 0.01$.

[b] Significantly greater than least two values ($P < 0.05$); [bb] $P < 0.01$.

[c] Significantly greater than least three values ($P < 0.05$); [cc] $P < 0.01$.

[d] Significantly greater than least four values ($P < 0.05$); [dd] $P < 0.01$.

[e] Significantly greater than all other values ($P < 0.05$).

RESULTS AND DISCUSSION

The growth, serum and bone data from the 2 trials were combined and treatment means are presented in table 2. Overt symptoms of rickets were evident in all of the pigs receiving no dietary vitamin D_2 by the end of either trial, but the severity did not appear as great as in former studies (14) and only one of the pigs from the first trial died. Growth rate was depressed in the vitamin D_2-deficient animals. Pigs receiving soy protein diets containing 50, 100 or 250 IU/kg of dietary vitamin D_2 grew at a slightly suboptimal rate, whereas pigs receiving the soy protein diet containing 500 IU/kg or casein diets containing 50 or 100 IU/kg of vitamin D_2 grew at an optimal rate. Efficiency of food utilization was greatest in those pigs which grew at an optimal rate with a correlation coefficient of 0.82 ($P < 0.01$) between daily gain and gain-to-food parameters (table 2) of pigs in this study.

Serum calcium concentration was depressed at the end of the trial in pigs receiving the casein diet with no vitamin D_2 and soy protein diets with 50 or 100 IU/kg of vitamin D_2. Pigs receiving casein diets containing 50 or 100 IU/kg or soy protein diets containing 250 or 500 IU/kg of vitamin D_2 had normal levels of serum calcium concentration throughout the study. Serum inorganic phosphorus data are not presented because values of serum P concentration were depressed in only those pigs receiving no dietary vitamin D_2. Serum magnesium concentration became rapidly depressed in pigs receiving no vitamin D_2 and less extensively depressed after 3 weeks of the trial in pigs receiving the casein diet containing 50 IU of vitamin D_2/kg and soy protein diets containing 50 or 100 IU of vitamin D_2/kg. Pigs receiving the other diets had normal serum magnesium concentrations throughout the study. Serum alkaline phosphatase activity was significantly elevated only in pigs receiving no dietary vitamin D_2; however, there was a tendency toward elevated activity also in the pigs receiving soy protein diets containing 50 or 100 IU/kg of vitamin D_2.

Humeral ash data indicate the depression in bone mineralization which occurs when vitamin D is limiting in the diet.

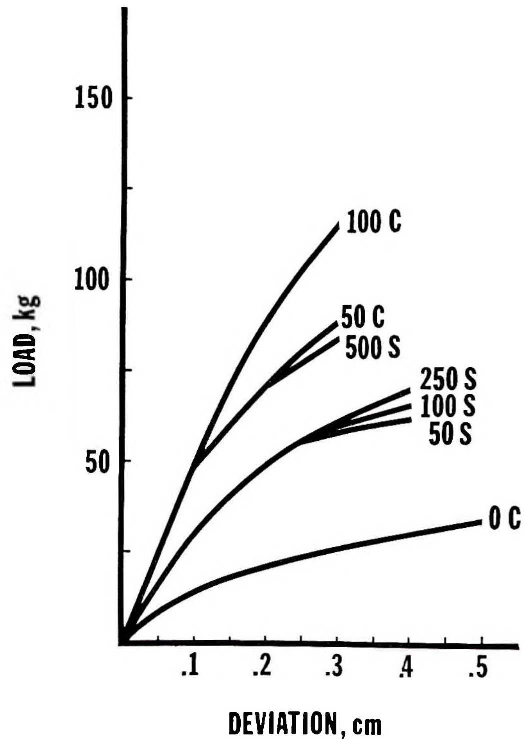

Fig. 1 Load-deviation curves of baby pigs receiving casein (c) or isolated soy protein (s) diets containing various levels of vitamin D_2 (IU/kg).

This is apparent from the values of humeral ash, calcium, phosphorus and magnesium of pigs receiving no dietary vitamin D_2 or pigs receiving the soy protein diet supplemented with 50 IU of vitamin D_2/kg. There is also a significantly reduced magnesium level in humeri of pigs receiving the casein diet supplemented with 50 IU of vitamin D_2/kg. Femur load-deviation curves obtained from breaking-strength studies are presented in figure 1. The degree of influence which dietary vitamin D_2 level in casein diets has upon these characteristic curves is pronounced. Much less pronounced is the influence of vitamin D_2 level in soy protein diets in differentiating the characteristic load-deviation curves. The strength of the femur is due largely to the diameter of the shaft and the thickness of the compact layer of bone beneath the periosteum. The pictures of cross-sections of the femurs of pigs in this study (fig. 2) reveal the tendency toward a greater degree of formation

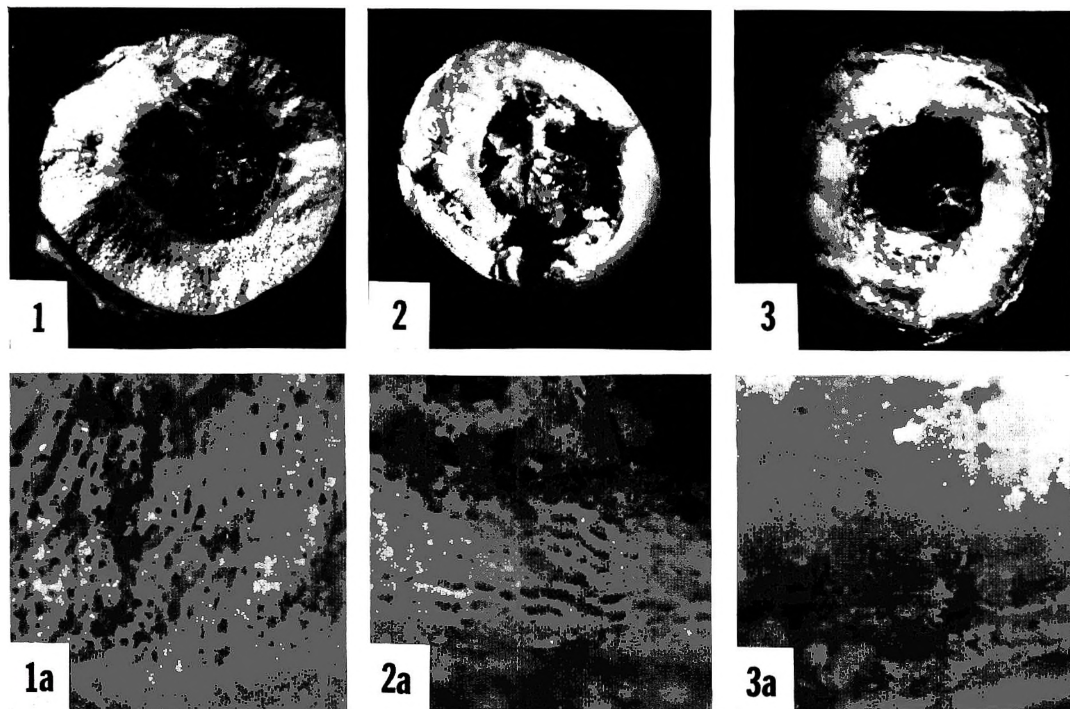

Fig. 2 The upper pictures (1–3) are photographs (2 ×) of cross-sections of the femurs from pigs receiving 1) casein diet with no dietary vitamin D_2, 2) soy protein diets with 100 IU of vitamin D_2/kg and 3) casein diet with 100 IU of vitamin D_2/kg. The lower pictures (1a–3a) are photomicrographs (20 ×) of segments of these sections. Note the essential absence of compact bone in 1 and 1a and the spongy appearance of both the compact and cancellous areas. Similar spongy areas, although less extensive, may be observed in 2 and 2a. The bone in 3 and 3a has a dense, nearly amorphous, appearance.

of compact bone by pigs receiving a casein diet containing 100 IU of vitamin D_2/kg than by pigs receiving a soy protein diet with an identical dietary vitamin D_2 level.

Results of the mineral balance studies are presented in table 3. Food intake and consequent mineral intake was less for pigs receiving no dietary vitamin D_2. Pigs consuming the casein diet containing 100 IU of vitamin D_2/kg exhibited Ca, P and Mg balance patterns similar to those of animals receiving adequate diets in previous studies (15, 20). Pigs receiving the soy protein diets excreted greater amounts of fecal Ca, P and Mg. Increasing the concentration of dietary vitamin D_2 was not effective in overcoming the excessive fecal mineral excretion. The cause of decreased apparent macromineral absorption from soy protein diets compared with casein diets containing adequate vitamin D as determined by other criteria must of necessity be ascribed to the nature of the protein. Isolated soy protein diets have been shown to be less permissive of apparent trace mineral absorption than similar diets in which casein is the principal protein source (6, 22, 23). The poorly utilized phytate of soy protein also effectively binds the macro cations, that is, Ca and Mg (24). The relative unavailability of the P of phytate compared with the availability of P from several inorganic sources has been demonstrated (25). On the other hand, the P of casein is fully as available as P from inorganic phosphate sources (26). In the present study the portion of dietary P supplied by either casein or soy protein amounted to about 23% since both the casein and soy proteins analyzed approximately 0.7% of P. Spitzer and Phillips (27) have shown that

TABLE 3

Daily calcium, phosphorus and magnesium excretion and retention as affected by type of protein and level of vitamin D_2

Type of protein	Casein	Casein	Soy	Soy	Soy
Dietary level of vitamin D_2, IU/kg	0	100	100	250	500
No. of collections	8	8	8	8	8
Daily food intake, g	288 ± 13 [1]	392 ± 30[aa]	403 ± 24[aa]	384 ± 26[aa]	412 ± 20[aa]
Ca balance,daily					
Ca intake, g	2.30 ± 0.10	3.13 ± 0.24[aa]	3.22 ± 0.19[aa]	3.08 ± 0.21[aa]	3.30 ± 0.16[aa]
Fecal Ca, g	0.65 ± 0.08	0.40 ± 0.07	0.83 ± 0.15[a]	0.73 ± 0.11[a]	0.97 ± 0.13[aa]
Urinary Ca, mg	3 ± 0	18 ± 6[a]	10 ± 2	23 ± 7[a]	19 ± 5[a]
Ca retention, g	1.65 ± 0.09	2.71 ± 0.19[aa]	2.34 ± 0.17[aa]	2.32 ± 0.20[aa]	2.31 ± 0.09[aa]
Ca retention, %	72 ± 3	87 ± 1[d]	74 ± 4	75 ± 3	71 ± 3
P balance, daily					
P intake, g	1.72 ± 0.08	2.35 ± 0.18[aa]	2.41 ± 0.14[aa]	2.30 ± 0.16[aa]	2.47 ± 0.12[aa]
Fecal P, g	0.30 ± 0.02	0.25 ± 0.03	0.62 ± 0.10[bb]	0.55 ± 0.08[aa,b]	0.74 ± 0.09[bb]
Urinary P, g	0.21 ± 0.03	0.34 ± 0.05[bb,c]	0.10 ± 0.04	0.11 ± 0.03	0.31 ± 0.04[bb]
P retention, g	1.21 ± 0.06	1.76 ± 0.13[aa]	1.69 ± 0.11[aa]	1.64 ± 0.12[aa]	1.62 ± 0.07[aa]
P retention, %	70 ± 4	77 ± 2	70 ± 4	72 ± 3	67 ± 3
Mg balance, daily					
Mg intake, mg	106 ± 5	145 ± 11[aa]	158 ± 10[aa]	150 ± 10[aa]	161 ± 9[aa]
Fecal Mg, mg	48 ± 4	63 ± 10	106 ± 16[aa,b]	83 ± 16[a]	93 ± 10[a]
Urinary Mg, mg	1 ± 0	4 ± 1[a]	2 ± 1	5 ± 2[a]	5 ± 2[a]
Mg retention, mg	57 ± 4	78 ± 5[b]	50 ± 11	62 ± 12	63 ± 4
Mg retention, %	53 ± 3[a]	56 ± 4[aa,b]	33 ± 7	41 ± 6	40 ± 2

[1] Mean ± SE.
[a] Significantly greater than least value ($P < 0.05$); [aa] $P < 0.01$.
[b] Significantly greater than least two values ($P < 0.05$); [bb] $P < 0.01$.
[c] Significantly greater than least three values ($P < 0.05$).
[d] Significantly greater than all other values ($P < 0.01$).

58% of the P in soybean meal is in the form of phytin or phytate which was readily available to the rat due to the activity of intestinal phytase (28). Singsen and Mitchell (29) observed that soybean meal rations for chicks need no supplemental inorganic P when phytase from field-cured leaf meal and vitamin D are provided. On the other hand McGinnis et al. (30) observed poor utilization of phytin P by chicks even when adequate phytase was available. Dietary vitamin D has been shown to be particularly essential in the utilization of phytate P (31). Intestinal phytase activity in the very young pig has not been measured. Data in the present study suggest that perhaps the intestinal phytase activity of baby pigs reared with purified diets is inadequate to digest the phytate present in soy protein, thus failing to make available for absorption the P of phytate as well as the cations which it effectively binds. Dietary vitamin D_2 does not appear to be effective in the absorption of Ca, P and Mg which are incorporated in or combined with phytate.

From the standpoint of obtaining optimal utilization of the Ca, P and Mg which are absorbed for optimal growth, serum and bone mineral levels and skeletal development, it must be concluded that the dietary vitamin D_2 requirement for baby pigs reared with purified diets containing casein as the protein source is confirmed to be 100 IU/kg (14) and that the minimal vitamin D_2 requirement is increased when isolated soybean protein replaces casein in the diet. The extent of this requirement increase appears to depend greatly upon the criteria of adequacy selected. The studies of Carlson and coworkers (12, 13) with poults also indicate that the dietary vitamin D requirement is dependent upon the dietary level of isolated soybean protein. Recent work [6] indicated that this is also true with the baby pig and that the method used in isolating

[6] Unpublished data.

the soybean protein may also be a factor in the vitamin D_2 requirement level of prepared diets. This work is presently receiving further study.

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Influence of Varying Levels of Dietary Minerals on the Development of Urolithiasis, Hair Growth, and Weight Gains in Rats

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ABSTRACT Calcium at varying levels (0.3 and 1.2%), phosphorus (0.3 and 1.2%), potassium (0.1, 0.2, and 0.9%), magnesium (0.05, 0.2, and 0.5%), and sodium (0.05 and 0.81%) were fed to rats in order to produce a magnesium-phosphate type of urinary calculi. A diet containing 0.3% calcium, 1.20% phosphorus, 0.1% potassium, 0.5% magnesium, and 0.81% sodium produced a high incidence of urolithiasis within a 70-day period. Urolithiasis did not occur when the level of any of the individual minerals was varied from the levels in the calculogenic diet. Phosphate damage to the kidneys occurred with the low calcium, high phosphorus diets. Specific patterns of hair growth were present on rats receiving the low potassium diets. Weight gains were significantly affected by calcium, phosphorus, and potassium levels. Significant interactions ($P < 0.01$) occurred between levels of calcium and phosphorus, calcium and potassium, phosphorus and potassium, and phosphorus and magnesium in their effect on growth.

The role of dietary constituents in the development of urolithiasis in animals is complicated in that the disease varies with the absolute amounts of these nutrients in the diet and may be related to interrelationships among the various nutrients. Also, dietary factors influence the composition of the uroliths in rats to the extent that calcium citrate calculi are produced by feeding diets low in phosphorus (1–3). Calcium oxalate calculi have been reported to occur in rats fed low phosphorus diets (4) or in rats fed vitamin B_6-deficient diets containing varying levels of phosphorus and magnesium (5). Dietary protein of poor quality has been implicated in the etiology of calcium stones (6). Rats fed diets high in phosphorus and magnesium have been prone to develop calculi of the magnesium-phosphate type (7, 8). Moreover, tissue calcification has occurred in rats and other animals under a variety of dietary conditions (9).

The purpose of the studies reported herein was to determine the effects of wide calcium-to-phosphorus ratios in the presence of high and low dietary intakes of magnesium and potassium on the development of urolithiasis in rats. Interactions among the levels of the dietary minerals on hair growth, kidney abnormalities, and weight gains are also discussed.

METHODS

Three trials were conducted with 226 male rats averaging 45 g initial body weight. The animals were assigned at random to treatments, with a maximal variation of 4 g allowed for any individual animal and a maximal age difference of 3 days. The rats were housed in individual cages in an air conditioned room maintained at 26°. Feed and tap water were provided ad libitum, and weekly weight and feed consumption records were maintained on each rat. The length of the experimental periods was 98, 70, and 84 days for trials 1, 2, and 3, respectively. At the end of each trial, the rats were killed and the kidneys and urinary tracts examined with a dissecting microscope for the presence of urinary calculi. Rats showing signs of urolithiasis were killed during the experimental periods. Isolated calculi from individual rats were air-dried, weighed, and combined into a composite

Received for publication December 7, 1964.

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TABLE 1

Composition of diets fed to rats in trials 1, 2 and 3

	%
Protein [1]	23.0
Soybean oil	5.0
Vitamin mix [2]	2.2
DL-Methionine	0.3
Cellulose [3]	2.0
Basal mineral mix [4]	0.012
Butylated hydroxytoluene	0.025
Additional minerals [5]	—
Cornstarch [6]	—

[1] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Minneapolis.

[2] When included in the diet at a level of 2.2%, the vitamin mix supplied the following vitamins in mg/kg of diet: riboflavin, 22; pyridoxine·HCl, 22; thiamine·HCl, 22; Ca pantothenate, 66; niacin, 99; p-aminobenzoic acid, 110; α-tocopherol, 110; ascorbic acid, 900; inositol, 110; choline chloride, 1600; menadione, 50; biotin, 0.4; folic acid, 1.9; and vitamin B_{12}, 0.03. Also vitamin A, 20,000 USP units and vitamin D, 2200 USP units/kg of diet.

[3] Solka-Floc (BW-40), Brown Company, Berlin, New Hampshire.

[4] When included in the diet at 0.012%, the mineral mixture contributed the following minerals in g/kg of diet: $FeSO_4 \cdot 7H_2O$, 1.005; KI, 0.03; $MnSO_4 \cdot 7H_2O$, 0.15; ZnO, 0.0060; $CuSO_4 \cdot 5H_2O$, 0.018; and $CoCl_2 \cdot 6H_2O$, 0.0007.

[5] Additional minerals were supplied to obtain the desired mineral levels in the diets by using a selected combination from the following reagent grade chemicals: $Ca_3(PO_4)_2$, K_3PO_4, NaH_2PO_4, $CaCO_3$, KCl, $NaH_2PO_4 \cdot H_2O$, KH_2PO_4, K_2HPO_4, $MgCO_3$, $MgCl_2$, H_3PO_4, and NaCl.

[6] The percentage of cornstarch varied according to the additional minerals added.

sample within a single treatment. The uroliths were ashed and chemical determinations were made for calcium (11), phosphorus (12), and magnesium (13).

The ingredients used in the experimental diets are shown in table 1. Chemical analyses (14) were made on the diets in trial 1 (table 2), and the mineral levels in trials 2 and 3 were calculated since the same sources of ingredients were used for all trials.

The factors tested in trial 1 (treatments 1–8, table 2) were calcium (0.30 and 1.20%) phosphorus (0.30 and 1.20%) and potassium (0.10 and 0.90%) in a 2^3 factorial arrangement of treatments. Two additional treatments were added to this trial. Treatments 9 and 10 were replications of treatments 2 and 7 except that the potassium level was raised to 0.20%. The data from these treatments (9 and 10) were not included in the statistical analysis. Trial 2 contained the same factors as trial 1 at their respective levels with magnesium added as another factor at levels of 0.05 and 0.50% and was designed as a 2^4 factorial arrangement of treatments. Trial 3 was designed as a 2^2 factorial arrangement of treatments to test the effect of sodium and chlorine variation on urolithiasis development. Treatment 21, which contained 0.81% sodium and 0.30% chlorine, was repeated in treatment 27. Sodium (0.05%) and chlorine (0.004% calculated) were tested in appropriate combinations in the remaining 3 diets of trial 3. The salt mixtures were added at the expense of cornstarch in these trials. The number of rats used per treatment was 9, 6, and 10 for trial 1, 2, and 3, respectively.

Analysis of variance was used for statistical evaluation of the data.

RESULTS AND DISCUSSION

Incidence and composition of calculi. Urolithiasis occurred in the rats in only 4 of the 30 treatments (table 2). Diets which were calculogenic contained the low levels of calcium and potassium and the high levels of phosphorus and magnesium. Twenty-three of the 26 rats fed the low calcium, low potassium, high phosphorus, high magnesium, and high sodium diets developed calculi, whereas only 1 of 20 rats had urolithiasis when the sodium content was reduced to 0.05%.

The calculi observed either in the kidneys or bladder had similar physical characteristics and appeared white and chalky after air-drying. A total calculi weight of 49 mg, 189 mg, and 408 mg was collected from rats receiving diets 21, 27, and 29, respectively. A small calculus in an animal fed diet 28 was not large enough to permit chemical analysis. The calculi were of similar chemical composition (table 3) and contained approximately 1.5% calcium, 15.9% phosphorus, and 10.5% magnesium. They were comparable in chemical composition to those which have been produced experimentally in sheep by the use of diets high in phosphorus and potassium (10). Vermeulen et al. (8) produced calculus formation of the magnesium-phosphate type in rats by implanting foreign bodies in the urinary bladder.

In the present studies, the development of calculi was dependent on a rather specific set of dietary mineral levels (Ca, 0.30%; P, 1.20%; Mg, 0.50%; K, 0.10%; and Na, 0.81%). These diets contained

TABLE 2

Mineral composition of diets, gross kidney observations and 70-day weight gains of rats in trials 1, 2 and 3

Trial	Treatment group	No. of rats/ treatment	Dietary mineral composition						Gross kidney observations						Avg wt gain
			Ca	P	Mg	K	Na	Cl	Norm [1]	C [2]	B [3]	P [4]	E [5]	C or B [6]	
			%	%	%	%	%	%							g
1	1	9	1.20	1.20	0.20	0.90	0.33	0.30	7	0	0	0	2	0	283
	2	9	1.20	1.20	0.20	0.10	0.46	0.30	7	0	0	0	2	0	200
	3	9	1.20	0.30	0.20	0.90	0.20	0.50	9	0	0	0	0	0	277
	4	9	1.20	0.30	0.20	0.10	0.29	0.30	9	0	0	0	0	0	207
	5	9	0.30	1.20	0.20	0.90	0.45	0.30	3	0	0	6	0	0	251
	6	9	0.30	0.30	0.20	0.90	0.20	0.50	9	0	0	0	0	0	287
	7	9	0.30	1.20	0.20	0.10	0.81	0.30	1	0	0	8	0	0	126
	8	9	0.30	0.30	0.20	0.10	0.20	0.30	9	0	0	0	0	0	231
	9	9	1.20	1.20	0.20	0.20	0.26	0.39	3	0	0	0	6	0	273
	10	9	0.30	1.20	0.20	0.20	0.61	0.39	0	0	0	9	0	0	213
2	11	6	1.20	1.20	0.50	0.90	0.33	0.30	6	0	0	0	0	0	276
	12	6	1.20	1.20	0.05	0.90	0.33	0.30	5	0	0	0	1	0	258
	13	6	1.20	1.20	0.50	0.10	0.46	0.30	6	0	0	0	0	0	219
	14	6	1.20	1.20	0.05	0.10	0.46	0.30	3	0	0	0	3	0	185
	15	6	1.20	0.30	0.50	0.90	0.40	0.50	6	0	0	0	0	0	211
	16	6	1.20	0.30	0.05	0.90	0.40	0.50	6	0	0	0	0	0	244
	17	6	1.20	0.30	0.50	0.10	0.29	0.30	6	0	0	0	0	0	226
	18	6	1.20	0.30	0.05	0.10	0.29	0.30	6	0	0	0	0	0	239
	19	6	0.30	1.20	0.50	0.90	0.45	0.30	6	0	0	0	0	0	258
	20	6	0.30	1.20	0.05	0.90	0.45	0.30	5	0	0	0	1	0	240
	21	6	0.30	1.20	0.50	0.10	0.81	0.30	0	2	2	4	1	4	162
	22	6	0.30	1.20	0.05	0.10	0.81	0.30	4	0	0	0	2	0	137
	23	6	0.30	0.30	0.50	0.90	0.20	0.50	6	0	0	0	0	0	260
	24	6	0.30	0.30	0.05	0.90	0.20	0.50	6	0	0	0	0	0	266
	25	6	0.30	0.30	0.50	0.10	0.20	0.30	6	1	0	0	0	1	223
	26	6	0.30	0.30	0.05	0.10	0.20	0.30	6	0	0	0	0	0	219
3	27	10	0.30	1.20	0.50	0.10	0.81	0.30	5	3	9	1	4	9	177
	28	10	0.30	1.20	0.50	0.10	0.05	0.30	10	0	1	0	0	1	208
	29	10	0.30	1.20	0.50	0.10	0.81	0.00	0	4	8	2	0	10 [7]	150
	30	10	0.30	1.20	0.50	0.10	0.05	0.00	10	0	0	0	0	0	188

[1] Norm indicates a normal gross appearance of kidneys.
[2] C indicates calculi observed grossly in kidney.
[3] B indicates calculi observed in the urinary bladder.
[4] P indicates that kidneys showed the gross appearance of phosphate damage.
[5] E indicates enlargement of kidneys without other signs of abnormality.
[6] C or B indicates calculi found either in the kidney or bladder or both.
[7] Seven animals died with ruptured bladders due to calculi blockage during the experiment.

levels of calcium, phosphorus, and magnesium which have been previously reported to be calculogenic (7). Unexpectedly, a low level of potassium was the critical factor in the development of urolithiasis in the rat instead of the high dietary potassium intake which increased the incidence of urolithiasis in sheep when combined with a high level of dietary phosphorus. However, a potassium deficiency has been shown to affect markedly the physiology of the rat kidney by causing the production of lysosomes in the cytoplasm of cells of the renal papillae with increased acid phosphatase in papillary tissue (15). Also, granules containing an acid mucopolysaccharide appear in the collecting tubule cells of potassium-deficient rats (16). Unfortunately, the previous work (15, 16) apparently was conducted with a salt mixture deficient in other minerals besides potassium. In the present studies careful attention was given to the preparation of the salt mixtures to obtain a ration low in potassium, yet adequate in all other nutrients. Mucopolysaccharides and other mucoid substances have been implicated in urolithiasis as part of the organic matrix of calculi (17).

The alternate levels of the minerals in the factorial treatments, when changed singly, did not result in calculogenic diets with the exception of the sodium level. The low sodium level (0.05%) signifi-

TABLE 3

Percentage ash, calcium, phosphorus and magnesium of calculi from rats in trials 2 and 3

Treatment group	Ash	Calcium	Phosphorus	Magnesium
	%	%	%	%
21 and 27	74.9	1.4	15.1	10.6
29	72.2	1.6	14.9	10.5

cantly lowered ($P < 0.01$) the calculogenicity of the diets in trial 3. A potassium deficiency has been shown to be exaggerated when sodium was fed at a level of 1.0% (18). The reduction of urolithiasis in rats fed the low sodium diets was, therefore, probably the result of a less severe deficiency of potassium in these animals than those fed the high sodium diets.

Gross kidney abnormalities. Phosphate damage of the kidneys was observed in each of the 3 trials (table 2). Abnormal appearance of the kidneys included overall enlargement with necrosis in the cortical areas and the outer surfaces showing numerous indentations. The renal structure exhibited marked lesions in the cortical and medullary areas as described previously

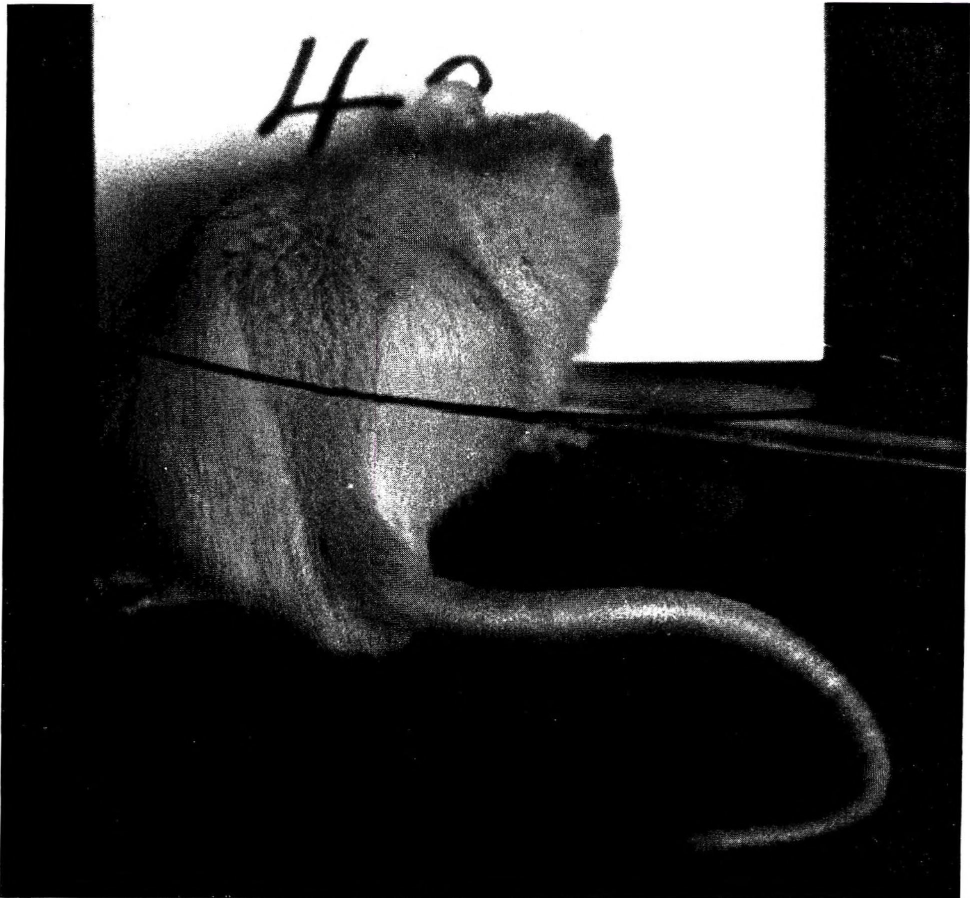

Fig. 1 Symmetrical hair pattern shown by low density area along the back of rat fed the low potassium diet for 32 days.

(19). A low calcium, high phosphorus ratio with the medium level of magnesium was more conducive to phosphate damage than the high level of magnesium. Renal damage was absent in rats fed diets containing 0.05% magnesium. Gross enlargement of the kidneys occurred most frequently in rats receiving calcium and phosphorus at the high levels. Kidney enlargement did not appear to be related to renal phosphate damage and neither of these conditions was related to the development of urolithiasis.

Hair growth patterns. Rats fed the low potassium diets developed a characteristic hair pattern as shown in figure 1. Other dietary factors did not influence these patterns. The manifestation of changes in hair growth on the head, back, and occasionally along the upper part of the sides was noticeable after the animals had been fed the diets for 2 to 3 weeks, and continued throughout the experimental periods to some degree. Histological sections made of biopsies taken at the end of trial 1 indicated fewer hairs per follicle group in the affected animals. This was especially evident where hairs in cross section were counted at the level of the sebaceous glands. The control animals averaged 10.4 hairs per follicular group as compared with an average of 5.6 hairs per follicular group for the animals fed the low potassium diets. Hairs and follicles in the longitudinal sections were less numerous in the low potassium groups. These pronounced changes both in the gross pattern of hair growth and the differences in follicular groups indicate that potassium is involved in the shedding and regeneration of hair and in the normal hair growth pattern. The abnormal hair coat was transitory and was probably due to the lower potassium requirement with increasing age and weight (20). Observation of these hair patterns should be helpful in detecting signs of a conditioned potassium deficiency when such deficiency would not likely be expected. Although a number of nutritional deficiencies have been implicated in abnormal hair growth (21), the hair condition as described appeared to be rather specific and more defined than previously reported.

Animal growth. Weight gains of the animals were influenced markedly by treatments (table 2). Average weight gains were calculated for the 70-day period so that comparisons could be made between the 3 experiments. Calcium, phosphorus, and potassium levels significantly affected weight gains (table 4). Animals fed at either the high calcium, low phosphorus, or the high potassium levels gained faster than the animals fed at the respective alternate levels in trials 1 and 2. Animal

TABLE 4

Summary of mean squares [1] from the analysis of variance of cumulative gain in trials 1 and 2

Source of variation	Degrees of freedom		Mean squares	
	Trial 1	Trial 2	Trial 1	Trial 2
Calcium (C)	1	1	37.65*	9.42*
Phosphorus (P)	1	1	162.33**	17.11**
Potassium (K)	1	1	1211.13**	571.98**
Magnesium (M)	—	1	—	0.76
CP	1	1	186.88**	63.91**
CK	1	1	12.08	44.08**
CM	—	1	—	2.02
PK	1	1	51.03**	78.90**
PM	—	1	—	35.30**
KM	—	1	—	4.81
CPK	1	1	7.81	1.09
Animals within CPK	56	—	6.96	—
CPM	—	1		1.84
CKM	—	1		0.17
PKM	—	1		0.44
CPKM	—	1		0.18
Animals within CPKM	—	80		1.66

[1] Mean squares divided by 1000.
* $P < 0.05$.
** $P < 0.01$.

growth was not affected by the 2 levels of magnesium in trial 2. The low level of potassium was not adequate for maximal growth as illustrated by the increased weight ($P < 0.01$) of the animals on treatment 9 as compared with treatment 2. The principal effects of the high and low levels of calcium, phosphorus and potassium were not unexpected since the respective levels had been deliberately fixed to provide a wide difference in mineral intakes. The levels of magnesium, however, would not be expected to influence growth since all were above the dietary requirement (20).

Certain significant interactions ($P < 0.01$) were present in trials 1 and 2. Animal growth was depressed by high phosphorus in the presence of low calcium levels, but normal growth rates were obtained when calcium levels were high irrespective of the phosphorus levels. A significant P × K interaction resulted from the large reduction in weight gains when high phosphorus levels were combined with the low potassium levels, indicating that these mineral levels were not acting independently. Animal growth was significantly depressed with the high phosphorus, low potassium diets and not as much with the low phosphorus, low potassium diets. In trial 2, a significant Ca × K interaction was observed which was not significant in trial 1. This resulted from the increased growth of rats receiving the high calcium in the presence of low potassium, whereas animals fed the high calcium gained less than those receiving the low calcium when potassium levels were high. Also, in trial 2, where magnesium was added at 2 levels, a significant P × Mg interaction occurred. Magnesium at the high level of intake was beneficial to animal growth in the presence of high phosphorus, but this effect was reversed in the low phosphorus groups.

In these studies, as in other nutritional studies involving the etiology of urolithiasis, the entire mineral content of the diet must be considered if urolithiasis is to be produced by dietary means. The present trials were designed to determine what mineral interrelationships existed at the levels investigated. To understand the resulting interactions on a physiochemical basis, would require more complex investigations as has been pointed out with a number of mineral interactions (9).

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Fatty Livers in Weanling Rats Fed a Low Protein, Threonine-deficient Diet [1,2]

I. EFFECT OF VARIOUS DIET FATS

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ABSTRACT Weanling albino rats were fed a diet containing 9% casein and 30% fat with and without supplements of threonine. The fat source in the diet was provided by corn oil, olive oil, cottonseed oil, hydrogenated vegetable oil, or corn oil hydrogenated to an iodine value of 74. Animals were maintained with their respective diets for 2 to 4 weeks. Feeding weanling rats a threonine-deficient diet containing 30% corn oil resulted in the appearance of fatty livers. Replacing the corn oil in this diet with either cottonseed oil or hydrogenated vegetable oil caused a significant reduction in liver fat concentration. Substituting olive oil for corn oil slightly increased liver fat levels. In every instance, the addition of threonine lowered liver fat levels. When hydrogenated corn oil was substituted for corn oil in threonine-deficient diets, liver fat concentrations decreased markedly after 2 weeks; this effect persisted after 4 weeks. Since all diets within a series were isocaloric and since no significant differences in food intake or growth were observed among any of the deficient groups, the "protective" action of some diet fats is apparently not mediated through a more equitable balance between the amino acid and calorie ratio.

Feeding weanling rats a 9% casein diet supplemented with methionine and tryptophan, but not threonine, induces fatty livers under specific dietary conditions (1–3). The basal diets used in these experiments contained corn oil (5% of the diet) as the fat source and sucrose as the carbohydrate source. Under these dietary conditions, the deposition of fat in liver tissues reaches a maximum after 2 to 3 weeks of feeding and then decreases slowly (4, 5).

Recently emphasis has been shifted to a study of interrelationships between the threonine-deficient state and the kind and proportion of other nutrients in the diet. In 1961 Sidransky and Clark (6) suggested that the pathological changes observed in young rats fed a threonine-devoid diet were due to an imbalance between the amino acid and the calorie intake. Later Sidransky and Verney (7) observed an accentuation of such pathological changes by increasing the corn oil content of the diet from 5 to 25%.

In addition to the quantity, the type of fat present in the diet has also been shown to influence the severity of lesions induced by nutritionally inadequate diets. Benton et al. (8) reported higher liver fat concentrations when 20% of butterfat or lard provided the diet fat source than when 20% of corn oil or margarine was used as the dietary fat in threonine-deficient diets. In all instances, the adddition of threonine significantly lowered liver fat levels. Benton et al. postulated that the long-chain fatty acid composition of the fat was the determining factor in regulating liver fat deposition. Carroll (9, 10), using different dietary conditions, also observed a marked effect of different diet fats on glycolysis and lipogenesis.

The experiments reported in the present paper were undertaken to determine the effects of various types of fats and oils on growth and liver composition of rats fed threonine-deficient diets.

METHODS

Weanling male rats of the Sprague-Dawley strain were used as the experimental animals. They were divided by weight into groups of ten and housed in individual screen-bottom cages in a temperature-controlled room. Data from replicate

Received for publication October 9, 1964.
[1] Journal article no. 3484 from the Michigan Agricultural Experiment Station.
[2] This investigation was supported in part by Public Health Research Grant no. A-6093 from the National Institute of Arthritis and Metabolic Diseases.

groups were combined at the close of the study. Food and water were supplied ad libitum except in one experiment when the paired-feeding technique was used.

The basal diet had the following percentage composition: sucrose, 25; casein, 9; salts W,[3] 4; fat,[4] 30; vitamin mix, 0.25; choline, 0.15; DL-methionine, 0.30; DL-tryptophan, 0.10; cellulose,[5] 31.20. The composition of the vitamin mix has been described previously (11). When threonine was added to the diet, 0.36% of the DL-mixture of the amino acid replaced an equal amount of sucrose. These diets, containing 30% fat, were isocaloric with the 5% fat diets used in previous experiments (4, 5); the cellulose was used to make up the weight differences. The animals were divided into the following experimental groups: group 1, basal diet (corn oil); group 2, corn oil + 0.36% DL-threonine; group 3, olive oil; group 4, olive oil + 0.36% DL-threonine; group 5, cottonseed oil; group 6, cottonseed oil + 0.36% DL-threonine; group 7, hydrogenated vegetable oil;[6] group 8, hydrogenated vegetable oil [7] + 0.36% DL-threonine; and group 9, hydrogenated corn oil.[8]

The rats were weighed at weekly intervals during the study, and food consumption records were kept. At the end of the experimental period the animals were lightly anesthetized with ether, approximately two-thirds of the tail was severed with a sharp razor blade, and blood samples were collected. By this method 1 ml of blood could be collected from very young rats (2 weeks postweaning). The blood samples were allowed to stand overnight and the serum was separated the next morning. The serums were stored frozen until analyzed for proteins by paper electrophoresis in a Spinco apparatus. The strips were scanned in a model RB Spinco Analytrol and results expressed as the percentage of total protein separated.

After collecting the blood, the rats were decapitated. Livers were excised, weighed, homogenized with distilled water, and dried at 90° to constant weight. The dried livers were weighed to determine moisture content, and ground in a Wiley mill. Fat content was determined on one-gram samples by continuous ether extraction for 3 hours on a Goldfisch apparatus. The concentration of fat in the tissues was expressed as percentage of the dry weight. The percentage of nitrogen was determined on the residue by the macro-Kjeldahl method and expressed on a fresh-weight basis.

Standard errors of the means were calculated for all data. Student's t test was used as a measure of significance. Only those differences with a probability of less than 0.01 were considered significant, and are so identified in the text.

RESULTS

Food intake and weight data for rats fed different types of fat in 9% casein diets with and without threonine supplements are summarized in table 1. No marked changes were observed in these parameters when the corn oil in the basal diet was replaced by olive oil, cottonseed oil, or hydrogenated vegetable oil. Likewise, there were no significant changes in food intake or weight gain when threonine was added to a diet containing any one of these fats. However, by changing the type of fat in the diet, or by the addition of threonine, significant changes in liver composition were induced (table 2).

The most marked effect of diet on liver composition centered in the lipid component (table 2). Replacing the corn oil in the basal diet (group 1) with either cottonseed oil (group 5) or hydrogenated vegetable oil (group 7) caused a significant reduction in liver fat concentration. Substituting olive oil (group 3) for corn oil (group 1) slightly increased the quantity of fat in livers of rats fed these diets (25.6% vs. 22.5%, respectively). In every instance, the addition of threonine significantly lowered liver fat levels (groups 1 vs. 2, 3 vs. 4, 5 vs. 6, 7 vs. 8).

In an effort to draw a more controlled comparison between the effects of corn oil and hydrogenated vegetable oils on threonine-deficient animals, corn oil was hy-

[3] Wesson, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, 75: 339.

[4] Containing 75 mg α-tocopheryl acetate/kg of diet.

[5] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[6] Distributed by Wesson Oil Sales Company, Fullerton, California. Iodine value = 74.

[7] See footnote 6.

[8] Corn oil hydrogenated by Procter and Gamble to iodine value of 74. The authors are indebted to the Procter and Gamble Company for their cooperation and assistance.

TABLE 1

Food intake and weight gain of rats fed 9% casein diets with and without threonine and containing different sources of dietary fat

Group	Diet [1]	No. animals/ group	Food intake	Wt gain
			g/week	*g/week*
1	Corn oil	50	70 ± 2 [2]	23 ± 2
2	Corn oil + threonine	40	70 ± 2	25 ± 1
3	Olive oil	10	68 ± 2	22 ± 1
4	Olive oil + threonine	10	70 ± 4	25 ± 2
5	Cottonseed oil	10	68 ± 2	20 ± 1
6	Cottonseed oil + threonine	10	64 ± 2	22 ± 1
7	Hydrogenated vegetable oil [3]	30	77 ± 4	27 ± 1
8	Hydrogenated vegetable oil [3] + threonine	30	81 ± 2	30 ± 1
9	Hydrogenated corn oil [4]	10	75 ± 1	22 ± 1

[1] Fat content of all diets = 30% w/w. Length of experimental period, 2 weeks.
[2] SE of mean.
[3] Maxim Brand shortening (iodine value = 74); manufactured by Hunt Foods and Industries, Inc., Fullerton, California. It is composed of approximately 90% hydrogenated soybean oil and 10% hydrogenated cottonseed oil. According to analyses kindly run by the manufacturer on a sample we submitted, the following is the percentage fatty acid composition: myristic, 0.3; palmitic, 12.7; stearic, 12.9; oleic, 57.5; and linoleic, 12.2.
[4] Corn oil was hydrogenated to an iodine value of 74 by the Procter and Gamble Company, Cincinnati.

TABLE 2

Liver composition of rats fed 9% casein diets with and without threonine and containing different sources of dietary fat

Group	Diet [1]	Moisture	Nitrogen	Fat
		%	*% wet wt*	*% dry wt*
1	Corn oil	68.4 ± 0.5 [2]	2.53 ± 0.04	22.5 ± 1.0
2	Corn oil + threonine	70.6 ± 0.3	2.69 ± 0.04	14.8 ± 0.5
3	Olive oil	68.1 ± 0.2	2.45 ± 0.02	25.6 ± 0.9
4	Olive oil + threonine	71.0 ± 0.4	2.62 ± 0.03	17.6 ± 1.2
5	Cottonseed oil	69.9 ± 0.2	2.60 ± 0.03	17.9 ± 0.9
6	Cottonseed oil + threonine	70.9 ± 0.3	2.76 ± 0.03	11.4 ± 0.5
7	Hydrogenated vegetable oil [3]	71.0 ± 0.2	2.47 ± 0.05	17.6 ± 0.8
8	Hydrogenated vegetable oil [3] + threonine	70.6 ± 0.7	2.62 ± 0.03	15.4 ± 0.5
9	Hydrogenated corn oil [4]	72.2 ± 0.2	2.53 ± 0.03	13.7 ± 0.4
9P	Hydrogenated corn oil [4] (pair-fed with group 1)	71.1 ± 0.3	2.74 ± 0.03	13.2 ± 0.3

[1] Fat content of all diets = 30% w/w. Length of experimental period, 2 weeks.
[2] SE of mean.
[3] Maxim Brand (iodine value = 74). See footnote 3, table 1, for description.
[4] Corn oil was hydrogenated to an iodine value of 74 by the Procter and Gamble Company, Cincinnati.

drogenated to the same iodine value determined for the hydrogenated fat used in groups 7 and 8. When hydrogenated corn oil was used as the diet fat in a threonine-deficient diet (group 9), liver fat levels were again significantly decreased below those in the control group (1). Since a slight difference in food consumption was observed between these 2 groups (table 1), the experiment was repeated using the paired-feeding technique. Liver fat levels in rats pair-fed a diet containing hydrogenated corn oil (9P) were still significantly lower than those in control animals (corn oil).

A more detailed study comparing groups 1 and 9 was initiated. Ten animals from each group were killed at 2 weeks and again at 4 weeks, because liver fat levels in threonine-deficient rats have been shown to vary with time (4). Results are presented in table 3. Replacing corn oil (group 1) with hydrogenated corn oil (group 9) in threonine-deficient diets significantly reduced liver fat levels after 2 weeks; this effect persisted for 4 weeks. The decreased fat concentrations in livers from rats in group 9 were accompanied by an increased concentration of nitrogen and

TABLE 3

Liver composition and serum proteins of rats fed threonine-deficient diets containing either corn oil or hydrogenated corn oil [1] for 2 or 4 weeks

Diet [2]	Group 1 (corn oil)		Group 9 (hydrogenated corn oil) [1]	
	2 Weeks	4 Weeks	2 Weeks	4 Weeks
Liver composition, %				
Moisture	69.9 ± 0.4 [3]	69.2 ± 0.4	72.2 ± 0.2	71.8 ± 0.3
Nitrogen	2.51 ± 0.02	2.63 ± 0.04	2.53 ± 0.03	2.80 ± 0.03
Fat	22.8 ± 0.8	20.2 ± 1.0	13.7 ± 0.4	12.3 ± 0.6
Serum proteins, %				
α_1-Globulin	13.2 ± 0.5	12.0 ± 0.8	9.9 ± 0.3	11.2 ± 0.8
α_2-Globulin	10.8 ± 0.7	8.5 ± 0.5	8.8 ± 0.6	8.3 ± 0.4
β-Globulin	15.0 ± 0.5	13.8 ± 0.4	12.7 ± 0.5	13.1 ± 0.5
γ-Globulin	4.3 ± 0.9	13.7 ± 1.9	3.7 ± 0.9	12.4 ± 1.6
Albumin	56.8 ± 2.0	52.0 ± 1.9	65.0 ± 1.7	55.2 ± 2.3

[1] Corn oil was hydrogenated to an iodine value of 74 by the Procter and Gamble Company, Cincinnati.
[2] Fat content of both diets = 30% w/w.
[3] SE of mean.

moisture after 4 weeks as compared with rats in group 1.

Significant changes with time were also observed in the serum protein patterns; the most striking change was observed in the γ-globulin fraction. This fraction increased approximately threefold between the second and the fourth weeks of the experiment. The magnitude of the increase was of the same order in both groups of rats, and was not related to diet. The increase in γ-globulin concentration was not observed in subsequent experiments and probably reflected a subclinical infection in both groups.

DISCUSSION

The data reported here support the contention that the severity of fatty livers associated with a threonine deficiency varies to a considerable extent with the chemical composition of the fat source in isocaloric diets. Of the various fats incorporated in threonine-deficient diets, olive oil produced the most severe fatty livers (25.6%) followed closely by corn oil (22.5%). Fat accumulation in livers from rats fed deficient diets containing cottonseed oil or hydrogenated vegetable oil was comparable (17.9% and 17.6%, respectively), and rats fed hydrogenated corn oil had the least severe fatty livers (13.7%).

Since all diets were isocaloric and since no significant differences in food intake or weight gain were observed among any of the these threonine-deficient groups except group 9, the "protective" action of some diet fats is apparently not mediated through a more equitable balance between the amino acid and calorie ratio. Food intake of rats in group 9 (hydrogenated corn oil) was significantly greater than that of the control group. However, when rats in this group were pair-fed with the control animals, liver fat levels were still significantly depressed below those in rats fed the basal diet. This observation substantiates the suggestion that the calorie intake is not of major importance in regulating the degree of fat accumulation in threonine-deficient animals.

Of the fats tested, hydrogenated fats tended to be more protective against fatty livers associated with threonine deficiency. Feeding threonine-deficient rats corn oil hydrogenated to the same iodine number as the commercial hydrogenated fat resulted in a significantly lower concentration of liver lipids as compared with those in rats fed the unhydrogenated corn oil. However, the oils did not offer equal protection to threonine-deficient animals; cottonseed oil was far more effective than was olive oil, even though the iodine value of olive oil is lower than that of cottonseed oil (12). Thus the degree of hydrogenation did not solely determine the effectiveness of the fat in limiting the development of fatty livers in threonine-deficient animals.

The data reported here do not support the suggestion made by Benton et al. (8)

and by Channon et al. (13) that the chain length of the constituent fatty acids determines the severity of fatty livers associated with threonine deficiency, since the hydrogenation of corn oil would not alter the length of the fatty acid chains. However, the hydrogenation process may have resulted in the appearance of isomeric forms which may actively reduce liver fats in threonine-deficient animals.

These data suggest that both the fatty acid composition and the degree of hydrogenation of the dietary fat serve to influence the degree of fat accumulation in threonine-deficient rats. The combination of these 2 factors apparently determines the metabolic disposition of a given fat in the liver. The presence of various isomers in the triglyceride may further delineate the metabolic route to be taken. Thus, an intricate interrelationship must exist between threonine and the metabolic path taken by dietary fat. Studies on this problem are in progress.

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Zinc-65 Absorption and Turnover in Rats

I. A PROCEDURE TO DETERMINE ZINC-65 ABSORPTION AND THE ANTAGONISTIC EFFECT OF CALCIUM IN A PRACTICAL DIET [1,2]

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ABSTRACT Zinc-65 as chloride or glycine complex was administered to growing rats receiving a practical diet in 6 experiments. A rapid fecal excretion of Zn^{65} occurred following administration via feed or gavage. Increased dietary calcium significantly increased ($P < 0.01$) the initial rate of Zn^{65} loss following oral administration and consistently and significantly decreased the percentage remaining in the body longer than 2 days. Conversely, body loss of injected Zn^{65} decreased significantly ($P < 0.01$) with increased calcium. Comparison of retention curves at 100 to 250 hours post-administration for injected and dietary or gavage-administered Zn^{65} and their extrapolation to zero time allowed the determination of the percentage of radiozinc absorbed. Calcium significantly decreased ($P < 0.01$) the percentage of Zn^{65} absorption and increased the biological half-life (decreased turnover) of Zn^{65} beyond 100 hours post-administration. Calcium did not affect body weight. The results are explained by a decreased absorption of stable and radiozinc with increased dietary calcium. Calcium also significantly increased ($P < 0.01$) carcass Zn^{65} in the femur and decreased it in liver, kidney and muscle at 28 days post-administration.

Many investigators have demonstrated that rations high in calcium cause a marked potentiation of the zinc-deficiency syndrome in swine, poultry and dogs (1–10). The site(s) of the calcium antagonism has not been elucidated. In swine, high calcium diets were shown to decrease the zinc content of certain organs such as the liver, but not of others such as skin, pancreas or intestine (4). This led to the postulation that calcium interfered with the absorption of zinc from the intestine. Substantiating this idea were experiments conducted in vitro which demonstrated that zinc was readily removed from solution during the precipitation of calcium phosphate compounds such as might occur in the intestinal lumen (11). Phytic acid complexes have also been shown to decrease the availability of dietary zinc (12).

Attempts to demonstrate directly a calcium antagonism of zinc absorption have been largely unsuccessful. Newland et al. (13) suggested that calcium increased endogenous zinc metabolism in swine. In balance studies with rats receiving semipurified diets, Forbes and Yohe (14) concluded that increased calcium did not reduce zinc absorption. Also, Roberts and Hoekstra,[2] working with rats fed purified diets, did not observe, at 3 days post-administration, a reduced body retention of Zn^{65} administered by gavage. In an overall review of the problem, Forbes (15) speculated that the calcium antagonism occurred at the cellular level.

In view of the confusion pertaining to the site of the antagonistic action of calcium on zinc, it was decided to reinvestigate the problem more directly with rats by using Zn^{65} and a whole-animal scintillation counter. A preliminary report of this research has been published.[3] The results of these experiments, as well as results of somewhat different experiments reported subsequently by others (12, 16) indicate that under appropriate dietary conditions,

Received for publication October 21, 1964.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station, Madison. Supported in part by Public Health Service Research Grant no. AM-05606 from the National Institute of Arthritis and Metabolic Diseases.

[2] Roberts, H. F., and W. G. Hoekstra, unpublished data.

[3] Preliminary reports of this research were presented at the 1963 annual meeting of American Society of Animal Science at Corvallis, Oregon, and some of the data was included in a review (1) presented at the 1964 annual meeting of the Federation of American Societies for Experimental Biology at Chicago, Illinois.

calcium does decrease zinc absorption or availability.

METHODS AND MATERIALS

Six experiments were conducted, using male albino rats of the Holtzman strain. There were 4 rats per dietary treatment group except in experiment 6 which had 8 in the groups receiving Zn^{65} in the feed or by gavage and 4 in the groups injected with Zn^{65}. The rats were obtained at 3 weeks of age and placed in individual galvanized cages with raised wire floors. They were weighed initially and at approximately weekly intervals. Distilled water was supplied ad libitum in glass water bottles.

The composition of the basal diets is given in table 1. Additional calcium at the 0.00, 0.60 or 1.76% level (also 0.30 and 0.90% in experiment 1) was provided as calcium carbonate. In each experiment a sample of the basal diet was analyzed for total calcium, phosphorus and zinc. The calcium was found to vary from 0.22 to 0.29% in air-dried samples of ration. The phosphorus content was from 0.96 to 1.01% in the first 3 experiments and 0.83 to 0.86% in experiments 4 through 6. Total zinc was determined by atomic absorption spectrophotometry and varied from 28 to 33 ppm except in experiment 1 where it was 51 ppm. Analyses of several currently available calcium carbonate sources showed none to contain over 28 ppm zinc. Of those analyzed and used in the experiments reported, none contained over 24 ppm zinc and experiments 5 and 6 utilized a source which assayed less than 5 ppm zinc.

TABLE 1

Composition of basal diet [1]

	g/kg
Ground corn	587.37
Soybean oil meal (44% protein)	287.00
Dried brewer's yeast	30.00
Alfalfa meal (17% protein)	50.00
Vitamin B_{12} (0.1% mix)	0.01
Irradiated yeast	0.12
Sodium chloride	12.50
Potassium phosphate (dibasic)	33.00 [2]
	1000.00

[1] Calcium-supplemented diets were obtained by adding calcium carbonate at the expense of the entire diet in experiments 1 through 5 and at the expense of ground corn in experiment 6.

[2] Changed to 25.00 g/kg in experiments 4, 5 and 6 with compensating increase in ground corn.

A ration of the type used, but with somewhat less soybean oil meal is often used in commercial swine feeding. Its adequacy for rats was indicated by the satisfactory growth obtained in all experiments.

A pre-experimental period varying from 13 to 21 days was allowed in all experiments to accustom the animals to their rations. During this and the experimental period, the respective rations were provided ad libitum except for a mild diet restriction just prior to administration of Zn^{65} via feed. The exact amount and duration of this restriction varied from experiment to experiment but feed intake was limited to about one-half the amount normally consumed during a 6- to 13-hour period immediately prior to administering the isotope. This restriction served to stimulate the appetite, thus insuring adequate isotope ingestion. Animals to receive the isotope via gavage or intramuscular injection were not subjected to any diet restriction.

In experiment 1, high specific activity Zn^{65} [4] was administered as its chloride in dilute HCl solution. Prior to administration in the other experiments the Zn^{65} chloride was complexed by adding glycine at a 4:1 (glycine:zinc) molar ratio and adjusting the pH to 7.4 with sodium hydroxide. Radioactive feed was prepared by adding an aliquot of the Zn^{65} solution to a sample of each ration. The samples were dried and thoroughly mixed, and each animal was offered a similar amount of radioactive feed varying in different experiments from 2.5 to 7.5 g. In the experiments where the isotope was administered by gavage, the animals were lightly anesthetized to facilitate insertion of the stomach tube. Animals injected with Zn^{65}-glycine intramuscularly in the rear leg received no special pre-administration treatment. Each animal received from 100,000 to 300,000 counts per minute (about 0.5 to 1.5 μc of Zn^{65}) irrespective of administration method. The exact amount was determined at the time of the initial measure of animal radioactivity.

[4] The radiozinc was obtained as zinc-65 chloride in HCl from Oak Ridge National Laboratory, Oak Ridge, Tennessee.

After allowing the animals to eat their radioactive feed for a 4- to 5- hour period, they were counted in a whole-animal gamma-scintillation detector [5] to determine the administered dose. Thereafter all rats received their normal rations for the duration of the experiment which varied from 250 to 1100 hours. During this period the animals were counted at intervals ranging from 12 hours to several days. A Zn^{65} standard was measured regularly and all values were corrected for background, isotope decay and any minor changes in counter efficiency. A 4- to 5-hour delay between administration and first counting was also allowed when the isotope was given via gavage or intramuscular injection. It was shown separately that essentially no loss of body radioactivity occurred during this time.

RESULTS AND DISCUSSION

Growth was not influenced during the duration of the experimental periods by the addition of calcium (table 2). However, level of calcium in the ration had a significant effect on the retention of ingested Zn^{65} (fig. 1). This figure illustrates the results from experiment 3, which are similar to those obtained in all other experiments. For each increase of calcium in the diet, the percentage of the ingested oral Zn^{65} which was retained decreased at

[5] Armac scintillation detector model 440 manufactured by Packard Instrument Company, Inc., La-Grange, Illinois.

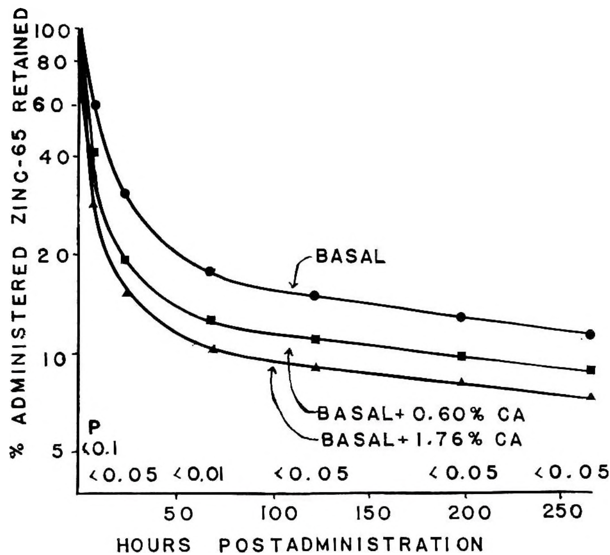

Fig. 1 Effect of dietary calcium on retention of Zn^{65} administered in the feed (exp. 3).

TABLE 2
Effect of dietary calcium on body weight

Exp.	Days fed experimental diet	Mean [1] final [2] body weight			Significance due to calcium
		Basal	+ 0.60% Ca	+ 1.76% Ca	
		g	g	g	
1 [3]	50	258	280	276	$P > 0.1$
2	24	149	160	150	$P > 0.1$
3	35	238	244	233	$P > 0.1$
4	34	220	219	231	$P > 0.1$
5	46	305	311	314	$P > 0.1$
6	49	301	299	296	$P > 0.1$

[1] Treatment means (dietary, injected and gavage Zn^{65} treatments combined where applicable).
[2] Initial body weights within an experiment were similar between treatments.
[3] Intermediate calcium levels omitted, but omitted values correspond to those listed.

each post-administration measurement of radioactivity. Early in the experiment (zero to about 50 hours post-administration) there was a rapid loss of body Zn^{65}. This represents primarily the passage of isotope through the intestine and its subsequent loss in the feces. This early loss of Zn^{65} was increased by increasing dietary calcium. After 50 hours, the loss of Zn^{65} was at a much slower rate representing excretion of Zn^{65} which had been absorbed from the intestine into the body proper. The later rate of loss was also influenced by calcium, but in the opposite direction from the effect on early Zn^{65} loss, as evidenced by converging of the retention curves with increasing time (the animals were counted for a longer post-administration time than illustrated in fig. 1 which, if plotted, would show converging). It appeared, therefore, that increased dietary calcium both increased the early loss of Zn^{65} by decreasing Zn^{65} absorption and decreased the rate of turnover of body Zn^{65}.

This effect of calcium on zinc turnover was substantiated when Zn^{65} was injected rather than fed. Increased calcium very significantly increased the retention of the injected Zn^{65} at all time periods (fig. 2). This is explained on the basis that increased calcium also reduced the absorption of stable dietary zinc thereby causing the Zn^{65} which was in the body to be replaced at a slower rate. This effect occurred regardless of whether the Zn^{65} had entered the body via injection or by absorption from the intestine.

TABLE 3

Effect of dietary calcium on fecal excretion of injected Zn^{65} (exp. 2)

Treatment	Body Zn^{65} lost/g dry feces [1]
	%
Basal	1.51
Basal + 0.60% Ca	1.11
Basal + 1.76% Ca	0.84
Significance due to Ca	$P < 0.01$

[1] Feces collected for 12 hours starting at 157 hours post-administration. Growth and feed intakes were similar between treatments.

The effect of calcium on the loss of body Zn^{65} was reflected by altered fecal excretion of Zn^{65} (table 3). This was expected because numerous workers have demonstrated that body zinc excretion via the urine is extremely small compared with fecal excretion, and any effect on urinary excretion could not greatly affect total body excretion of zinc.

When Zn^{65} retention was plotted on a logarithmic scale against time on a linear scale (fig. 1), the retention curves were essentially linear in all experiments between 100 and 250 hours post-administration. Furthermore, the slopes of these lines were the same whether Zn^{65} was injected or administered orally. It was thought, therefore, that by comparing the retention curves for the 2 methods of administration a procedure might be developed for calculating the actual percentage absorption of

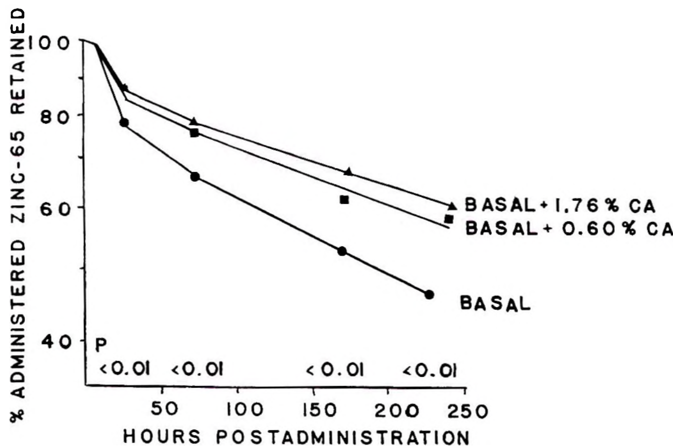

Fig. 2 Effect of dietary calcium on retention of intramuscularly injected Zn^{65} (exp. 2).

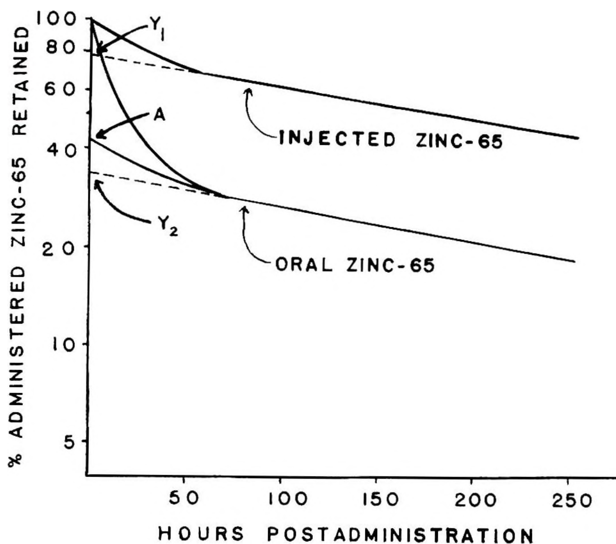

Fig. 3 A comparison of retention curves for Zn^{65} administered in the feed and by intramuscular injection (exp. 2). Percentage absorption (A) of oral Zn^{65} is calculated by dividing Y_2 (the y intercept of the extrapolated retention curve for oral Zn^{65}) by Y_1 (the y intercept of the extrapolated retention curve for injected Zn^{65}) and multiplying by 100.

orally administered isotope. Figure 3 shows the separate retention curves for Zn^{65} when it was administered orally and injected. At a single level of calcium, both methods of administration produced linear retention curves 100 to 250 hours postadministration whose slopes did not differ significantly in any of several experiments.

The procedure and rationale for calculating percentage Zn^{65} absorption (fig. 3) was as follows. 1) The retention curve of the injected isotope showed an initial rapid decrease but followed a simple exponential curve during the period 100 to 250 hours. Extrapolation of this linear portion of the injected isotope curve to zero time gave 79% of the injected dose. 2) The retention curve for orally administered isotope was similar except for the much greater early loss of body radioactivity (prior to 100 hours). Extrapolation of the linear portion of the oral isotope retention curve (100 to 250 hours) to zero time gave a value of 34% of the dose. 3) Therefore, comparison of the retention curves for oral versus injected isotope indicated that the y intercept value of 34% of the dose for the oral dose represented 79% of the isotope actually entering the body (the y intercept for the injected dose). 4) Division of 34% by 79% and multiplication by 100 gave 43% which represents that portion of the oral Zn^{65} which was absorbed from the intestine (i.e., that portion which was handled in the body similarly to an injected dose). Since the y intercept values for injected Zn^{65} were influenced somewhat by calcium, it was desirable to use similar calcium levels for oral and injected Zn^{65} in determining the percentage of Zn^{65} absorbed. Cotzias et al. (17) proposed a similar method to determine the absorption of Zn^{65} in mice. However, their method did not utilize any injected animals and individual variation was found to be excessive with 6 mice. This was not the case with rats fed a practical diet in the present study.

Curves as described above were developed for each animal with every level of calcium in each of the 6 experiments. Experiments 1, 3 and 4 included only oral Zn^{65} treatments. Since the above method was developed after experiment 4 was completed, data from the injected rats of experiment 2 were used to determine the percentage absorption in the other 3 experiments. Experiments 5 and 6 were conducted to verify the procedure used to determine the percentage Zn^{65} absorption.

The Zn^{65} absorption data from the 6 experiments calculated as described are presented in table 4. Increased dietary calcium significantly reduced the percentage absorption of Zn^{65} in all experiments except for isotope administered via feed in experiment 5 in which the trend was the same, but the differences were not statistically significant. Variations in the percentage zinc absorption in the different experiments were noted. When 100 ppm of stable zinc were added to the diet, the percentage absorption of oral Zn^{65} was markedly reduced, but the effect of calcium was still significant ($P < 0.05$). This observation is not in agreement with Berry et al. (18) who reported that the addition of calcium to a swine ration containing 100 ppm supplemental zinc increased the retention of oral Zn^{65}. Calcium significantly reduced Zn^{65} absorption whether it was administered in the feed or by gavage; however, there was a greater percentage absorption of Zn^{65} administered by gavage. The magnitude of the effect of calcium was greater between the low and middle calcium levels than between middle and high levels regardless of administration method.

The retention curves for Zn^{65} through 250 hours post-admistration could be resolved into 2 linear components as described by Comar (19) and the biological half-lives were calculated. Also, experiments extending beyond 250 hours showed additional logarithmic components of zinc turnover representing zinc compartments in the body which had slow turnover rates.

Table 5 shows the biological half-lives of the 3 different components in experiment 6. There was a significant decrease in the half-life of the earliest component (zero to 60 hours) due to calcium. (These values were obtained after correcting for the sec-

TABLE 4

Effect of calcium on absorption of Zn^{65}

Exp.	Method of Zn^{65} administration	Administered Zn^{65} absorbed			Significance due to calcium
		Basal	+ 0.60% Ca	+ 1.76% Ca	
		%	%	%	
1 [1]	Diet	28.2 A [2]	20.6 B [2]	16.1 B [2]	$P < 0.01$
2	Diet	42.8 A	20.6 B	15.7 C	$P < 0.01$
3	Diet	24.1 A	15.4 B	12.6 B	$P < 0.01$
3 [3]	Diet	8.7 A	7.8 AB	6.3 B	$P < 0.05$
4	Diet	28.9 A	19.2 B	13.3 C	$P < 0.01$
5	Diet	29.8 A	21.8 A	21.8 A	$P > 0.1$
6	Diet	30.9 A	23.1 B	20.3 B	$P < 0.01$
5	Gavage	36.3 A	30.6 B	27.3 C	$P < 0.01$
6	Gavage	36.7 A	27.2 B	25.6 B	$P < 0.01$

[1] Intermediate calcium levels omitted, but omitted values fall between those listed.
[2] Treatment means. Values not followed by same letter within the same row are significantly different ($P < 0.05$).
[3] Diets supplemented with $ZnCO_3$ to provide 100 ppm added zinc.

TABLE 5

Influence of dietary calcium on zinc turnover (exp. 6)

Period post-administration	Method of Zn^{65} administration	Biological half-life of Zn^{65}			Significance due to calcium
		Basal	+ 0.60% Ca	+ 1.76% Ca	
hours		*hours*	*hours*	*hours*	
0–60	Diet	8.8 A [1]	7.2 B [1]	6.3 B [1]	$P < 0.01$
	Gavage	8.4 A	7.8 A	5.7 B	$P < 0.01$
100–250	Diet	316 A	432 B	492 C	$P < 0.01$
	Intramuscular injection	325 A	434 B	500 B	$P < 0.01$
	Gavage	338 A	428 B	483 C	$P < 0.01$
300–661	Diet	721 A	1042 B	1053 B	$P < 0.01$
	Gavage	713 A	992 B	1129 C	$P < 0.01$

[1] Treatment means. Values not followed by same letter within the same row are significantly different ($P < 0.05$).

ond component.) This occurred whether the Zn^{65} was administered in the feed or by gavage. Graphic analyses of the data from the first 4 experiments substantiated this observation. Calcium significantly increased the half-life or body Zn^{65} during the period 100 to 250 hours post-administration regardless of whether the isotope had been administered orally, by injection or by gavage. When all methods of isotope administration were considered, there was only one case in the 6 experiments in which the half-life of Zn^{65} during this period was not significantly increased by adding 0.60% calcium to the basal diet. In this isolated case (injected isotope in experiment 5) it was necessary to add 1.76% calcium to statistically increase the half-life of body Zn^{65}. In most cases adding 1.76% calcium further increased the Zn^{65} half-life over 0.60% added calcium, but many of these differences were not statistically significant. These results substantiate those illustrated in figure 2. Calcium was also shown to increase the half-life of Zn^{65} which remained in the body longer than 250 hours.

Table 5 also shows that at a single level of calcium there was no difference in the 100 to 250 hour half-life regardless of whether the Zn^{65} was administered in the diet, by intramuscular injection or by gavage. A separate experiment showed that the intramuscularly injected Zn^{65}-glycine moved rapidly away from the site of injection and only about 8% of the administered dose remained at or near the injection site by 24 hours post-administration. This had decreased to 4% by 4 days. These observations are important in justifying the previously described procedure for determining the percentage absorption of oral Zn^{65}.

Dietary calcium not only affected Zn^{65} absorption and turnover, but also altered the distribution of Zn^{65} in the carcass at 28 days post-administration as shown in figure 4. Calcium decreased the Zn^{65} content of the soft tissues as represented by liver, kidney and muscle and increased it in the bones as represented by the femur. This was true regardless of whether Zn^{65} was expressed per organ or per gram of dry organ, although not all the soft tissue differences on a concentration basis were statistically significant.

The mechanism by which calcium acts to reduce zinc absorption is unknown. Calcium may effect zinc-binding in the intestinal contents or some other type of competition between calcium and zinc may exist either in the intestine or elsewhere in the body. The observation that increased calcium decreased the half-life of orally administered Zn^{65} during the period zero to 60 hours post-administration indicates an accelerated fecal loss of intestinal Zn^{65}. An explanation of this observation might be that calcium is simply increasing the rate of passage of zinc through the intes-

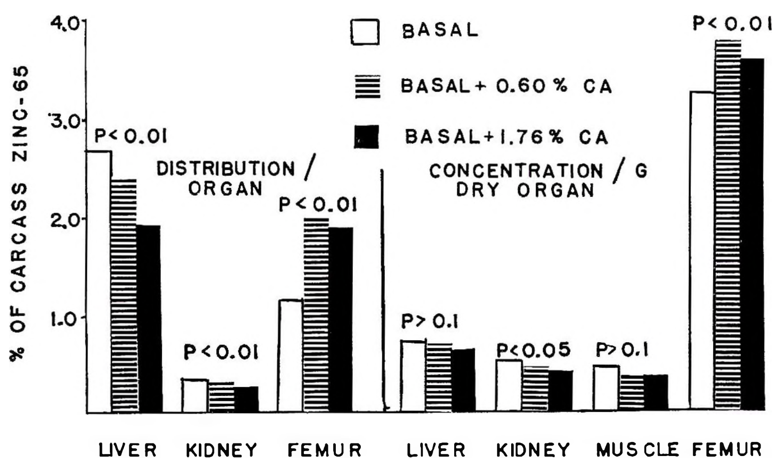

Fig. 4 Effect of dietary calcium on distribution of carcass Zn^{65} at 28 days post-administration (exp. 5).

tine and thus decreasing zinc absorption per se. Another explanation might be that calcium allows normal absorption of zinc, but accelerates the rapid recycling of recently absorbed zinc back into the intestine (possibly via the liver and pancreas) thus decreasing the body retention which would result in a decreased net absorption. Either of these conditions would result in a decreased biological half-life during the early period post-administration. The effect of calcium in decreasing Zn^{65} turnover at extended periods post-administration probably relates to the continued action of calcium on the absorption of stable dietary zinc.

The factor in the corn-soybean diets which interferes with zinc absorption in the presence of calcium has been speculated to be phytic acid (12). However, under our experimental conditions we have not been able to potentiate a zinc deficiency in chicks or decrease the absorption of oral Zn^{65} in rats by the addition of phytic acid to purified diets containing animal proteins or protein hydrolysates. In the present studies phytic acid and vitamin D were constant within each experiment. Our results on the effect of added calcium on Zn^{65} absorption with purified diets have been inconsistent. However, under the experimental conditions employed with the practical diet, increased dietary calcium consistently and significantly reduced absorption of oral Zn^{65} and decreased its turnover in the body.

ACKNOWLEDGMENTS

The authors are indebeted to Donald Willet, Feed and Fertilizer Section, General Laboratory Division, Wisconsin Department of Agriculture, for his analyses of calcium and phosphorus in the rations. The authors are also indebted to Wayne Becker for his assistance in preparing the electronic computor program which was used in the analysis of a portion of the data.

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Vitamin A Activity of Fermentation β-Carotene for Swine[1]

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ABSTRACT Two experiments were conducted with 171 weanling pigs to provide an estimate of the biopotency of fermentation carotenoids produced by *Blakeslea trispora*, and to estimate the vitamin A and β-carotene requirement of the depleted 50-kg pig. Vitamin A depletion for 7 weeks lowered serum vitamin A concentration from 23 to 4 μg/100 ml, and lowered liver vitamin A concentration from 35 to 2 μg/g of dry tissue. Repletion levels of fermentation β-carotene ranged from 0.5 to 3.5 mg/kg of diet and of all-*trans* vitamin A_1 palmitate from 250 to 2000 IU/kg of diet. Using total liver vitamin A after 9 weeks of repletion as the criterion, one mole of fermentation β-carotene had 11.5% of the biopotency of one mole of all-*trans* vitamin A_1 palmitate. The minimal requirements for gains from 50 to 100 kg body weight did not exceed 0.5 mg of fermentation β-carotene or 250 IU of vitamin A palmitate/kg of diet. However, 3.5 mg of fermentation β-carotene or 1000 IU of vitamin A palmitate/kg diet were required to restore serum vitamin A concentration to predepletion levels. Liver vitamin A concentration or total liver vitamin A increased linearly with increasing dietary increments of either fermentation β-carotene or vitamin A palmitate. This relationship was represented by the equation $Y = -0.636 + 0.002X$ for fermentation β-carotene and by $Y = -4.87 + 0.0174X$ for all-*trans* vitamin A_1 palmitate, where Y = milligrams of total liver vitamin A after 9 weeks repletion and X = international units of fermentation β-carotene or of vitamin A palmitate per kilogram of diet.

All-*trans*-β-carotene can be produced readily by a fermentative process involving the mating of opposite types of the heterothallic mold, *Blakeslea trispora* (1). The β-carotene is produced and retained in the mycelium in a concentration approximating 1 to 2% of the dry solids. Because carotene produced by fermentation may be used as a source of vitamin A activity for foods and feedstuffs as well as a coloring agent, the biological activity of the carotenoids produced is of considerable importance.

The following experiments were conducted to provide more exact estimates of the biopotency of fermentation carotenoids, and to estimate the vitamin A and β-carotene requirement of the depleted 50-kg pig.

MATERIALS AND METHODS

The sources of vitamin A activity used in this study were as follows.

Vitamin A palmitate. This material was obtained commercially as a vitamin A feed supplement.[4] It contained 250,000 IU all-*trans* vitamin A_1 palmitate/g plus edible tallow, gelatin, glucose, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and soybean feed. Particle surfaces were treated with sodium silico aluminate.

Synthetic β-carotene. This material, obtained from a commercial source, contained 2.4% total β-carotene.[5] It was a dispersion of β-carotene and vegetable oil in a matrix of gelatin and carbohydrate, with high stability and designed primarily for food coloring.

Fermentation β-carotene. This material was produced by the U.S.D.A. Northern Regional Research Laboratory and sup-

Received for publication November 2, 1964.

[1] Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal article no. 3493. This research was conducted under contract no. 12-14-100-6850(71) with the Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Peoria, Illinois.

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[4] PGB-250 Dry Vitamin A Feed Supplement, Distillation Products Industries, Rochester, New York.

[5] Roche Dry Beta-Carotene Beadlets, Type 2.4-S, Water Dispersible, Hoffmann-LaRoche, Inc., Nutley, New Jersey.

plied as dry, fermentation solids (milled to pass a 20-mesh screen) consisting largely of mycelia containing the β-carotenes. The solids from 2 fermentations were used in these experiments. Analyses, using the methods described by Bickoff et al. (2, 3) and Bickoff (4) for separation and determination of carotene isomers and stereoisomers indicated that they contained approximately 94 to 96% all-*trans*-β-carotene and 4 to 6% neo-B-β-carotene.

Feed premixes and feeds. All products were diluted (on the basis of total vitamin A or total β-carotene content) to the appropriate premix concentration with soybean meal (milled to pass a 20-mesh screen) containing 2% of a 2:3 mixture of BHA and BHT. These premixes were analyzed and then stored in the dark under helium at − 40° until added to the experimental diets as indicated below. Vitamin A analyses were performed according to AOAC procedures (5), except that a hot water extraction was made to liquefy the gelatin. No chromatographic separations were performed.

β-Carotene analyses were performed by adapting AOAC procedures (5). Samples of premix (0.2 g) and feed (40 g) were placed directly on Celite: Sea Sorb 43 (1:1) or Hyflo Super Cel: Sea Sorb 43 (1:1) columns (10 × 1.8 cm), followed by elution with 10% acetone in pentane-hexane. The eluate was appropriately diluted and the optical density compared with an all-*trans*-β-carotene standard[6] in a Beckman B spectrophotometer.

Experiment 1. This experiment was designed to establish the usefulness of the depletion diet and to permit regular examination of serum and liver vitamin A concentration as depletion and repletion were effected.

Seventy-two weanling, Hampshire and crossbred pigs were assigned to the study. Blood samples were obtained from the anterior vena cava of all pigs for serum vitamin A assay, and 6 pigs were killed immediately to establish initial liver vitamin A concentrations. The remainder were grouped in 6 lots on the basis of weight, sex and litter relationship and placed on the basal depletion diet shown in table 1.

Additional blood samples for serum vitamin A assay were obtained from 6 pigs in each lot after 3 and 5 weeks of depletion and from all pigs after 7 weeks of depletion. Six pigs (one pig from each lot) were killed for liver vitamin A assay during the depletion period, two of these after 3 weeks and four after 7 weeks of depletion.

The remaining 60 pigs were assigned to 6 treatments. The diets were prepared by adding appropriate amounts of the vitamin A and β-carotene supplements and the soybean meal-antioxidant diluent to the basal depletion diet. The intended and assay concentrations of these experimental diets are shown in table 2.

Two batches of feed were mixed for each lot during the treatment period, and samples for assay were taken at the time of mixing and when the last of the batch

TABLE 1

Composition of basal diet

	%
Ground white milo	78.3
Soybean meal, 50% crude protein	19.0
Dicalcium phosphate	0.7
Limestone	1.1
Trace mineral salt [1]	0.5
B-vitamin supplement [2]	0.1
B_{12} supplement (13 mg/kg)	0.15
Vitamin D_2 supplement (9000 IU/kg)	0.005
Zinc oxide	0.01
Antibiotic supplement [3]	0.1

[1] Contained not less than: (in per cent) NaCl, 94.5; Zn, 0.80; Mn, 0.52; Fe, 0.27; Cu, 0.05; Co, 0.01; and I, 0.01 (Morton Salt Company, Chicago).
[2] Four milligrams riboflavin, 9 mg pantothenic acid, 20 mg nicotinic acid, and 200 mg choline chloride/kg.
[3] Eleven grams procaine penicillin, and 33 g streptomycin sulfate/kg.

TABLE 2

Intended and assay concentrations of 6 repletion diets (exp. 1)

Lot	Treatment	Concentration	
		Intended	Assay
1	Basal	0	0
2	Fermentation β-carotene, mg/kg	0.9	0.9
3	Fermentation β-carotene, mg/kg	1.8	1.8
4	Vitamin A palmitate, IU/kg	590	590
5	Vitamin A palmitate, IU/kg	1180	1140
6	Synthetic β-carotene, mg/kg	1.8	1.9

[6] Supplied by Hoffmann-LaRoche, Inc., Nutley, New Jersey.

was fed. The feed was mixed in a darkened room and stored in cloth sacks in a darkened area. The mean environmental temperature where the feed was stored and fed in experiment 1 was – 2°.

Blood serum samples for vitamin A analysis were collected from 5 pigs in each lot after 2 and 4 weeks on treatment. At 4 weeks, 2 additional pigs in each lot were bled and then killed for liver vitamin A assay. Similarly, at 5, 6, 8 and 9 weeks on treatment, 2 pigs in each lot were bled and killed.

Experiment 2. This experiment was designed to provide a basis for estimating the relative biopotency of fermentation β-carotene as compared with preformed vitamin A, and to permit estimation of β-carotene and vitamin A requirements for maximal weight gains and restoration of predepletion serum vitamin A levels.

Ninety-nine weanling, Hampshire and crossbred pigs were assigned to 9 lots on the basis of weight, sex and litter relationship. They were fed the depletion diet shown in table 1 for a period of 8 weeks. Blood samples were obtained from the anterior vena cava of 5 pigs in each lot initially and at 2, 4, 6 and 8 weeks of depletion.

The pigs were then assigned to 9 treatments. The diets were prepared as described for experiment 1. The intended and assay concentrations of these experimental diets are shown in table 3.

Five batches of feed were mixed for each lot during the treatment period, and samples for assay were taken at the time of mixing and when the last of the batch was fed. The feed was mixed in a darkened room and stored in cloth sacks in a darkened area. The mean environmental temperature where the feed was stored and fed in experiment 2 was – 1°.

Blood serum for vitamin A analysis was obtained from 5 pigs in each lot after 2, 4 and 6 weeks of treatment and from all pigs after 8 weeks of treatment. All pigs were killed after 9 weeks on treatment and liver samples were taken for vitamin A analysis.

Analytical procedures. Serum vitamin A concentrations were determined using the antimony trichloride method according to Embree (6). The section of liver removed for assay was taken from the lower one-half of the left central lobe. Liver vitamin A concentrations were determined according to the method of Gallup and Hoefer (7) using a Bausch and Lomb Spectronic 20 spectrophotometer rather than an Evelyn colorimeter.

Statistical analysis. Mean differences were compared using Duncan's multiple range test (8).

TABLE 3

Intended and assay concentrations of 9 repletion diets (exp. 2)

Lot	Treatment	Concentration	
		Intended	Assay
1	Basal	0	0
2	Fermentation β-carotene, mg/kg	0.5	0.5
3	Fermentation β-carotene, mg/kg	1.0	0.9
4	Fermentation β-carotene, mg/kg	2.0	2.0
5	Fermentation β-carotene, mg/kg	4.0	3.5
6	Vitamin A palmitate, IU/kg	250	250 [1]
7	Vitamin A palmitate, IU/kg	500	500 [1]
8	Vitamin A palmitate, IU/kg	1000	1000 [1]
9	Vitamin A palmitate, IU/kg	2000	2000 [1]

[1] Calculated on the basis of the supplement premix assay.

RESULTS AND DISCUSSION

Experiment 1. The effects of depletion and repletion upon weight gains and feed use are shown in table 4. All treatments produced significantly greater weight gains during repletion than were evident in the basal lot. There were no significant differences between treatments, however, and it appeared that 0.9 mg of fermentation β-carotene, 590 IU of vitamin A palmitate or 1.9 mg of synthetic β-carotene/kg of diet were all adequate to support normal weight gains from 48 to 99 kg body weight.

The effects of depletion and repletion upon serum vitamin A concentration are shown in table 5. Serum vitamin A concentration decreased 30% during the first 3 weeks of depletion. Subsequent 2-week

TABLE 4

Weight gain and feed use of pigs fed a vitamin A free diet for a 7-week depletion period and then repleted with diets containing fermentation β-carotene, vitamin A palmitate or synthetic β-carotene (exp. 1)

	Depletion, 7 weeks
Initial body weight, kg	14.1(72) [1]
Average daily gain, kg	0.7(60)
Feed/gain	2.7(60)

Repletion, 9 weeks

Lot	1	2	3	4	5	6
Diet	Basal	Basal + fermentation β-carotene		Basal + vitamin A palmitate		Basal + synthetic β-carotene
(Assay conc)	(0)	(0.9 mg/kg)	(1.8 mg/kg)	(590 IU/kg)	(1140 IU/kg)	(1.9 mg/kg)
	Repletion, 9 weeks					
Initial body weight, kg	52.9(10) [1]	50.7(10)	52.8(10)	51.5(10)	52.9(10)	51.8(10)
Average daily gain, kg	0.6	0.7[a]	0.8[a]	0.8[a]	0.8[a]	0.8[a]
Feed/gain	4.3	3.8	3.9	3.7	3.8	3.8

[1] Numbers in parentheses indicate number of pigs.
[a] Significantly greater than least value ($P < 0.05$).

TABLE 5

Serum vitamin A concentration of pigs fed a vitamin A-free diet for a 7-week depletion period and then repleted with diets containing fermentation β-carotene, vitamin A palmitate or synthetic β-carotene (exp. 1)

	Depletion, 7 weeks, serum vitamin A conc, μg/100 ml
Initial value (72) [1]	23
3 weeks (36)	16
5 weeks (30)	8
7 weeks (60)	4

Repletion, 9 weeks, serum vitamin A conc

Lot	1	2	3	4	5	6
Diet	Basal	Basal + fermentation β-carotene		Basal + vitamin A palmitate		Basal + synthetic β-carotene
(Assay conc)	(0)	(0.9 mg/kg)	(1.8 mg/kg)	(590 IU/kg)	(1140 IU/kg)	(1.9 mg/kg)
	μg/100 ml	*μg/100 ml*	*μg/100 ml*	*μg/100 ml*	*μg/100 ml*	*μg/100 ml*
2 weeks (5) [1]	2	8[a]	12[a]	16[b]	23[e]	12[a]
4 weeks (7)	3	11[a]	19[b]	16[b]	19[b]	17[b]
5 weeks (2)	3	12[a]	17[a]	14[a]	22[b]	18[a]
6 weeks (2)	2	12	18[a]	14	14	12
8 weeks (2)	2	10[a]	18[b]	13[a]	17[b]	12[a]
9 weeks (2)	2	12[a]	13[a]	11	22[b]	10

[1] Numbers in parentheses indicate number of pigs per value.
[a] Significantly greater than least value at this stage of repletion ($P < 0.05$).
[b] Significantly greater than two least values at this stage of repletion ($P < 0.05$).
[e] Significantly greater than five least values at this stage of repletion ($P < 0.05$).

values were each 50% of the immediately preceding value until a low of 2 μg/100 ml was reached. All treatments resulted in significant increases in serum vitamin A levels within 2 weeks to levels which did not increase further during the 9 weeks of repletion. Only in lot 5, where 1140 IU of vitamin A palmitate/kg of diet were provided, did serum vitamin A concentration reach predepletion levels. In some instances, but not in all, serum vitamin A concentration was significantly higher in those pigs receiving 1.8 mg of fermentation β-carotene, 1.9 mg synthetic β-carotene, 590 IU or 1140 IU of vitamin A palmitate/kg of diet than in those receiving 0.9 mg of fermentation β-carotene /kg of diet.

The effects of depletion and repletion upon liver vitamin A are shown in table 6. A pronounced depletion of liver vitamin A was apparent by 3 weeks, and by 7 weeks assay values were extremely low. The initial liver vitamin A depot was decreased by 93 μg/day during depletion. This is about one-half the daily decrease (180 μg) noted in unsupplemented weanling pigs by Hjarde et al. (9). All treatments appeared to produce an increase in liver vitamin A although the differences observed were not always statistically significant. The 2 higher dietary carotene levels (1.8 mg fermentation β-carotene and 1.9 mg synthetic β-carotene/kg of diet) were approximately equal in their effects on liver vitamin A, and the lower level of dietary vitamin A (590 IU/kg of diet) tended to support greater liver vitamin A storage than the lower level of dietary carotene (0.9 mg fermentation β-carotene/kg of diet). The higher dietary vitamin A level (1140 IU/kg of diet) tended to support the greatest liver vitamin A storage of all.

Experiment 2. The effects of depletion and treatment upon weight gains and feed use are shown in table 7. Daily gains during depletion were normal. All lots of pigs (except lot 7) receiving supplemental levels of β-carotene or vitamin A gained significantly faster than the basal lot. There were no significant differences between treatments except that lot 8 pigs gained faster than pigs in lot 7. Two pigs in lot 7 gained rather poorly during repletion but appeared healthy and no lesions were noted at slaughter.

Since maximal weight gains were supported by either 0.5 mg fermentation β-carotene (833 IU) or 250 IU of vitamin A palmitate/kg of diet, it appears that the minimal requirements for gains in swine from approximately 50 to 100 kg body weight do not exceed these values. Both values are slightly below the requirement estimates of Braude et al. (10) who reported that 40 μg of β-carotene or 7.7 μg of vitamin A/kg of body weight daily were adequate for good growth but allowed little liver storage. These values are approximately equivalent to 0.7 mg of β-carotene and 380 IU of vitamin A/kg of diet. Hentges et al. (11) suggested that 25 μg of purified carotene/kg of body weight was the minimal daily requirement for depleted 8-week-old pigs to restore initial blood plasma vitamin A levels and to provide some vitamin A liver storage. Ten micrograms per kilogram of body weight per day supported good weight gains. If the assumptions of Lucas and Lodge (12) are correct relative to food consumption, these levels are equivalent to 0.4 and 0.16 mg/kg of diet.

Using depleted baby pigs from 1 to 8 weeks old, Frape et al. (13) observed normal weight gains when the diet contained no more than 220 IU of vitamin A palmitate/kg. These workers concluded that a better estimate of requirement was 1760 IU of vitamin A palmitate/kg of diet based on cerebrospinal fluid pressure and plasma and liver vitamin A concentration. A subsequent report by Frape et al. (14) indicated that maximal weight gains to 13 weeks of age were supported by 220 to 880 IU of vitamin A palmitate/kg of diet.

Nelson et al. (15) observed that, for the pig to 14 weeks of age, 17.6 to 35.2 μg of vitamin A/kg of body weight daily produced normal plasma vitamin A concentrations, some liver storage and low cerebrospinal fluid pressure. Based upon the weight-feed intake relationship in their study, this was approximately equivalent to 1350 to 2700 IU/kg of diet. Maximal gains were produced with the lowest supplemental level, approximately 380 IU/kg of diet.

Our data do not permit the estimation of the relative biopotency of fermentation β-carotene for weight gains because no dietary levels of β-carotene or vitamin A palmitate were used which produced weight gains intermediate between the maximal value and the minimal gain obtained with the basal diet. The small difference in gain between those animals receiving no dietary vitamin A activity and those receiving 0.5 mg of fermentation β-carotene or 250 IU of vitamin A palmitate/kg of diet makes it difficult to produce such an estimate. Experience with rats suggests that the growth response to selected levels of vitamin A (16) or all-*trans*-β-carotene (17) closely parallels liver vitamin A storage.

TABLE 6

Liver vitamin A values for pigs fed a vitamin A-free diet for a 7-week depletion period, and then repleted with diets containing fermentation β-carotene, vitamin A palmitate or synthetic β-carotene (exp. 1)

Depletion, 7 weeks, liver vitamin A

	Concentration, dry basis	Total
	μg/g	mg
Initial value (6) [1]	35	5.0
3 weeks (2)	13	2.4
7 weeks (4)	2	0.5

Repletion, 9 weeks, liver vitamin A

	Concentration, dry basis						Total					
Lot	1	2	3	4	5	6	1	2	3	4	5	6
Diet	Basal	Basal + fermentation β-carotene		Basal + vitamin A palmitate		Basal + synthetic β-carotene	Basal	Basal + fermentation β-carotene		Basal + vitamin A palmitate		Basal + synthetic β-carotene
(Assay conc)	(0)	(0.9 mg/kg)	(1.8 mg/kg)	(590 IU/kg)	(1140 IU/kg)	(1.9 mg/kg)	(0)	(0.9 mg/kg)	(1.8 mg/kg)	(590 IU/kg)	(1140 IU/kg)	(1.9 mg/kg)
	μg/g	μg/g	μg/g	μg/g	μg/g	μg/g	mg	mg	mg	mg	mg	mg
4 weeks (2) [1]	< 1	< 1	6	1	14[c]	2	0.1	0.3	2.5	0.4	5.6	0.7
5 weeks (2)	< 1	2	9	13	29[c]	12	0.2	1.2	4.1	5.0	13.5[c]	5.3
6 weeks (2)	< 1	2	5	4	13[a]	7	0.3	0.8	2.2	2.1	5.6[a]	2.7
8 weeks (2)	< 1	2	10[b]	5	20[e,bb]	6	0.1	0.8	4.0[b]	2.1	7.5[e,bb]	2.4
9 weeks (2)	< 1	3	12	8	24[c]	14[a]	0.1	1.3	4.9	3.2	10.2[e,bb]	4.6

[1] Numbers in parentheses indicate number of pigs per value.
[a] Significantly greater than least value at this stage of repletion ($P < 0.05$).
[b] Significantly greater than 2 least values at this stage of repletion ($P < 0.05$); [bb] $P < 0.01$.
[c] Significantly greater than 3 least values at this stage of repletion ($P < 0.05$).
[e] Significantly greater than 5 least values at this stage of repletion ($P < 0.05$).

TABLE 7

Weight gain and feed use of pigs fed a vitamin A-free diet for an 8-week depletion period, and then repleted with diets containing fermentation β-carotene or vitamin A palmitate (exp. 2)

Depletion, 8 weeks									
Lot [1]	1	2	3	4	5	6	7	8	9
Initial body wt, kg	11.5	11.5	11.6	11.6	11.5	11.5	11.6	11.6	11.6
Final body wt, kg	50.0	52.7	50.1	51.4	46.7	50.0	49.3	51.6	51.0
Avg daily gain, kg	0.7	0.7	0.7	0.7	0.6	0.7	0.7	0.7	0.7
Avg daily feed, kg	1.7	1.9	1.6	1.8	1.6	1.5	1.7	1.8	1.8
Feed/gain	2.6	2.7	2.4	2.6	2.6	2.3	2.6	2.6	2.6

Repletion, 9 weeks

Lot [1]	1	2	3	4	5	6	7	8	9
Diet	Basal	Basal + fermentation β-carotene				Basal + vitamin A palmitate			
(Assay conc)	(0)	(0.5 mg/kg)	(0.9 mg/kg)	(2.0 mg/kg)	(3.5 mg/kg)	(250 IU/kg)	500 IU/kg)	1000 IU/kg)	(2000 IU/kg)
Final body wt, kg	88.3	96.4	95.7	95.0	89.2	93.9	94.9	93.0	98.1
Avg daily gain, kg	0.6	0.7[aa]	0.7[aa]	0.7[a]	0.7[a]	0.7[a]	0.7[aa]	0.7	0.8[aa,b]
Avg daily feed, kg	2.3	2.9	2.9	2.8	2.8	2.9	3.0	3.0	2.9
Feed/gain	4.2	3.8	3.9	4.0	4.1	4.1	4.1	4.6	3.8

[1] Eleven pigs per lot.
[a] Significantly greater than least value ($P < 0.05$); [aa] $P < 0.01$.
[b] Significantly greater than lots 1 and 8 ($P < 0.05$).

TABLE 8

Serum and liver vitamin A levels of pigs fed a vitamin A-free diet for an 8-week depletion period, and then repleted with diets containing fermentation β-carotene or vitamin A palmitate (exp. 2)

	Depletion, 8 weeks, serum vitamin A, μg/100 ml
Initial value (45) [1]	24
2 weeks (45)	20
4 weeks (45)	14
6 weeks (45)	11
8 weeks (45)	6

Repletion, 9 weeks

Lot	1	2	3	4	5	6	7	8	9
Diet	Basal	Basal + fermentation β-carotene				Basal + vitamin A palmitate			
(Assay conc)	(0)	(0.5 mg/kg)	(0.9 mg/kg)	(2.0 mg/kg)	(3.5 mg/kg)	(250 IU/kg)	(500 IU/kg)	(1000 IU/kg)	(2000 IU/kg)
	Serum vitamin A, μg/100 ml								
Initial value (5) [1]	6	5	7	6	6	7	5	6	5
2 weeks (5)	7	11	18[bb]	18[bb]	19[bb,d]	14[aa]	13[aa]	23[dd]	27[gg]
4 weeks (5)	6	8	16[bb,c]	17[dd]	20[dd,f]	12[aa,b]	12[aa,b]	23[gg]	23[gg]
6 weeks (5)	5	8	15[bb,c]	16[cc]	22[ff]	13[aa,b]	11[aa]	21[ff]	25[ff]
8 weeks (11)	4	8[a]	13[bb]	17[ee]	20[ee]	12[bb]	12[bb]	18[ee]	22[gg]
	Liver vitamin A conc, dry basis (11),[1] μg/g								
	1	2	7[a]	16[dd]	30[ee]	3	9[aa,c]	27[ee]	76[hh]
	Total liver vitamin A (11),[1] mg								
	0.4	0.8	2.7[a]	6.1[dd]	11.0[ee]	1.1	3.4[aa,c]	10.4[ee]	30.9[hh]

[1] Numbers in parentheses indicate number of pigs per value.
[a] Significantly greater than least value at this stage of repletion ($P < 0.05$); [aa] $P < 0.01$.
[b] Significantly greater than 2 least values at this stage of repletion ($P < 0.05$); [bb] $P < 0.01$.
[c] Significantly greater than 3 least values at this stage of repletion ($P < 0.05$); [cc] $P < 0.01$.
[d] Significantly greater than 4 least values at this stage of repletion ($P < 0.05$); [dd] $P < 0.01$.
[e] Significantly greater than 5 least values at this stage of repletion ($P < 0.05$); [ee] $P < 0.01$.
[f] Significantly greater than 6 least values at this stage of repletion ($P < 0.05$); [ff] $P < 0.01$.
[g] Significantly greater than 7 least values at this stage of repletion ($P < 0.05$); [gg] $P < 0.01$.
[h] Significantly greater than 8 least values at this stage of repletion ($P < 0.05$); [hh] $P < 0.01$.

Serum and liver vitamin A values are presented in table 8. Serum levels declined to 25% of the initial values after 8 weeks of depletion. Treatment resulted in significant increases in serum vitamin A in all lots, except lot 2, after 2 weeks of repletion. These levels did not increase further. Predepletion serum vitamin A concentrations were achieved only in lots which received at least 3.5 mg of fermentation β-carotene or 1000 IU of vitamin A palmitate/kg of diet.

Whether the theory is favored that vitamin A is derived from β-carotene primarily by central cleavage (18, 19) or by a step-by-step breakdown involving β-oxidation (20, 21), the sources of vitamin A activity used here may be compared on a mole per mole basis by expressing dietary concentrations of β-carotene or vitamin A palmitate in international units and calculating the regression of total liver vitamin A or liver vitamin A concentration upon dietary vitamin A activities. This assumes that daily feed intakes in each treatment group were equal. By dividing the regression coefficient of the β-carotene source by the regression coefficient of the reference material (all-*trans* vitamin A_1 palmitate) and multiplying by 100, the potency of the carotene source relative to the potency of the reference material can be calculated. The procedure using the rat (6) has been somewhat modified, and the liver vitamin A values of pigs that continued to be fed the basal diet during the repletion period were not used in calculating the regression values.

Using total liver vitamin A after 9 weeks of repletion as the criterion, the relative biopotency of fermentation β-carotene was 11.5% compared with that of all-*trans* vitamin A_1 palmitate. The relative biopotency of fermentation β-carotene, when using liver vitamin A concentration as the criterion, was 11.4%. Previous estimates (10) of the conversion of β-carotene into vitamin A alcohol by swine indicated an efficiency no greater than 30 to 40% on a mole per mole basis.

The regression equations fitted to the data by the method of least squares (fig. 1) are as follows:

(1) $Y = -4.87 + 0.0174\,X$
where Y = mg vitamin A/liver, and X = IU of all-*trans* vitamin A_1 palmitate/kg diet

(2) $Y = -0.636 + 0.002\,X$
where Y = mg vitamin A/liver, and X = IU of fermentation β-carotene/kg diet

(3) $Y = -11.4 + 0.0429\,X$
where Y = μg vitamin A/g dry liver, and X = IU of all-*trans* vitamin A_1 palmitate/kg diet

(4) $Y = -1.08 + 0.00488\,X$
where Y = μg vitamin A/g dry liver, and X = IU of fermentation β-carotene/kg diet.

The data presented here indicate that 1 mg fermentation β-carotene is equivalent to 192 IU of all-*trans* vitamin A_1 palmitate for the support of liver storage in the depleted pig from 50 to 100 kg of body weight. The relationship currently accepted by the National Research Council (NRC) (22) is that "1 mg of carotene equals 533 IU vitamin A for the pig." The difference between these values may be a reflection of relatively lower utilization of fermentation β-carotene as compared with the carotene serving as the basis for the NRC recommendations. Just as likely, is the possibility that the vitamin A reference material used here was more efficiently utilized by the pig than the vitamin A standard upon which the NRC recommendations were based. In any case, a judgment of the usefulness of preformed vitamin A and provitamins A is dependent upon the available isomers present (17, 23, 24) and how carefully these are protected against change. The isomers were carefully characterized in this study and no measurable change was noted in their distribution or concentration in the experimental feeds from the time of mixing until they were consumed. Therefore, the use of a single expression relating carotene and vitamin A activity for the pig is erroneous and an expression of relationship must identify the stereoisomeric forms being compared.

ACKNOWLEDGMENTS

We wish to acknowledge the assistance of B. E. Brent, D. C. Brown, C. H. Long, B. H. Vincent and C. L. Zutaut.

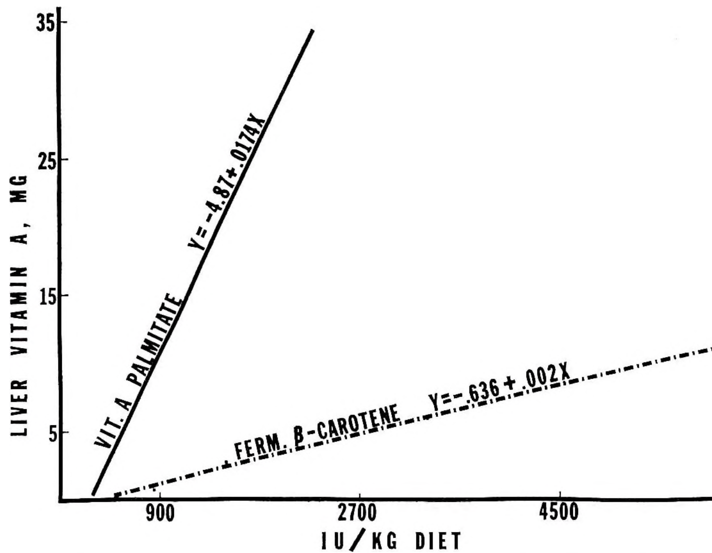

Fig. 1 Regression equations relating total liver vitamin A and dietary concentration of all-*trans*-vitamin A_1 palmitate or fermentation β-carotene.

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Applicability to Chicks of the Carcass Analysis Method for Determination of Net Protein Utilization

II. EFFECT OF PROTEIN, CALORIE AND FIBER LEVEL

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ABSTRACT Feeding experimental diets at 4 protein levels (7.5, 10, 12, 5 and 15%) had no significant influence on the regression relationship between carcass nitrogen and water content of chicks. A study of the effect of protein level on net protein utilization (NPU) and net protein ratio (NPR) involving 5 different protein sources, each fed at 4 supplementation levels, showed that the diet containing 10% protein consistently resulted in the highest NPU and NPR values for all the proteins under test. The conversion factor required to calculate protein retention efficiency (PRE) from NPR was found to be independent of the protein level in the experimental diet. A distinct difference exists between the conversion factor to be used for rats and chicks, respectively. The practical importance of PRE derived from weight gains during a 9-day experimental period as an estimate of NPU is discussed. The effect of varying levels of starch, (10, 20 and 30%), oil (4, 8 and 12%) and cellulose (zero and 4%) on the NPU value of a 10% casein diet was tested in a factorial experiment. The NPU value was not influenced significantly by either calorie level or fiber level. Substitution of 20% of sucrose in the experimental diet with cornstarch resulted in significantly higher NPU and NPR values.

Results obtained by De Muelenaere et al. (1) have shown that the carcass analysis method for determining net protein utilization (NPU) could be applied successfully to chicks. Summers and Fisher (2) confirmed these observations and suggested that proteins under test should be supplemented at the 13% level and not at 7% as used in the earlier study (1). They ascribed the difference in the factor required to convert net protein ratio (NPR) to protein retention efficiency (PRE), found by these 2 groups of workers, to the different protein levels used in the experimental diet. De Muelenaere et al. (1) had reported such a difference in the NPR-PRE conversion factor to exist between rats and chicks and suggested it was the result of breed difference.

Ascarelli and Gestetner (3), working with a different breed of chicks, fed a 10% protein diet, reported a NPR-PRE conversion factor of 17.1. The factor was obtained from the regression between NPU and NPR as originally suggested by Bender and Doell (4). The factor of 18 suggested by Summers and Fisher was derived from the average carcass protein.

The nitrogen and water content of carcasses of chicks of the same age was found to be highly correlated and a regression equation was formulated which could be used to estimate carcass nitrogen from the water content (1) Summers and Fisher (2) assigned 4 replicates to each test diet. They determined the nitrogen-to-water ratio of the pooled chick carcasses in one replicate and used this ratio to estimate the carcass nitrogens of the chicks in the remaining replicates.

Later reports by Fisher and his coworkers (5, 6) have established the practical importance of the carcass retention method to assess protein value for chicks.

In the present study NPU, NPR and PRE values of several proteins fed at different levels are reported. The effect of dietary protein level on the NPR-PRE conversion factor was investigated for both rats and chicks. The effect of calorie level, fiber content and cource of carbohydrate, present in the experimental diet, on these values was also studied.

EXPERIMENTAL TECHNIQUES AND METHODS

Experimental animals. All chicks used in these investigations were females from an autosexing cross between New Hamp-

Received for publication November 17, 1964.

shire (laying strain) males and interbred F_1 New Hampshire × Light Sussex females. The one-day-old chicks were given a standard chick mash for 4 days. On the fifth day they were wing-banded, weighed and allocated at random to the different treatments. The average weight of chicks at that age was 44.1 g. After the chicks had been fed the experimental diet for 9 days, they were starved for 18 hours prior to killing. Water was withdrawn 8 hours before the animals were killed.

Weanling male rats (Wistar strain) weighing, on the average, 35 g were allocated to the experimental diets. The experimental period, as in the case of the chicks, was of 9 days' duration. The rats were also starved for 18 hours before killing but water was not withdrawn.

Experimental diets. The experimental diet used in this study can be classified into 3 categories. The first group of diets was used in an experiment designed to test the effect of protein level on the regression between carcass nitrogen and water content. A mixed protein concentrate composed of fish meal, 50 parts; carcass meal, 20 parts; and blood meal, 10 parts, was added to the experimental diet to give 7.5, 10.0, 12.5 and 15.0% of protein content. The other dietary components were as follows: (in per cent) salts (7), 5; cod liver oil (containing 15 mg tocopherol/50 ml of oil), 1; choline chloride, 0.15; inositol, 0.1; vitamin mixture (1),[1] 1; maize oil, 4; and maize starch to make the ration up to 100%. The regression between carcass nitrogen and water was established in 2 independent experiments for both the 7.5 and 15% protein ration. These experiments were carried out at different times to test whether the nitrogen-water relationship would differ with the season.

In the second trial the basal diet was similar to the previous one except for the protein source. Fishmeal, carcass meal, groundnut meal, sunflower meal and commercial casein were each added to the respective experimental diets to contain 7.5, 10, 12.5 and 15% of protein. The nitrogen free diet was identical to the basal diet (without added protein concentrate) described above.

In the last experiment, which was designed to test the effect of calorie level, fiber content and source of carbohydrate on the NPU value of 10% casein, 18 different rations were prepared. These were obtained by combinations of 3 levels of maize starch (10, 20, and 30%) 3 levels of maize oil (4, 8, and 12%) and 2 levels of cellulose powder (zero and 4%). The remaining components of the diets were identical with those in the basal mixture described above except that sucrose was used to make the diets up to 100%.

Analytical methods and experimental design. After killing the experimental animals, the carcasses were wrapped in circular filter paper (24-cm diameter), placed in Petri dishes and dried in a forced-draft oven at 105°. After the carcasses had been dried to consistent weight they were predigested with concentrated HCl on a sandbath for 24 hours. The suspension was made up to volume and Kjeldahl determination (using mercuric oxide as catalyst) carried out on triplicate aliquots.

No nitrogen determinations were performed on the rat's carcasses. These values were estimated from the age and water content by using the following multiple regression equation $y = 184.53 + 43.0362\ x_1 + 3.5736\ x_2$ where y is the estimate of nitrogen content of the carcass in milligrams, x_1 the water content in grams and x_2 the age of the rat in days (8). NPU was determined by the method of Bender and Doell (4):

$$\mathrm{NPU} = \frac{B_f - B_k + I_k}{I_f} \times 100$$

where B_f and I_f denote, respectively, carcass nitrogen and nitrogen intake of rats fed the protein-containing diet. B_k and I_k represent the carcass nitrogen and nitrogen intake of rats fed the protein-free diet. NPR was determined by the following formula (4):

$$\frac{\text{Weight gain of animals fed test diet} + \text{Weight loss of animals fed protein-free diet}}{\text{Protein consumed}} = \mathrm{NPR}.$$

Then, PRE = NPR × conversion factor. The PRE was calulated as described by

[1] The vitamin mixture contained the following: (in milligrams) *p*-aminobenzoic acid, 500; thiamine, 30; riboflavin, 60; pyridoxine, 40; Ca pantothenate, 200; biotin, 2; folic acid, 10; menadione, 50; vitamin B_{12}, 0.2; and (in grams) nicotinic acid, 1; and glucose to make 100 g.

Bender and Doell (4). The conversion factor used was the regression coefficient for NPU on NPR, assuming that the regression line goes through the origin.

The NPU and NPR values in trial 2 were determined for the respective test proteins using chicks and rats. For the chicks each value was determined in quadruplicate, each replicate consisting of 5 animals. The values for the rats were obtained by triplicate determinations, each replicate containing 4 rats. Unless otherwise indicated in the text the weight changes used for NPR determination were those obtained after the animals had been starved for 18 hours just prior to killing.

In the third trial the diets consisted of a factorial set. The 3 factors were starch, oil and cellulose with 3, 3 and 2 levels, respectively, of application. Each of the 18 diet combinations was allocated to a group of 6 chicks in each replicate of a randomized block design with 3 replicates.

RESULTS

Effect of protein level on the correlation between water and nitrogen content of chicks. The influence of dietary protein level on body composition and regression between nitrogen and water content is shown in table 1. Analysis of variance of the respective regression equations (9) reveals no significant difference between the regression lines. Furthermore there is no significant evidence of any difference in estimates obtained in 2 separate experiments carried out at the 7.5 and 15% levels, thus supporting the view that the regression relationship does not alter from experiment to experiment. The nitrogen and water contents of the 211 chicks fed at the different levels of protein were therefore pooled to give the following regression equation:

$$x_1 = 130.5 + 36.5\ x_2$$

where x_1 is the estimate of the carcass nitrogen in milligrams and x_2 the water content of the carcass in grams. The range of the data from which this equation was derived lies between 22.15 and 63.9 g of carcass water. The standard error of a single estimated x_1 would lie between 80.9 and 83.0 mg. This combined regression equation is used to estimate the carcass nitrogen in all the subsequent chick experiments carried out in the present study.

Effect of protein level on NPU, NPR, PRE and NPR-PRE conversion factor. In table 2 are shown the NPU, NPR and PRE values as determined with chicks for the 5 protein sources each fed at 4 different protein levels. All the NPU's determined at the 10% level are significantly higher than values obtained at any of the other dietary protein levels. A closer inspection of the NPU and NPR values reveals a high degree of association between these 2 values. The regressions to estimate NPU from NPR at the 4 different protein levels did not show any significant difference: 7.5% protein, $y = 15.75\ x$; 10% protein, $y = 15.43\ x$; 12.5% protein, $y = 15.59\ x$; and 15% protein, $y = 15.38\ x$, when y is the estimate of NPU and x the NPR value. On analysis of variance of these regression equations no significant

TABLE 1

Carcass composition and regression of nitrogen on water content of chicks fed rations containing protein at varying levels

Protein levels, %	7.5	10	12.5	15
No. of animals	68	36	35	72
Water content, %	67.4 ± 0.2 [1]	66.6 ± 0.3	67.0 ± 0.4	68.2 ± 0.2
Protein content, %	17.1 ± 0.4	16.9 ± 0.4	16.7 ± 0.2	17.0 ± 0.4
Nitrogen content, % of dry carcass	8.15 ± 0.09	8.10 ± 0.09	8.15 ± 0.08	8.38 ± 0.08
Ratio of water, (g)/N(g)	24.9 ± 0.2	24.6 ± 0.2	25.1 ± 0.2	25.3 ± 0.1
Regression equation, x_1	$124.41 + 36.34x_2$	$233.97 + 34.04x_2$	$152.01 + 35.91x_2$	$141.42 + 36.35x_2$

[1] SE of mean.

TABLE 2

Net protein utilization (NPU), net protein ratio (NPR) and protein retention efficiency (PRE) values of proteins fed at different levels as determined with chicks

Protein content of ration	Protein source	NPU	NPR	PRE
%				
7.5	Fish meal	47.9 ± 2.2 [1]	2.72 ± 0.27	42.3 ± 1.8
	Carcass meal	33.7 ± 3.0	2.23 ± 0.28	34.6 ± 3.4
	Groundnut meal	45.1 ± 2.9	2.74 ± 0.52	42.6 ± 5.4
	Sunflower meal	53.0 ± 1.5	3.52 ± 0.33	54.7 ± 3.0
	Casein	38.4 ± 1.6	2.56 ± 0.49	39.7 ± 2.7
10	Fish meal	62.0 ± 5.1	4.09 ± 0.38	63.5 ± 4.7
	Carcass meal	53.6 ± 2.2	3.47 ± 0.07	53.9 ± 0.7
	Groundnut meal	49.8 ± 1.2	3.06 ± 0.15	47.5 ± 2.3
	Sunflower meal	56.5 ± 1.1	3.65 ± 0.10	56.7 ± 1.6
	Casein	58.2 ± 0.8	3.78 ± 0.15	58.7 ± 2.3
12.5	Fish meal	49.5 ± 1.0	3.17 ± 0.12	49.2 ± 1.0
	Carcass meal	35.2 ± 2.9	2.33 ± 0.22	36.1 ± 2.6
	Groundnut meal	30.1 ± 1.5	1.68 ± 0.35	26.1 ± 2.7
	Sunflower meal	40.5 ± 1.0	2.49 ± 0.20	38.7 ± 1.8
	Casein	38.3 ± 1.2	2.64 ± 0.17	40.9 ± 2.5
15	Fish meal	43.2 ± 1.6	2.64 ± 0.24	41.0 ± 3.6
	Carcass meal	30.1 ± 0.8	2.10 ± 0.11	32.6 ± 1.6
	Groundnut meal	33.2 ± 3.8	2.09 ± 0.14	32.4 ± 0.9
	Sunflower meal	40.8 ± 0.4	2.58 ± 0.20	40.1 ± 3.4
	Casein	34.8 ± 1.0	2.48 ± 0.13	38.5 ± 2.2

[1] SE of mean.

difference was found to exist between them. By definition, the multiplication of NPR by b, which is the slope of the regression line, gives the PRE. The slope of the 4 lines, also referred to as the NPR-PRE conversion factors are not significantly different from each other. The regression equation of PRE in terms of NPR calculated from the pooled data gives the following conversion ration PRE = 15.53 NPR. The PRE's obtained by using this conversion factor are shown in table 2.

TABLE 3

NPR-PRE [1] conversion factors for rats and chicks

Protein level	Chicks		Rats	
	A [2]	B [3]	A	B
%				
7.5	15.75	13.92	18.09	15.51
10	15.43	14.16	17.59	15.47
12.5	15.59	13.11	18.50	15.73
15	15.38	—	—	—

[1] NPR indicates net protein ratio; PRE indicates protein retention efficiency.

[2] Values in column A calculated from change in weight after animals had been fasted for 18 hours prior to killing.

[3] Values in column B calculated from change in weight, with final weight changes recorded just before the animals were fasted.

In table 3 the NPR-PRE conversion factors obtained with chicks are compared with those calculated from an experiment in which rats were fed the respective diets used in the chick study. In the table, 2 conversion factors, referred to under column A and B, are shown for the chicks and rats. The factors in column A are calculated from the change in weight after the animals had been fasted for 18 hours just prior to killing. The values in column B are calculated in an identical manner as for A except that the final weight changes were recorded just before the animals were fasted. Since the weight changes will be less in method A the NPR values are lower and hence the slope of the regression lines steeper, or the NPR-PRE conversion factor higher. The NPR-PRE conversion factor for rats is consistently higher than in the case of chicks fed the same diets. The conversion factors were, however, not significantly influenced in either species by the dietary protein level.

Effect of calorie, fiber and starch levels on the NPU and NPR values. The mean NPU and NPR values of the factorial ex-

TABLE 4

Mean net protein utilization (NPU) and net protein ratio (NPR) values of factorial experiment

Effect of oil			Effect of starch			Effect of cellulose		
Level	NPU [1]	NPR [2]	Level	NPU [1]	NPR [2]	Level	NPU [3]	NPR [3]
%			%			%		
4	48.86	3.16	10	45.81	3.09	nil	46.42	3.18
8	47.28	3.18	20	50.25	3.32	4	48.62	3.22
12	46.42	3.28	30	46.52	3.20	—	—	—

[1] SE, 1.027; least significant difference at 5% level, 2.95; at 1% level, 3.96.
[2] SE, 0.0516; least significant difference at 5% level, 0.14; at 1% level, 0.19.
[3] SE, NPU, 0.838; SE, NPR, 0.0421.

periment are shown in table 4. There is evidence that the NPU and NPR values are significantly affected by the level of starch. The highest values are obtained at the 20% level. The increase in value from 10 to 20% is significant for both NPU and NPR but the decrease in value from the 20 to 30% level does not attain significance in the case of NPR, although the trend is similar for both NPU and NPR. Different levels of cellulose or oil have no significant effect on the NPU and NPR values. Some evidence can be found for an interaction between cellulose, oil and sucrose for the NPR values, a component of this interaction attaining significance at the 5% level.

DISCUSSION

From the study involving analyses of a large number of individual chicks, it was found that the protein and water content of chick carcasses is not significantly influenced by dietary protein levels. Although the values published by Summers and Fisher (2) show an increase with increasing dietary protein, such a tendency was not observed in the present study. However, the dietary protein in the present study ranged from 7.5 to 15% as compared with 12 to 27% in Summer's study. The chicks were also older and the initial weights larger in the latter study.

Although the nitrogen and water content does not correspond in the respective studies the water-to-nitrogen ratios are in close agreement. This constancy of the water-to-nitrogen ratio is indirectly substantiated by the fact that on analysis of variance of the regression of nitrogen on water content of chicks no significant difference could be found between the respective regression equations. As previously emphasized (1, 4) the use of this ratio to estimate the nitrogen content of the chick from its water content eliminates the laborious nitrogen determination and makes the whole method relatively simple. If the water-to-nitrogen ratio obtained from the analysis of 5 pooled birds of one replicate is used to calculate the nitrogen content of the other replicates, as suggested by Summers and Fisher (2), all information of individual variation is lost. In such a case it is impossible to calculate the extent of error introduced by estimating carcass nitrogen from the water content. The use of the regression equation to estimate carcass nitrogen is therefore to be preferred from the statistical point of view.

Although it is generally accepted (4, 10) that biological value and NPU determinations should be carried out with rations containing 10% of protein, the 2 previous studies (1, 2) dealing with the applicability of the carcass analysis method to chicks do not comply with this practice. In the first study in this field, which was concerned mainly with NPU values of maize, a diet containing 7% protein had to be used because the cereal protein level was too low to reach the 10% level. This low level was criticized by Summers and Fisher (2) and they proposed the use of an experimental diet containing 13% protein. From the NPU values obtained for 5 different protein sources (table 2) it appears that 10% dietary protein is the optimal level for NPU determinations with chicks.

The high correlation found to exist between NPU and NPR values is in accord with results obtained for rats (4). The estimation of NPU from the NPR is referred to as the PRE value in order to differentiate it from NPU which is the value obtained from the nitrogen retention. It

is evident that there is good agreement between the 2 values but the standard errors are in general higher for the PRE's than for the NPU's. This is in line with the observation that growth methods are subject to more individual variation than balance methods (11). The fact that PRE, a value derived from growth, can be equated with the product of digestibility and biological value has a great advantage over any of the other growth methods used to assay protein quality for chicks. This is of practical importance since no separation of urine from feces is required, the determination does not involve any chemical analysis and the experimental period is of only 9 days' duration.

Some disagreement still appears to exist concerning the NPR-PRE conversion factor. Summers and Fisher attempted to explain the difference found between their conversion factor of 18 and that of 13 reported by De Muelenaere et al. as being the result of difference in protein level in the experimental diets used by the respective workers. From results obtained in this study no such relationship can be observed; analysis of variance of the respective regression lines did not show a difference in slope. In table 3, two NPR-PRE conversion factors are listed. The lower values for NPR-PRE conversion factor for chicks are similar to the value reported in an earlier publication (1) and are obtained from the NPR calculation which uses the weight gain of chicks before the final starvation period. This is the procedure described by Bender and Doell (4) in their original paper on NPR determination with rats. Because both Summers and Fisher (2) and Ascarelli and Gestetner (3) calculated their NPR values from weight gains recorded after fasting their experimental animals, a similar procedure was applied in the present study for comparative purposes. The new method of NPR calculation results in a considerably higher value for the conversion factor but it is still lower than the value obtained by these other workers. The values for rats listed in column B are very close to those reported by Bender and Doell (4), whereas the new method of calculation yields a higher value. However, whatever method of calculation is adopted, the conversion factor for rats remains consistently higher than for the chicks.

The observation that both NPU and NPR, as determined with chicks, are lower at the 7.5% protein level than at the 10% level, is not in line with the general accepted view that NPU decreases with increasing protein level in the experimental diet. In the experiment with rats fed identical diets no such decrease in NPU values for the 7.5% protein level was observed. It therefore appears that chicks behave differently.

The results of the factorial experiment agree with the generally accepted fact that biological value is not influenced by the calorie value of the ration, provided it is adequate (12, 13). Concerning the effect of different levels of starch, it appears that chicks behave differently from rats. Where NPU values (by carcass analysis) obtained with rats fed diets containing either starch or sucrose or a combination of the two, as source of carbohydrate, did show no consistent differences (14), the NPU's for chicks appear to be influenced by the source of carbohydrates.

ACKNOWLEDGMENT

The authors are indebted to Anita Mays, B. W. Martinaglia and R. Bunicich for technical assistance throughout these studies.

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Protein Reserves and Growth of the Walker Carcinosarcoma [1] in Rats

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ABSTRACT In rats bearing a Walker 256 carcinosarcoma, carcass weight and serum albumin decreased, whereas liver weight, RNA, protein and free amino acids increased. When the tumor-bearing animals were fed a protein-free diet for 3 days before autopsy, the levels of liver RNA and protein decreased. However, there was no effect upon tumor size or composition. When the protein reserves of the host were reduced by feeding a protein-free diet for 2 weeks prior to the implantation of the tumor, the growth rate, RNA-to-DNA ratio and protein content of the tumor decreased markedly. To test the hypothesis that the tumor utilizes the amino acids of the protein reserves for its rapid growth, an experiment was performed which compared the turnover rate of radioactive serum in tumor-bearing and control rats. In the tumor-bearing rats, the catabolic rate of serum globulin and albumin was increased, as was the biosynthetic rate of globulin but not that of albumin. The data suggest that there is a correlation between the magnitudes of the host's protein reserves and free amino acid pool size and the growth rate of the Walker 256 carcinosarcoma. On the basis of the foregoing information a model was proposed to explain protein metabolism in the tumor-bearing animal.

As the Walker 256 carcinosarcoma develops, there is a marked reduction in the weight of the carcass and in the concentration of serum albumin (1–5). From this it has been concluded that rapidly developing tumors grow at the expense of the protein reserves of the body (1). The protein reserves have been defined as those proteins which can be reversibly depleted during conditions of nutritional stress (6).

When animals were fed a protein-free diet, there was a reduction in the protein reserves of the organism (6). In other studies (7–9) tumors did not grow as rapidly in rats that were fed a protein-free diet prior to implantation. It has also been reported (7) that a lower ribonucleic acid (RNA) content prevailed in tumor tissue from rats that were fed a protein-free diet as compared with tumors from animals fed a protein diet. However, when rats with well-established tumors were fed a protein-deficient diet, there was no change in the growth rate or RNA content of their tumor tissue (10, 11). In these latter experiments, it appeared that the protein reserves of the host supported normal tumor development. In the present study, concentrations of RNA, protein and free amino acids were measured in the tumors and livers of rats that were fed a protein-free diet for various time periods before and after implantation of the Walker 256 carcinosarcoma. These results were correlated with the protein reserves and metabolic activities of the tumor-bearing hosts.

MATERIALS AND METHODS

Dietary regimen and care of animals. Male Wistar rats (100 to 150 g) were divided into 4 groups of 10 animals each. The first group was fed a protein-free diet (12) for 2 weeks, after which a homogenate of the Walker 256 carcinosarcoma was implanted subcutaneously in a lateral position of the rat. After implantation, these animals were fed the protein-deficient diet for an additional 15 days. The second and third groups of rats were fed an 18% casein diet (12) for 2 weeks and then implanted with Walker tumor. The casein diet was continued for 12 days following implantation, after which group 2 was fed a protein-free diet, whereas group 3 was maintained with 18% casein for the next 3 days. The fourth group, which served as

Received for publication November 16, 1964.

[1] This work was supported in part by Public Health Service Research Grant no. AM-04341 and by the American Cancer Society Grant IN 20E.

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non-tumor controls, was fed the 18% casein diet throughout the experimental period. Fifteen days after implantation, the tumor-bearing and control rats were killed, and samples of liver, tumor and serum were taken for chemical analyses. All animals were housed at a temperature of 26 ± 1° with a relative humidity of 40%. Animal quarters were illuminated daily from 6 AM to 6 PM. Daily food intakes and weekly body weights were recorded for each animal throughout the experiment.

Analyses. Liver and tumor tissues of each rat were homogenized with ice-cold distilled water into a 20% suspension. This suspension was used to determine the concentrations of total protein, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) as described previously (13). Samples of serum and tissue homogenates from each animal in a group were pooled and precipitated with picric acid. The resulting filtrate was used for the determination of free amino acids by ion exchange chromatography (14). Serum from each animal was separated into various component protein fractions (7) by paper electrophoresis.

Turnover rate of serum proteins. In another experiment, the turnover rates of serum proteins were determined in control and Walker 256 carcinosarcoma-bearing rats. Throughout the experiment all animals were fed an 18% casein diet. At 7 days after implantation of the Walker tumor, a tracer dose (2.4×10^6 count/min/rat) of biologically labeled S^{35} serum protein was injected intracardially. At 1, 3, 5, 7 and 10 days after injection of the radioactive serum, blood samples were collected via heart puncture. The serum proteins were separated by means of paper electrophoresis, and the radioactivity was measured by a previously described technique (15). The half-life, catabolic rate, change in pool size and synthetic rate of total serum, albumin and globulin protein fractions were calculated by the method of Jeffay and Winzler (16).

Standard errors were determined for all values. If the probability value was less than 0.01, the difference between the means was considered significant.

RESULTS

As illustrated in table 1, the rapid growth of the Walker tumor could be correlated with a significant decrease in the weight of the carcass (body weight minus the weight of the tumor) and in serum albumin concentration, and also, with an increase in liver weight of the tumor-bearing host. Although the recorded change in carcass weight was for a 15-day period after implantation of the tumor, previous experiments (1) have indicated that the host loses the most weight during the terminal phase of rapid growth of the tumor. When tumor-bearing rats that were fed a protein-free diet for 3 days prior to killing, were compared with the tumor-bearing animals fed a casein diet throughout the experimental period, the carcass weight, liver size and concentration of serum albumin were markedly decreased. However, there was an insignificant decrease in tumor size. Even though the liver was smaller in the tumor-bearing animals fed the deficient diet, it was still significantly

TABLE 1

Carcass,[1] tumor, and liver weights and concentration of serum albumin of tumor-bearing animals fed a protein-free diet for various lengths of time

Treatment	Change in carcass wt	Tumor wt	Liver wt	Serum albumin
	g/15 days	*g*	*g*	*g/100 ml*
Control				
18% Casein diet	62 ± 5 [2]	—	8.96 ± 0.23	3.52 ± 0.14
Tumor-bearing				
18% Casein diet	43 ± 3	15.1 ± 3.8	11.78 ± 0.24	2.91 ± 0.17
18% Casein + 3-day protein-free diet	25 ± 6	13.0 ± 1.5	9.76 ± 0.37	2.50 ± 0.15
Protein-free diet	−7 ± 2	1.5 ± 0.4	5.36 ± 0.29	1.49 ± 0.08

[1] Carcass weight equals body weight minus tumor weight.

larger than that in non-tumor-bearing control rats. If the rats were fed the protein-free diet for 2 weeks prior to implantation of the tumor, the weight of the tumor was significantly less than that of tumors from animals that were fed casein throughout the experiment. As would be expected in these protein-depleted animals, the carcass weighed less and the size of the liver and the concentration of serum albumin were markedly reduced as compared with the rats that were fed a diet containing proteins (table 1).

In the liver, the RNA-to-DNA and protein-to-DNA ratios were higher in the tumor-bearing animals that were fed casein (solid bars, fig. 1) than in the non-tumor-bearing controls (open bars, fig. 1). When animals with established tumors were fed a protein-free diet for 3 days, the RNA-to-DNA and protein-to-DNA ratios (stippled bars, fig. 1) of the liver were significantly reduced below the levels noted in the tumor-bearing rats that were fed a diet containing casein, but were still above the values for non-tumor-bearing animals. Livers of the rats that were pre-fed the protein-free diet (bars with slanted lines, fig. 1) had a concentration of cellular RNA and protein which was below that observed in the liver of non-tumor-bearing animals fed a diet containing casein. The concentrations of cellular RNA and protein were closely correlated with liver size.

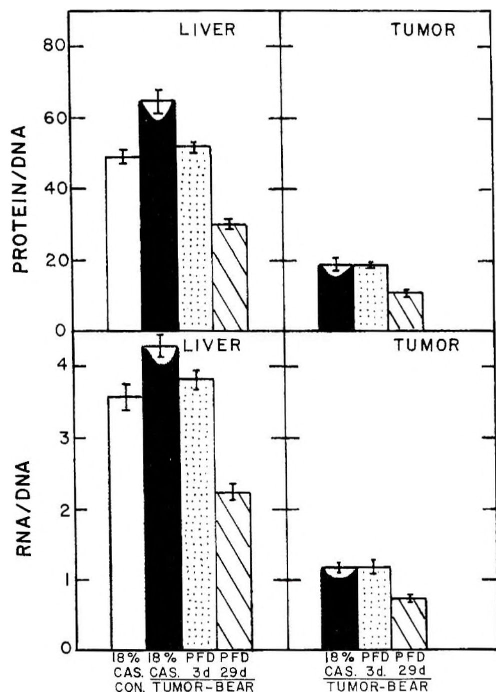

Fig. 1 RNA-to-DNA and protein-to-DNA ratios of liver and tumor from non-tumor-bearing controls (open bars), tumor-bearing rats fed 18% casein (solid bars), tumor-bearing rats fed a protein-free diet (PFD) for 3 days prior to autopsy (stippled bar) and tumor-bearing rats that were pre-fed a protein-free diet (bars with slanting lines). The vertical line in the center of each bar represents the standard error of the mean of 10 animals.

In the tumors of animals fed either casein or a protein-free diet for 3 days prior to being killed, the concentrations of RNA and protein were similar. However, when the rats were fed a protein-free diet throughout the entire experimental period, they developed tumors which, when compared with those from animals that were fed a diet containing protein, had a significantly decreased concentration of cellular RNA and protein (fig. 1). As in liver, the concentration of cellular RNA and protein could be correlated with the mass of tumor tissue.

In the tumor-bearing animals, the levels of essential and non-essential free amino acids of the liver and serum were higher than those observed for the non-tumorous controls (fig. 2). When rats were fed a protein-free diet, the levels of free amino acids of serum and liver decreased below those observed for the animals fed a diet that contained protein. The cellular concentrations of free amino acids in Walker tumors were unaffected by feeding a protein-free diet for 3 days prior to autopsy but were reduced in the tumors that dedeveloped in rats that were severely depleted in body protein (bars with slanted lines, fig. 2). In addition, the liver had a concentration of free amino acids per milligram of DNA which was about 10 times greater than that observed in a Walker tumor.

In rats implanted with the Walker 256 carcinosarcoma, the half-life of the total serum, albumin and globulin protein fractions was significantly decreased as compared with the non-tumorous controls (table 2). In the tumor-bearing animals, this decrease in the half-life of the serum

TABLE 2

Half-life, catabolic rate, change in pool size and synthetic rate of serum protein fraction from control and tumor-bearing rats

Serum protein fraction	Treatment group	Half-life ($t_{1/2}$)	Catabolic rate (a)	Change in pool size (b)	Synthetic rate ($a + b$)
		days	*g/day*	*g/day*	*g/day*
Total	control	3.0±0.2 [1]	0.129±0.025	+0.0090±0.0025	0.138±0.010
	tumor-bearing	1.5±0.1	0.216±0.041	−0.0084±0.0007	0.208±0.025
Albumin	control	3.4±0.3	0.057±0.003	+0.0045±0.0004	0.062±0.003
	tumor-bearing	2.0±0.2	0.073±0.008	−0.0215±0.0021	0.051±0.004
Globulin	control	2.4±0.1	0.081±0.008	+0.0045±0.0006	0.086±0.007
	tumor-bearing	1.1±0.3	0.162±0.020	+0.0033±0.0007	0.165±0.012

[1] SE of the mean of 10 animals.

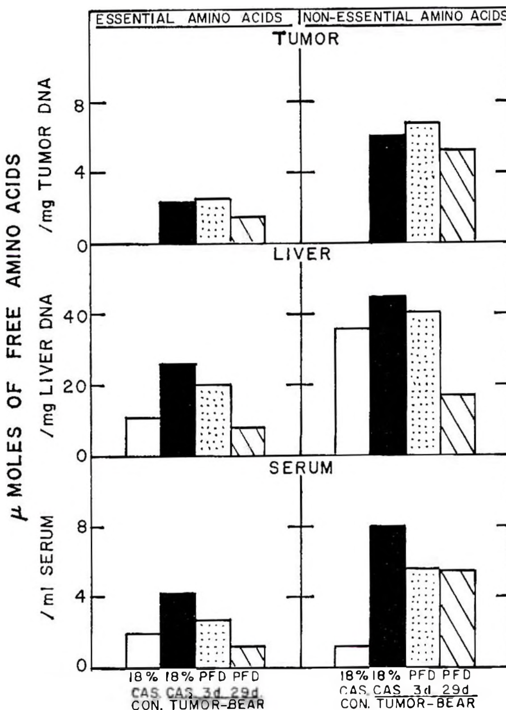

Fig. 2 The essential and non-essential free amino acids of serum, liver and tumor from non-tumor-bearing controls (open bars), tumor-bearing rats fed 18% of casein (solid bars), tumor-bearing rats fed a protein-free diet (PFD) for 3 days prior to autopsy (stippled bars) and tumor-bearing rats pre-fed a protein-free diet (bars with slanting lines). The values are expressed in micromoles of free amino acids per milligram of tissue DNA or per milliliter of serum.

protein fractions was correlated with a significant increase in the rate at which these proteins were catabolized. In growing non-tumorous rats, there was a net increase in the size of the body pool (grams of protein per animal) of serum albumin and globulin proteins. As the Walker tumor developed, the amount of circulating serum albumin decreased significantly and the serum globulin fractions increased. In the calculations, the catabolic rate (a) plus the change in the size of the protein pool (b) was equal to the rate of protein synthesis ($a + b$). Tumor-bearing rats as compared with the controls showed significant increase in the synthesis of the globulin fraction and a slight decrease in the rate of anabolism of the serum albumin (table 2). Thus, in the tumor-bearing animals, the data could be interpreted to mean that the decrease in serum albumin concentration was the result of an increased catabolism and a decreased anabolism of this protein fraction.

DISCUSSION

To better elucidate the significance of these experiments, a hypothetical model for protein metabolism in the tumor-bearing animal is proposed (fig. 3). Since amino acids are the building blocks from which proteins are synthesized, the free amino acid pool of the body should play a significant role in a scheme of protein metabolism. This free amino acid pool can be maintained by dietary protein and by the protein reserves of the body. In the non-tumor-bearing animals, the free amino acids can be utilized for the synthesis of 2 classes of body proteins: 1) the so-called "essential proteins" or those proteins which are essential for the maintenance of the organism and are not reduced during conditions of protein deprivation, and 2) the so-called "protein reserves" or those proteins which have various functions in the

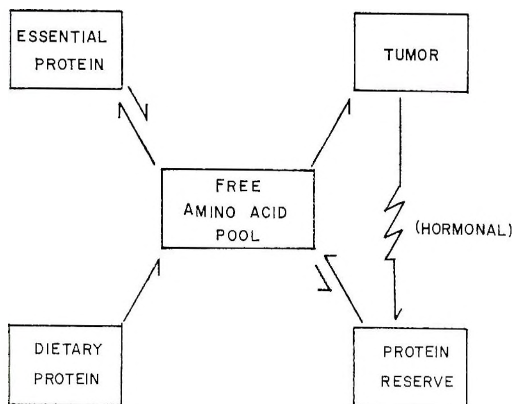

Fig. 3 A hypothetical scheme of protein metabolism in tumor-bearing animals.

body but can be reversibly depleted during conditions of nutritional stress.

In a growing animal that is receiving adequate dietary protein and calories, the anabolic activity of the body proteins (both essential and reserve proteins) exceeds the catabolic activity, which will result in a net gain in body nitrogen. When a rapidly growing tumor is placed in these animals, there is a marked flow of amino acids toward the tumor (4, 17). Since tumors of this type are considered as nitrogen traps (4, 17, 18), then the rapid growth of this tissue could result in a reduction in the free amino acid pool of the body. However, in the tumor-bearing animals that were fed adequate dietary protein, there was, as noted previously (19–22), a marked increase in the free amino acid content of serum and liver. Associated with this increase in the free amino acid pool was a decrease in the weight of the carcass and in the concentration of serum albumin and an increase in weight, RNA and protein content of the liver. When animals possessed a rapidly growing tumor, several other investigators noted a similar increase in the weight (3, 23, 24), RNA (10, 19, 25–27) and protein (3, 23, 24, 28) content of the liver. When rats were injected with adrenal corticoids, there was a decrease in the body weight and protein content of the muscle and an increase in liver size, protein and free amino acid concentrations of the cell in the liver (29, 30). In the tumor-bearing animals, these results can be correlated with a hyperfunction of the adrenals (31) and with an increase in the corticoid level in the serum and adrenals (32). Thus, in the hypothetical scheme outlined in figure 3, this stress response to growth of the tumor would result in an increased breakdown of the protein reserves (mostly muscle and skin)[4] and a flow of amino acids toward viscera and tumor. Under these conditions the dietary proteins would maintain and even increase the size of the body pool of free amino acids, so that the tumor could continue to grow and structural proteins continue to be synthesized at a rapid rate.

The biological half-life of serum albumin and globulins was evaluated in both tumorous and control rats. As reported previously (33), in the tumor-bearing animals the catabolism of serum albumin and globulin proteins increased. There was a concomitant increase in the protein biosynthetic rate and in the size of the body pool of serum globulins, but the synthesis and the pool size of serum albumin decreased. Thus, this experiment demonstrated how the so-called "protein reserves," as exemplified by serum albumin, can maintain and increase the free amino acid pool of the tumor-bearing animal. The serum globulins, with their increased turnover rate and concentration, were examples of the so-called "essential proteins."

As reported previously (10, 11), a slight reduction in the size of the host's free amino acid pool caused a decrease in the concentration of cellular RNA and protein of the liver but had no effect upon these parameters in tumor tissue. In the present study a similar response was noted in the tumors of rats fed a protein-free diet for 3 days prior to being killed. These terminally protein-depleted rats still had greater liver and serum free amino acid pools than non-tumor controls.

When the protein reserves of the rats were reduced prior to implantation of the tumor, the continued feeding of a protein-free diet resulted in a marked decrease in the size of the free amino acid pool of the

[4] Unpublished data, J. B. Allison and R. W. Wannemacher, Jr., 1964.

host. Under these conditions, the tumor had a decreased cellular concentration of free amino acids, RNA and protein. These changes in the composition of the tumor could be correlated with its slower rate of growth. Thus, although the tumor was less susceptible to minor changes in the size of the host's free amino acid pool (which may be related to the tumor's lower cellular free amino acid level as compared with the liver), it could be affected by a marked reduction in protein reserves and dietary protein. These data emphasize the positive correlation between the concentration of cellular free amino acids, RNA and protein and the growth rate of the Walker 256 carcinosarcoma.

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Relationship between Cholesterol and Vitamin A Metabolism in Rats Fed at Different Levels of Vitamin A [1]

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ABSTRACT The relationship between cholesterol and vitamin A metabolism was studied in 3 replications using a total of 225 weanling male rats. Following vitamin A depletion, each animal was assigned at random to an experimental group, receiving one treatment from each of the following 3 variables: 0.0 or 0.5% cholesterol mixed in the basal purified diet; 10, 150 or 1000 IU vitamin A acetate daily administered orally; and fed 7, 18 or 28 days. Feed was available approximately 18 hours daily. Serums were assayed for carotene, vitamin A and free and total cholesterol, and livers, for vitamin A, free and total cholesterol, total fat and moisture. Increasing the dietary vitamin A decreased the serum and liver total cholesterol concentrations when cholesterol was fed. The rats given 10 IU vitamin A daily had significantly higher ($P < 0.01$) liver total cholesterol values than those given either 150 or 1000 IU. Cholesterol-fed rats given 10 IU also had significantly higher ($P < 0.01$) mean serum cholesterol values than those given 1000 IU. Dietary cholesterol significantly lowered ($P < 0.05$) mean liver vitamin A concentrations on a dry weight basis. Liver vitamin A and free cholesterol were found to be positively correlated ($P < 0.01$) on either a percentage or per liver basis.

Positive correlations ($P < 0.01$) between serum vitamin A *and* serum free and total cholesterol as well as serum carotene *and* serum free and total cholesterol were found in 15- and 16-year-old boys and girls studied in 3 selected communities of Idaho (1). Similar observations were made in the same age group by Utah workers [3] and for the correlations between serum carotene *and* free and total cholesterol by Washington workers (2). However, the former group (3) reported a significant negative correlation between vitamin A intake and serum cholesterol ($P < 0.05$).

Reports in the literature concerning the relationship between cholesterol and vitamin A metabolism are limited and conflicting. Some of the conflicting evidence is probably caused by species differences. Schoenheimer and Breusch (4) observed that large amounts of dietary carotene had no effect on cholesterol synthesis in mice. Other workers (5, 6) also reported similarly that the blood cholesterol level of rabbits was not affected or only slightly increased by dietary vitamin A. In contrast, Wood (7) reported that in chicks vitamin A prevented hypercholesterolemia induced by dietary cholesterol. Kantiengar and Morton (8) and Green et al. (9) reported that dietary cholesterol significantly lowered vitamin A storage in livers of male rats (8, 9), whereas the opposite effect was noted by earlier workers (10).

The purpose of the present study was to investigate the effect of different levels of dietary vitamin A for various time periods upon the interrelationship between the vitamin A and cholesterol metabolism of white male rats.

EXPERIMENTAL

Weanling male rats totaling 225 of the Sprague-Dawley strain and 102 of the Holtzman strain were caged individually and fed a purified diet complete in all nutrients but vitamin A. The Holtzman rats are descended from the Sprague-Dawley strain. The Sprague-Dawley strain was used in 2 replications and the Holtzman strain in the third replication. Thus the 2 strains of animals were distributed pro-

Received for publication August 10, 1964.

[1] Published with the approval of the Director of the Idaho Agricultural Experiment Station as Research Paper no. 628. This study was supported in part by funds from Regional Projects W-4 and W-44 of the Western Agricultural Experiment Stations.

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portionately, approximately 2 Sprague-Dawley rats to 1 Holtzman rat, in all treatments. The percentage composition of the basal diet was similar to the A_1 diet described by Mayer and Krehl (11): powdered sucrose, 65.7; vitamin-free casein,[4] 25.0; cottonseed oil,[5] 5.0; salt mixture,[6] 4.0; cystine,[7] 0.2; and choline chloride,[8] 0.1. The following vitamins were added to each kilogram of diet: (in milligrams) α-tocopherol, 100; menadione, 5; viosterol, 0.1; thiamine·HCl, 5; pyridoxine·HCl, 5; riboflavin, 5; niacin, 50; Ca-pantothenate, 50; biotin, 0.5; folic acid, 0.5; inositol, 100; *p*-aminobenzoic acid, 100; and vitamin B_{12}, 0.03.[9] This vitamin A-deficient diet was given ad libitum from approximately 3:00 PM to 9:00 AM daily until growth ceased or decreased for 3 consecutive days and vitamin A deficiency symptoms appeared (12). These criteria were met in approximately 3 to 5 weeks, after which each animal was assigned at random to an experimental group in a 2 × 3 × 3 factorial design. The pattern of feeding remained the same. One-half of the animals received 0.5% cholesterol mixed in their diet, while the other half received none. One-third of each of these groups received 10, 150 or 1000 IU of vitamin A acetate daily, administered orally by medicine dropper in 0.1 ml of cottonseed oil [10] at approximately the same time each afternoon. The basal diet was the same as fed during the depletion period except that α-tocopherol was eliminated and given with the vitamin A supplement at the level of 1.5 mg/daily dosage. In 2 replications food cups were removed from the animals about 24 hours before killing and in the third replication, about 12 hours before killing. Vitamin A supplements were withheld about 42 hours from all animals before killing. At the end of 7, 18 or 28 days, animals from each subgroup were anesthetized with nitrous oxide, using a technique described previously.[11] Maximal blood was removed from the posterior vena cava for serum preparation. Livers were removed, blotted, weighed and packaged before freezing. All serum and livers were quick-frozen and held at −17° until analysis was performed. Ten or more animals were also killed at the end of the depletion period for each replication, to serve as negative controls.

Serum and liver samples were assayed for free and total cholesterol by the method of Galloway et al. (13). The modifications of this method for liver analyses are decribed by Wilcox and Galloway (14). Serum carotene and vitamin A were assayed by the method of Bessey et al. (15) and liver vitamin A by the Carr-Price reaction as modified by Vavich and Kemmerer (16). Moisture content of livers was determined by drying at 100–105°. Total fat is reported as the loss in weight after extracting the dried ground liver 24 hours with Skelly-Solve B. The liver vitamin A, moisture and total fat assays were determined at the University of Arizona.

RESULTS

Growth and food consumption were similar for all groups of animals except for being somewhat lower for all animals in the 7-day group and significantly lower ($P < 0.01$) for those fed 10 IU of vitamin A daily for 7 days. Cholesterol feeding did not appear to affect growth or food intake.

Mean liver and serum values are summarized in table 1 by group. The data of the 3 replications were combined because an analysis of variance revealed no clear-cut trend in differences among replications. The effects of dietary cholesterol level, dietary vitamin A level and period were determined by an analysis of variance for unequal subclass numbers. Logs of the liver vitamin A and total cholesterol values were used in this analysis. The least square analysis was adjusted for differences in numbers per treatment. When

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[4] "Vitamin-Free" Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[5] Wesson Oil, purchased in one lot; Wesson Oil Sales Company, Fullerton, California.

[6] As required in biological test diets listed in U. S. Pharmacopoeia XIV, 1950; the salt mix contained per kg of diet: (in grams) $AlNH_4(SO_4)_2 \cdot 12H_2O$, 0.09; $CaH_4(PO_4)_2 \cdot H_2O$, 112.80; $CaCO_3$, 68.60; $Ca_3(C_6H_5O_7)_2 \cdot 4H_2O$, 308.30; $CuSO_4 \cdot 5H_2O$, 0.08; $FeC_6H_5O_7 \cdot 3H_2O$, 15.26; $MgCO_3$, 35.20; $MgSO_4$, 38.30; $MnSO_4 \cdot H_2O$, 0.20; KCl, 124.70; KI, 0.04; K_2HPO_4, 218.80; NaCl, 77.10; and NaF, 0.50 (obtained from General Biochemicals, Inc., Chagrin Falls, Ohio).

[7] The authors gratefully acknowledge gifts of vitamins and other nutrients from Merck Sharp and Dohme, Rahway, New Jersey.

[8] See footnote 7.

[9] See footnote 7.

[10] See footnote 5.

[11] Bring, S. V., and T. B. Keith 1958 Blood sampling technique for white rats. Federation Proc., 17: 472 (abstract).

a significant difference was found where 3 treatments were involved, the significance of mean comparisons was examined by Duncan's multiple range test (17) as modified by Kramer (18).

Mean liver weights. The livers of the Holtzman animals weighed significantly less than those of the Sprague-Dawley rats ($P < 0.01$). This same difference was reflected in the animal weights at the time

TABLE 1

Mean liver and serum values

Daily vitamin A intake, IU	10	10	10	150	150	150	1000	1000	1000
Experimental period, days	7	18	28	7	18	28	7	18	28
Minimum no. of observations	16	15	14	15	15	14	16	14	15
Mean weekly wt gains, g									
Without cholesterol [1]	10.0	29.5	31.7	28.6	34.0	33.0	29.1	36.7	36.4
With cholesterol [2]	15.8	32.6	29.4	30.2	35.4	35.0	30.7	38.4	35.3
Liver									
Weight, g									
Without cholesterol	6.0	8.2	9.2	7.8	8.4	9.6	7.3	8.8	10.4
With cholesterol	6.9	9.8	10.8	8.2	9.8	11.4	8.4	10.5	11.5
Moisture, %									
Without cholesterol	68.8	72.5	72.2	71.9	72.8	70.5	70.1	66.4	69.9
With cholesterol	69.5	64.2	63.8	69.8	66.8	66.8	67.6	68.1	65.7
Total fat, % dry wt									
Without cholesterol	17.4	14.1	13.5	20.5	11.5	13.8	20.3	22.0	16.5
With cholesterol	24.4	37.7	35.9	22.3	27.4	25.3	28.8	28.0	28.3
Total cholesterol, % moist wt									
Without cholesterol	0.37	0.31	0.30	0.34	0.29	0.29	0.35	0.29	0.29
With cholesterol	1.98	3.07	3.80	1.68	2.33	2.55	1.77	2.15	2.26
Total cholesterol, % dry wt									
Without cholesterol	1.25	1.22	1.11	1.28	1.07	1.00	1.22	0.91	1.02
With cholesterol	6.67	8.67	10.62	5.61	7.08	7.74	5.52	6.67	6.77
Total cholesterol, mg/liver									
Without cholesterol	22	26	28	26	24	28	26	26	29
With cholesterol	137	300	412	136	226	293	148	226	264
Free cholesterol, % moist wt									
Without cholesterol	0.21	0.20	0.21	0.21	0.22	0.21	0.19	0.20	0.19
With cholesterol	0.24	0.24	0.25	0.22	0.22	0.22	0.24	0.23	0.23
Free cholesterol, mg/liver									
Without cholesterol	12	16	19	16	18	20	14	17	20
With cholesterol	17	24	27	18	22	25	20	24	26
Vitamin A, μg/g moist wt									
Without cholesterol	3.3	3.1	3.0	11.4	23.6	22.2	67.7	183.6	219.9
With cholesterol	3.1	3.1	2.3	11.7	20.7	29.7	91.9	138.3	196.6
Vitamin A, μg/g dry wt									
Without cholesterol	11	14	12	47	88	75	239	600	848
With cholesterol	11	9	5	39	65	90	288	454	586
Vitamin A, μg/liver									
Without cholesterol	20	27	29	85	196	211	502	1547	2281
With cholesterol	22	32	25	93	200	333	762	1402	2186
Serum									
Total cholesterol, mg/100 ml									
Without cholesterol	97	84	83	112	89	88	106	84	81
With cholesterol	154	154	129	129	113	110	120	98	91
Free cholesterol, mg/100 ml									
Without cholesterol	17	14	14	23	16	15	19	14	12
With cholesterol	26	23	18	19	15	15	20	14	15
Vitamin A, μg/100 ml									
Without cholesterol	50	38	27	84	66	64	82	78	69
With cholesterol	34	30	34	85	57	63	81	67	62

[1] No dietary cholesterol.
[2] 0.5% dietary cholesterol.

of killing. A fast of approximately 24 hours compared with 12 hours apparently did not have the depressing effect on liver weights that a fast compared with no fast evidenced in other laboratories (19, 20). Feeding cholesterol significantly increased ($P < 0.01$) the mean liver weights, which agrees with the observations of Green et al. (9). In general, the liver weights increased slightly as the vitamin A administered increased. The exceptions to this general trend were: 1) the slightly decreased mean liver weight of rats fed 1000 IU of vitamin A daily compared with 150 IU for only 7 days without cholesterol; and 2) the absence of change between rats fed 10 or 150 IU of vitamin A daily for 18 days with cholesterol. The rats fed 10 IU vitamin A daily had significantly lower ($P < 0.01$) mean liver weights than those fed either other level with the aforementioned exception.

Liver moisture. The feeding of cholesterol decreased the moisture concentration ($P < 0.01$) with the exception of a slight increase effected by dietary cholesterol on the rats supplemented daily with 10 IU of vitamin A for 7 days or 1000 IU for 18 days. The greatest depressive effect (12%) occurred in those rats receiving 10 IU of vitamin A daily for 18 or 28 days.

Total liver fat. Feeding cholesterol significantly increased the liver fat deposition on both a moist and dry weight basis ($P < 0.01$) at all periods and levels of dietary vitamin A. There appeared to be no consistent interrelationship between cholesterol and vitamin A levels and period in their effect on liver fat concentration.

Liver total cholesterol. The analysis of variance revealed an inverse relationship between dietary vitamin A level and liver total cholesterol when expressed on either a concentration or content basis. The 10 IU level of dietary vitamin A produced both a significantly higher concentration and content of total cholesterol compared with the 150 or 1000 IU levels when cholesterol was fed. These comparisons were highly significant ($P < 0.01$) except on a content per liver basis between 10 and 150 IU ($P < 0.05$). On a dry weight basis, the liver total cholesterol concentration was significantly higher ($P < 0.05$) in those rats fed 150 IU of vitamin A daily than in those fed 1000 IU. The liver total cholesterol increased significantly ($P < 0.01$) as the experimental period lengthened when cholesterol was fed. The rate at which feeding cholesterol increased the liver total cholesterol on both a percentage and per liver basis decreased as the dietary vitamin A level increased. However, cholesterol feeding increased both the concentration and content at every level of dietary vitamin A. This partially explained the significant interaction ($P < 0.01$) between cholesterol and vitamin A levels. It was also caused by the different dietary vitamin A levels effecting little change in the liver total cholesterol concentration and content when no cholesterol was fed.

Liver free cholesterol. Those rats given 10 IU of vitamin A daily had significantly higher concentrations of free cholesterol than those given 1000 IU ($P < 0.01$) with the exception of animals in the 7-day group that were fed cholesterol. The differences between actual values was very small, however. The liver content of free cholesterol per liver significantly increased with the experimental period ($P < 0.01$). This probably at least partially reflected the direct relationship between liver weights and length of experimental period.

Liver vitamin A. In general, cholesterol feeding decreased the concentration but increased the content of liver vitamin A. However, the only statistically significant effect of dietary cholesterol was its depressing effect on the concentration of vitamin A on a dry liver weight basis ($P < 0.05$). As the level of dietary vitamin A was increased, the liver content and concentration of vitamin A also significantly increased ($P < 0.01$) as would be expected. This relationship was almost linear. As the length of experimental period increased, the liver vitamin A content increased significantly ($P < 0.01$) for all animals given the 2 higher levels of vitamin A supplement. A similar significant increase ($P < 0.01$) in concentration of liver vitamin A with each increase of period occurred in all animals given the supplement of 1000 IU of vitamin A and in those given the supplement of 150 IU of vitamin A plus cholesterol. A few of the livers of the animals receiving 1000 IU of vitamin A for 18 or 28 days were higher

in vitamin A than were the other livers. These data were included, however, as there was no obvious explanation for eliminating them. Microscopic examination of some of these livers indicated they were filled with unevenly distributed fat globules. However, those livers with extreme vitamin A concentration fell well within the range of the group is cholesterol and fat concentrations.

Serum total and free cholesterol. The concentration of serum total cholesterol was inversely related to the level of dietary vitamin A when cholesterol was fed. This agrees with the observations of Utah workers, who observed a negative correlation between vitamin A intake and serum cholesterol in adolescent boys and girls eating their usual mixed diet (3). The cholesterol-fed rats given 10 IU of vitamin A daily had significantly higher serum total and free cholesterol levels than those given 1000 IU ($P < 0.01$). The peak in these serum levels for animals given no dietary cholesterol occurred at the 150 IU level of dietary vitamin A. These serum levels were also inversely related to the length of the experimental period. The animals maintained with the experimental diet 7 days had significantly higher concentrations of serum free and total cholesterol than those continued for 18 or 28 days ($P < 0.01$).

Serum vitamin A. The most common effect of feeding cholesterol was to decrease the serum vitamin A level, but not significantly. However, the mean serum vitamin A levels of those animals fed 10 IU of vitamin A daily for 28 days or 150 IU for 7 days were increased by cholesterol feeding. The serum vitamin A levels of rats in the 7-day group were significantly higher than those of rats in the 18- or 28-day groups ($P < 0.01$). The one exception to this significant trend was the absence of change in those rats fed cholesterol and 10 IU of vitamin A daily for 28 days compared with rats in the 7-day group that were treated similarly.

The serum of these rats was found to contain no carotene, as expected. However, it seemed worthwhile to check this possibility.

The rats given no vitamin A (negative controls) had very low mean amounts of this vitamin in their livers and serum: 15 μg/liver and 7 μg/100 ml, respectively.

Correlations. The pooled correlation coefficients corrected for other treatment effects among all comparisons of liver and serum values, total food intake and total weight gain were computed. Correlation coefficients corrected for other effects were also determined separately for each cholesterol level, vitamin A level and period. Only those correlation coefficients that were highly significant ($P < 0.01$) are discussed.

Those animals receiving 1000 IU of vitamin A daily showed a highly positive correlation ($r = 0.32$) between serum vitamin A concentration and free cholesterol content per liver when corrections were made for other treatment effects.

Although liver total cholesterol and vitamin A were not significantly correlated, liver free cholesterol and vitamin A were. There was a highly significant positive correlation between liver free cholesterol and vitamin A on the basis of concentration per unit of moist tissue ($r = 0.31$) or dry tissue ($r = 0.36$) and on the basis of content per liver ($r = 0.32$) when the data were combined. A highly significant correlation ($r = 0.44$) existed between liver vitamin A and free cholesterol on a dry weight basis among the rats fed cholesterol as well as among those not fed cholesterol ($r = 0.50$). When correlation coefficients were determined separately for the 3 experimental periods with corrections made for the other treatment effects, liver vitamin A and free cholesterol were again positively correlated ($r = 0.32$, 0.34 and 0.40 for 7, 18 and 28 days, respectively) on a dry weight basis. Those rats maintained with their respective diets for 7 days also had positive correlations ($P < 0.01$) between liver vitamin A and free cholesterol on a moist weight basis ($r = 0.31$) and per liver basis ($r = 0.36$); whereas those fed diets for 28 days had a positive correlation ($r = 0.39$) between liver vitamin A and total cholesterol on a dry weight basis. When correlations were determined separately for the 3 levels of dietary vitamin A with corrections for other treatment effects, it was found that all correlations between liver vitamin A and free cholesterol were significant or nearly significant for

animals fed 10 IU vitamin A daily ($r = 0.21$ to 0.47).

DISCUSSION

Cholesterol feeding significantly increased the weight and fat concentration of livers, as reported by Green et al. (9); but it also significantly decreased the moisture concentration. As a significant inverse relationship existed between the moisture and fat concentration in the liver, it appears likely that a variable that increased the one liver component probably decreased the other. The increased mean liver weight due to exogenous or dietary cholesterol was probably a reflection of the increased fat concentration.

Results from this study suggest that increasing amounts of dietary vitamin A acetate depress the serum and liver total cholesterol concentrations when cholesterol is included in the diet. The reason for this effect is not clear. It may be caused by a direct or indirect influence of vitamin A on 1) cell permeability in the liver or intestinal wall; 2) some unidentified factor that affects cholesterol metabolism; or 3) the possible accumulation of cholesterol elsewhere than the liver and serum in other reactions. Although vitamin A may be necessary for complete synthesis of cholesterol, as noted by Weber et al. (21), it may have a different regulating effect on formed cholesterol whether exogenous or endogenous. This might explain the fact that dietary cholesterol appeared to emphasize any depressing effect that increasing dietary vitamin A had on free and total cholesterol concentrations in serum and liver.

Some possible causes of exogenous cholesterol decreasing liver vitamin A concentrations on a dry weight basis might be either 1) an increased requirement for vitamin A because of its more rapid metabolism as suggested earlier by Green and co-workers (9); or 2) a competition between the absorbed cholesterol and vitamin A for storage in the liver. However, the significant positive correlation found between liver free cholesterol and vitamin A concentration would tend to discredit this latter possibility.

No significant correlation was observed between serum vitamin A and serum free or total cholesterol when compared separately for each dietary vitamin A level, cholesterol level, period or combined as found earlier in adolescent boys and girls (1). This might be explained as a species difference or a difference between a controlled laboratory and uncontrolled population type study.

The significant positive correlation between liver vitamin A and free cholesterol on all 3 bases suggest that whatever may be regulating the storage of one may be regulating the other. The significant positive correlation between serum vitamin A level and the amount of free cholesterol per liver in the animals fed 1000 IU of vitamin A daily may indicate that at this high level of supplementation there is also a common regulating factor or factors between these 2 components.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to the following: Dr. A. R. Kemmerer, Agricultural Biochemistry Department, University of Arizona, for directing the assays of rat livers for vitamin A, moisture and total fat content with the assistance of Dr. N. Raica, Jr. and D. L. Schneider; Lyndell Graue, Ruth Shane, Kay Swenson and Carol Thilenius for technical help; Drs. W. B. Ardrey, T. B. Keith and A. C. Wiese for their advice at various phases of the study; Dr. Dale Everson for statistical advice; and the Poultry Science Department for use of their mixing facilities.

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Effect of Protein Depletion and Repletion in the Rabbit on the Activity of S^{35} from Methionine in Serum Proteins and Urine [1,2,3]

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ABSTRACT Sulfur-35 from injected methionine was measured in serum protein and urine of adult rabbits after dietary protein depletion and after repletion. Five rabbits fed a 4% casein diet for 39 days developed a hypochromic microcytic anemia, lost weight and were in negative nitrogen balance; however serum protein concentrations and fractions and blood volumes showed no significant change. During the same period there were no significant differences in these parameters between 2 groups of rabbits fed either a 20% casein ration or standard chow. In the depleted animals serum protein S^{35} activity 8 hours after injection was higher, specific activity of the albumin fraction increased over 18 days, the percentage of the total activity persisting in the α-globulin fraction was greater and more S^{35}/mg of urinary nitrogen was excreted although the total urinary S^{35} was less. When the depleted animals were fed the 20% casein ration for 34 days, differences in nitrogen balance, red cell indexes, body weight and the utilization of the isotope disappeared. These results indicate that the isotope detected alterations in serum proteins that were not apparent by changes in concentration or albumin and globulin fractions.

Before protein deficiency results in functional impairment and symptoms become clinically manifest, adaptive mechanisms are utilized by the body in an effort to maintain homeostasis (1). These mechanisms serve to preserve the normalcy of traditional indicators of protein deficiency until severe deficiency is present. Blood volume changes may mask alterations in serum protein concentrations, serum fractions and red cell indexes. Loss of body weight may be obscured by edema. Positive nitrogen retentions occur in the presence of depleted nitrogen stores (2). The difficulty in evaluating marginal deficiency is documented by the inconsistency of results in studies attempting to evaluate protein nutriture. Even when the subject is used as his own control for observations on protein deficiency, changes may not be detected by the more commonly employed tools of evaluation (3).

For the past 10 years radioactive tracers have been used for compartmental analysis and for study of the rates of synthesis and degradation of serum proteins (4, 5). Garrow (6, 7) was one of the first to employ a tracer amino acid, S^{35}-L-methionine, for the purposes of clinical evaluation of protein nutriture.

Allison et al. (8) reported an increase in the specific radioactivity of serum proteins in tumor-bearing and protein depleted dogs. Others (9–11) have also reported the preferential incorporation of S^{35}-L-methionine into certain tissues in states of deprivation.

Alteration in the patterns of incorporation of S^{35} from methionine by the serum proteins may be apparent before the usual signs of protein deficiency occur. If so, protein deficiency would be detectable before the appearance of the more profound changes necessary at present to classify a person or animal as protein-deficient. The purpose of the present study was to develop mild-to-moderate protein deficiency in rabbits and to observe the effects of this on the rate of uptake of

Received for publication September 11, 1964.

[1] The material presented is taken in part from the dissertation submitted by Gladys W. Strain in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Western Reserve University.

[2] This work was supported in part by Public Health Service Research Grant no. HD-00669.

[3] This work was presented in part at the annual meeting of the Federation of American Societies for Experimental Biology at Chicago, Illinois, 1964.

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S^{35} from methionine by the serum proteins, the incorporation of the tagged amino acid into the serum protein fractions, and the urinary excretion pattern of the radioactive label.

METHODS

Fifteen adult male New Zealand rabbits 6 to 8 months of age and weighing approximately 3 kg were equally divided by the use of a table of random numbers into 3 dietary groups, A, B, and C, after a 28-day control period during which all animals were fed a standard laboratory chow.[5] Following the control period and after an 8-day period for diet transition, a depletion period started on day 37 and continued through day 75; a repletion period extended from day 76 through day 165. The rabbits in group A were fed a depletion diet during the depletion period, while the animals in groups B and C were fed adequate diets and served as controls. The rabbits in groups A and B were changed from the standard chow to a purified ration during the diet transition period by gradually increasing the proportion of a 20% casein diet (table 1) mixed with the standard chow. On day 37, rabbits in group A were fed a 4% casein diet (table 1) exclusively and the rabbits in group B were fed the 20% casein diet. The diet for group A was continued through day 75 (depletion period), when the 20% casein diet was substituted for the 4% casein diet. The rabbits in group A were fed the 20% casein diet for 55 days through experimental day 130. The rabbits in group B were continuously fed the 20% casein diet for 94 days from day 36 through day 130. The diet in both groups A and B[6] was changed to the standard laboratory chow after day 130 and observations were continued for another 35 days until day 165, the end of the experiment. Rabbits in group C were fed the standard laboratory chow throughout the 165 days of observation.

TABLE 1

Composition of the adequate protein and low protein purified rabbit diets [1]

	Type of diet	
	Adequate	Depletion
	%	%
Vitamin-free test casein	20	4
Non-nutritive fiber	12	12
Hydrogenated vegetable oil [2]	8	8
Salt mix, USP XIV	6	6
Cane sugar	26	34
Cornstarch	26	34
Agar	2	2
Vitamin supplement [3]		

[1] Supplied to specification by General Biochemicals, Inc., Chagrin Falls, Ohio.
[2] Primex, Procter and Gamble Company, Cincinnati.
[3] Plus complete vitamin supplement as follows: (in mg/kg of ration) α-tocopherol, 224.8; Ca pantothenate, 45.0; choline chloride, 5993.4; *i*-inositol, 299.7; menadione, 2.2; niacin, 599.3; pyridoxine·HCl, 21.0; riboflavin, 21.0; thiamine, 21.0; vitamin D conc (400,000 USP units/g), 0.6; and vitamin A conc (200,000 USP units/g), 23.7.

A. Standard laboratory procedures. Each rabbit was weighed to the nearest 10 g and blood samples were obtained at 10-day to 2-week intervals. Hematocrits (12), hemoglobin content (13), and red cell counts on the Coulter counter were determined on each blood specimen. Blood volumes were measured using Radioiodinated Human Serum Albumin (RISA I^{131}) during the control period, at the time of maximal depletion and during repletion. Nitrogen gains and losses were determined by metabolic techniques for three 3-day continuous periods: 1) at the time of stabilization for all the rabbits (control period); 2) after the purified diets had been fed for 5 weeks for groups A (4% casein) and B (20% casein); at 10 weeks when the 2 groups were fed the 20% casein diet. Stainless steel metabolism cages[7] were used for the collections. The rabbits were not collared.

Serums were analyzed for their total protein content by the biuret method (14) with micro-Kjeldahl standards. Serum fractions were obtained by paper electrophoresis using a Spinco durrum-type cell.[8]

B. Sulfur-35 methionine studies. After 5-weeks of protein depletion 100 μc of S^{35}-L-methionine[9] per 2.5 kg of body weight were injected into the marginal ear veins of the rabbits in groups A and B and 2 rabbits from group C. Post-injection

[5] Rockland Rabbit Ration, A. E. Staley Manufacturing Company, Decatur, Illinois. Average analysis: (in per cent) protein, 17.4; fat, 1.6; fiber, 14.9; carbohydrates, 65.8; and ash, 6.0.
[6] One rabbit in group B died on day 129.
[7] Partsco Company, Inc., Columbus, Ohio.
[8] Beckman Technical Bulletin no. 6051A (1958). A method for serum proteins using bromphenol blue dye in alcoholic solution. Spinco Procedure B. Beckman Instruments, Inc., Palo Alto, California.
[9] The specific activity of S^{35}-L-methionine was not less than 61.77 μc/mg.

blood samples were obtained from the opposite ear at 1 hour and from alternate ears at 2, 5 and 7 hours.

The activity of the total serum protein in the samples was obtained by precipitating the protein from 0.25 ml serum with trichloroacetic acid and redissolving the precipitate in 1 ml of M Hyamine.[10] To this solution were added 20 ml of a dioxane base scintillation fluid. Samples were cooled to 4°, dark-adapted and counted in a liquid scintillation counter.[11] Post-injection radioactivity was measured in each of 5 successive 3-day urine pools by placing 0.2-ml aliquots directly into the dioxane scintillation fluid for counting. The rabbits in group A continued to consume the deficient diet for 5 days following administration of the S^{35}-methionine and were then rehabilitated with the 20% casein purified ration. After a 5-week period of refeeding the depleted animals, the rabbits in groups A and B and the 2 rabbits from group C received a second injection of S^{35}-methionine administered on the same weight basis and 2 additional rabbits fed the stock diet (group C) received a primary injection of the tagged amino acid. Pre-injection blood samples were obtained to measure residual activity and post-injection samples were drawn at 1, 2, 4, 6, and 8 hours and processed as described above. The activity in the urine was measured for the following 9 days by the procedure described after the original injection.

Incorporation of the S^{35} label into the serum albumin and the α-, β-, and γ-globulins was studied. After the first injection, serum samples obtained at 4 to 5 hours and at 5, 18 and 32 days were analyzed. After the second injection, the 4- to 5-hour serum sample and samples obtained 6 and 21 days post-injection were analyzed. According to the pack principle described by Armstrong et al. (15), strips of cellulose acetate,[12] 5 cm by 20 cm, were placed in packs of four in a horizontal water-cooled cell. Four-tenths milliliter of serum was applied approximately 6 cm from the cathode in an even line by a micropipette leaving side margins of 1 cm. One hundred-sixty volts were used for 18 hours to fractionate the serum. A barbital buffer (pH 8.6 and ionic strength 0.075) was used to give separations consistent with those obtained by paper electrophoresis, which was used as a standard.

Strip no. 3 of each pack was air-dried and stained in 0.2% Ponceau S Red for 10 minutes. The remaining strips were cut to the pattern of the stained strip for the albumin, α-, β-, and γ-globulins. These 4 portions were dissolved directly in a dioxane scintillation fluid for counting.

The radioactivity in the serum fractions is reported in terms of the percentage of the total activity present in each fraction and the specific activity adjusted by the protein concentration in the fractions.

C. Statistical evaluation. The statistical significance of the results was determined by the use of the t test for small numbers (16). Differences in actual mean values or the mean change between dietary groups were examined. A P value of 0.05 or less was taken as significant.

RESULTS

During the 7 days of change from the stock to the purified diet the rabbits in groups A and B showed less weight gain than the control animals fed the stock diet. The rabbits in group B, fed the 20% casein ration, began to gain weight at a rate similar to that of the animals fed the stock diet continuously. When the rabbits in group A were changed to the 4% casein ration, weight loss resulted (fig. 1). The average weight loss during the depletion period was 6% of the average weight at the beginning of the period. However, the average weight was 13% below the weight predicted for these animals if the same rate of increase in weight observed during the control period had continued during the period of depletion. At the completion of the rehabilitation period the rabbits in group A had achieved an average body weight similar to the average weights in groups B and C.

The average values during the control, depletion, and repletion periods for total serum protein, blood volume, red blood cells, hemoglobin and hematocrit in the

[10] Packard Instrument Company, La Grange, Illinois.

[11] Packard Series 314E. Tri-Carb Liquid Scintillation Counter.

[12] Sepraphore III obtained from Gelman Instrument Company, Ann Arbor, Michigan.

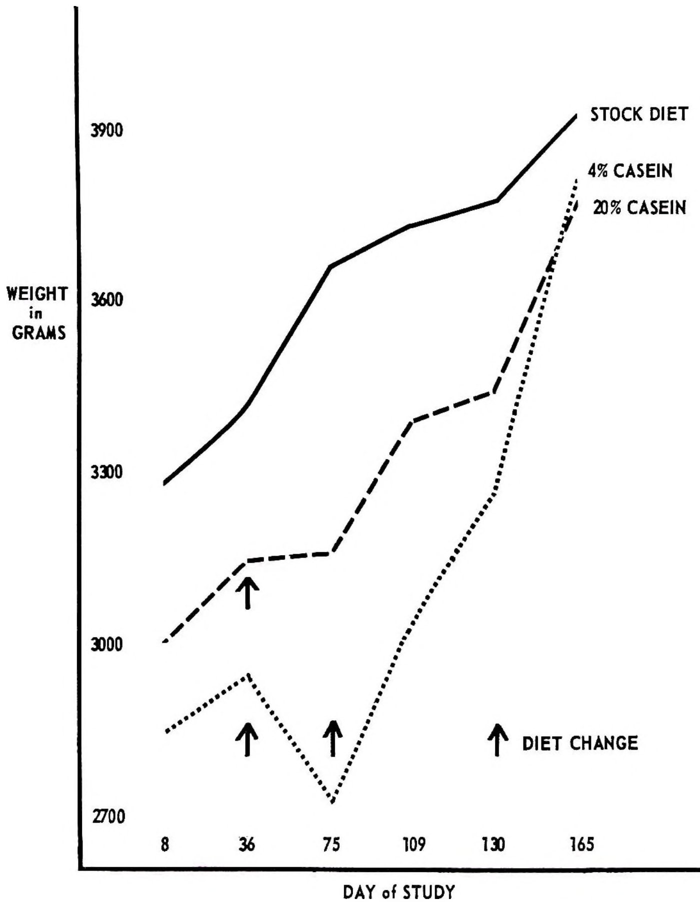

Fig. 1 Average weights of 3 groups of rabbits fed different diets during protein depletion-repletion studies.

3 groups of animals are shown in table 2. Total protein had decreased in group A (4% casein) by day 75, the end of the depletion period; however, this change was not significant by *t* test. Technical variations in blood volume measurement from one observation period to another make it difficult to compare changes over time in a single group of rabbits. When blood volume is related to body weight there were no significant differences between groups A and B during the depletion period or among all 3 groups during the control and repletion periods. A hypochromic microcytic anemia developed in the rabbits fed the 4% casein diet. This is shown by the significant decreases in red blood cell count, hemoglobin, and hematocrit on day 75 in table 2. Changes in the red cell indexes occurred in all groups and this is likely related to the repeated blood sampling during the study. The low values on day 115 were coincident with the end of a week of repeated bleedings. Significant differences among the groups were no longer present by day 130. There were no significant changes among the groups in serum protein fractions as measured by paper electrophoresis during the period of observation (table 3).

Nitrogen retentions, similar for the rabbits fed the 20% casein ration and the stock laboratory diet, averaged approximately 650 mg/day. Rabbits fed the 4% casein diet were losing an average of 220 mg of nitrogen per day at the time of maximal depletion. With the change to the 20% casein diet nitrogen retentions

TABLE 2

Average values for total serum protein, blood volume, red blood cells, hemoglobin, and hematocrit at various intervals in 3 groups of rabbits fed different diets

Study group	Study periods						
	Control	Depletion		Repletion			
	Day 28	Day 43	Day 75	Day 109	Day 115	Day 130	Day 165
			Total protein, g/100 ml blood				
A	6.16 [1]	5.94	5.44	6.70	6.59	6.43	6.04
B	6.05 [2]	6.25	6.36	6.27	6.55	6.39 [1]	5.98 [1]
C	6.11	6.17	6.12	6.05	6.46	6.16	5.82
			Blood volume, total ml				
A	167.2	167.6 [1]	113.4	— [3]	137.2 [1]	131.0	182.7 [1]
B	184.0 [1]	182.8	137.5	—	153.4	157.9 [2]	184.5 [2]
C	166.6	—	—	—	150.2 [1]	—	191.1 [1]
			Blood volume, ml/kg body wt				
A	58.9	58.0 [1]	42.2	—	43.7 [1]	40.0	48.3 [1]
B	60.4 [1]	59.5	44.1	—	44.8	45.5 [2]	49.5 [2]
C	56.2	—	—	—	39.8 [1]	—	48.0 [1]
			Red blood count, millions/mm^3				
A	5.99	6.20 [1]	4.87	5.19	4.73	4.54	5.80
B	5.71	5.97	5.90	5.60	4.98	4.82 [1]	6.01 [1]
C	5.77	—	—	6.28	5.38	5.17	6.50
			Hemoglobin, g/100 ml blood				
A	15.5	15.3 [1]	10.5	12.2	10.9	13.9	13.5
B	15.0	14.3	13.7	13.4	11.6	14.1 [1]	13.7 [1]
C	15.5	—	—	13.9	12.9	14.9	13.9
			Hematocrit, %				
A	37.3	41.7 [2]	27.8	34.0	34.8	36.2	39.4
B	39.6	39.4	36.6	37.0	35.6	37.4 [1]	39.8 [1]
C	39.5	—	—	38.0	38.8	40.5	39.8

[1] Four animals only.
[2] Three animals only.
[3] Value not determined.

for group A averaged between 800 and 1000 mg/day for the observed periods during repletion.

The serum was analyzed for the S^{35} present in the 4 basic protein fractions, namely, albumin and α-, β-, and γ-globulins. The trend of incorporation of the radioactive sulfur in the serum protein fractions over a month's study from depletion to repletion was observed. The percentage of the total original activity initially in the albumin fraction was 43.7 for those fed the 4% casein diet and 37.9 for the rabbits fed the 20% casein ration for 5 weeks (table 4). The percentage activity in the albumin fraction increased until the eighteenth day post-injection when activities were 68.8 and 64.2% in the depleted and control rabbits, respectively.

For the α-globulins an average decrease of 12% activity was noted between the original observations and the observations 5 days post-injection for the animals fed the 20% casein diet in contrast with an average decrease of 4.5% for the animals consuming 4% casein (table 4 and fig. 2). This difference in mean change was not significant ($t = 2.13$; $P > 0.05$). On the fifth day post-injection the diet was changed from 4% to 20% casein; a sharp decrease in the percentage activity in the α-globulin is seen in figure 2. This is similar to the original decrease in activity observed for the animals fed the 20% casein diet following the injection. The decrease from day 5 to 18 in the 4% casein group was significantly different from the decrease in the 20% casein group ($t = 2.34$;

TABLE 3

Average percentage of total serum protein in serum fractions at various intervals in 3 groups of rabbits fed different diets

Serum fraction	Study group	Control: Day 28	Depletion: Day 43	Depletion: Day 75	Repletion: Day 115	Repletion: Day 130	Repletion: Day 165
		%	%	%	%	%	%
Albumin	A	71.9 [1]	73.3	73.6	72.4	81.9	73.7
	B	74.7 [2]	70.7	71.5	76.9	80.3 [1]	71.8 [1]
	C	74.5	— [4]	75.9 [3]	73.2	75.0	70.2
α_1-Globulin	A	2.0 [1]	2.8	2.7	3.2	2.2	3.2
	B	1.7 [2]	2.6	2.1	2.6	1.9 [1]	3,1 [1]
	C	2.1	—	2.2 [3]	3.8	3.7	3.9
α_2-Globulin	A	7.6 [1]	7.7	9.0	7.2	3.7	6.2
	B	5.3 [2]	7.5	7.0	5.8	4.8 [1]	5.7 [1]
	C	7.4	—	6.6 [3]	7.3	6.4	5.8
β-Globulin	A	11.5 [1]	9.1	7.7	8.5	5.9	7.2
	B	9.9 [2]	10.7	9.4	7.6	6.7 [1]	10.4 [1]
	C	8.8	—	7.4 [3]	7.8	7.0	8.9
γ-Globulin	A	7.0 [1]	7.1	7.7	8.7	6.4	9.3
	B	8.4 [2]	8.6	9.4	7.2	6.3 [1]	9.1 [1]
	C	7.2	—	7.4 [3]	7.8	7.9	9.7

[1] Four animals only.
[2] Three animals only.
[3] Two animals only.
[4] Value not determined.

TABLE 4

Average percentage of total S^{35} activity in the serum protein fractions at the end of dietary depletion and during repletion

Serum fraction	Study group	Experimental day: 70 [1]	75	88	102	109 [1]	115	130
		%	%	%	%	%	%	%
Albumin	A	43.7	52.3	68.8	70.8	56.2	65.1	66.6
	B	37.9	55.5	64.2	71.2	51.2	59.2	67.5
α-Globulins	A	23.6	19.1	9.0	11.7	20.1	15.8	15.2
	B	27.0	15.2	11.3	11.5	24.6	17.2	15.9
β-Globulin	A	23.4	18.8	12.1	10.3	15.3	11.6	9.2
	B	25.1	17.9	12.1	10.3	17.2	13.6	9.2
γ-Globulin	A	9.3	9.8	10.1	7.5	8.4	7.5	9.1
	B	10.0	11.5	12.4	7.1	7.1	10.1	7.4

[1] Days of injection of S^{35}-methionine.

$P < 0.05$). The percentage activity in the β-, and γ-globulins for all groups decreased until day 18 and then stabilized. Data on rabbits fed the stock diet throughout the study showed no significant variation from the animals fed the 20% casein diet in any of the serum protein fractions.

After a 5-week period of repletion a second injection of S^{35}-methionine was given. No significant differences in the incorporation of the tracer were observed for dietary groups A and B (table 4 and fig. 2).

An analysis of the specific activity present in the serum fractions is shown in figure 3. Over the 18-day period analyzed, there was an actual increase in the specific activity in the albumin fraction for the rabbits depleted in protein. The control animals of group B demonstrated a gradual decrease in the specific activity of the

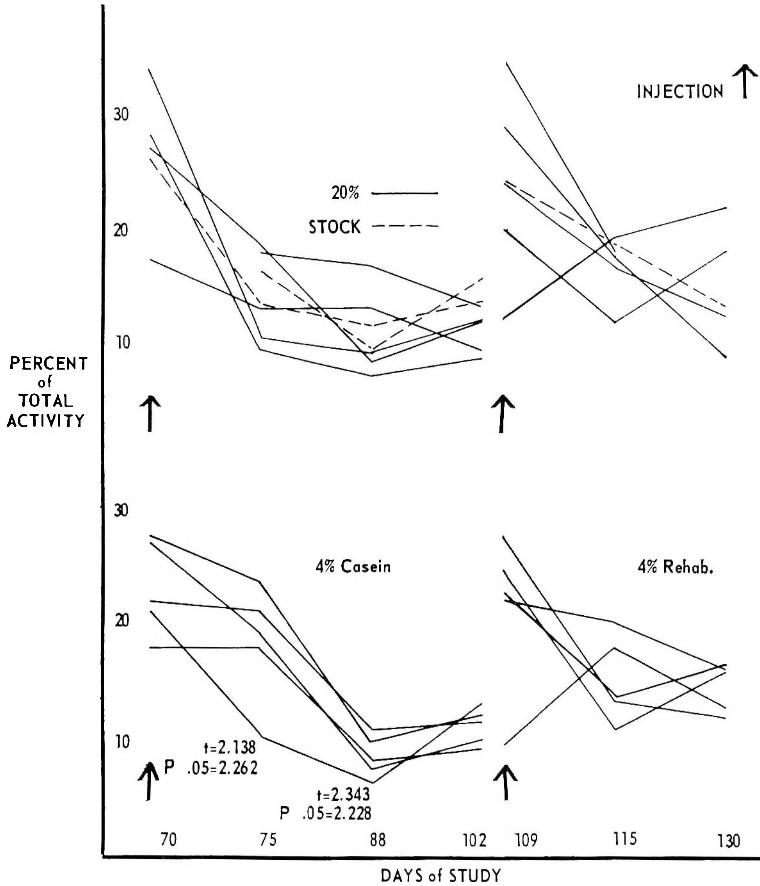

Fig. 2 Percentage of the total S^{35} activity in the α-globulin fractions following intravenous injection of S^{35}-L-methionine. The first injection was given 5 days before the end of the depletion diet and the second injection was given after 34 days of feeding an adequate diet.

albumin fraction which is in agreement with the anticipated decay of the isotope and half-life of the albumin fraction. The analysis of the percentage of activity in the α-fraction (fig. 2) demonstrates a persistence of the label in the depleted animals compared with the control animals but the loss in specific activity (fig. 3) is similar in the 2 groups.

The β- and γ-globulin fractions demonstrate a decrease in specific activity for both the depleted and the control animals. However, the actual counts per unit of protein were higher in the depleted than in the control rabbits.

S^{35} excretion patterns. Average urinary excretion of S^{35} per day per mg of urinary nitrogen in the 3-day pools are presented in table 5 for the rabbits fed the 4% casein diet and the 20% casein diet. In pool 1, collected at the time of maximal depletion, the urinary excretion of S^{35}/mg of urinary nitrogen was double that of the excretion in the normal animals. When total counts of S^{35}/day in the urine were measured, however, the normal animals excreted in excess of twice as much total S^{35} activity as the protein-depleted rabbits.

The difference in counts per milligram of N was still apparent in pool 2 collected for the 3 days immediately following the dietary change for group A from 4 to 20% casein. The excretion patterns gradually became more similar until by the sixth day of refeeding the differences in the total counts excreted were not significant

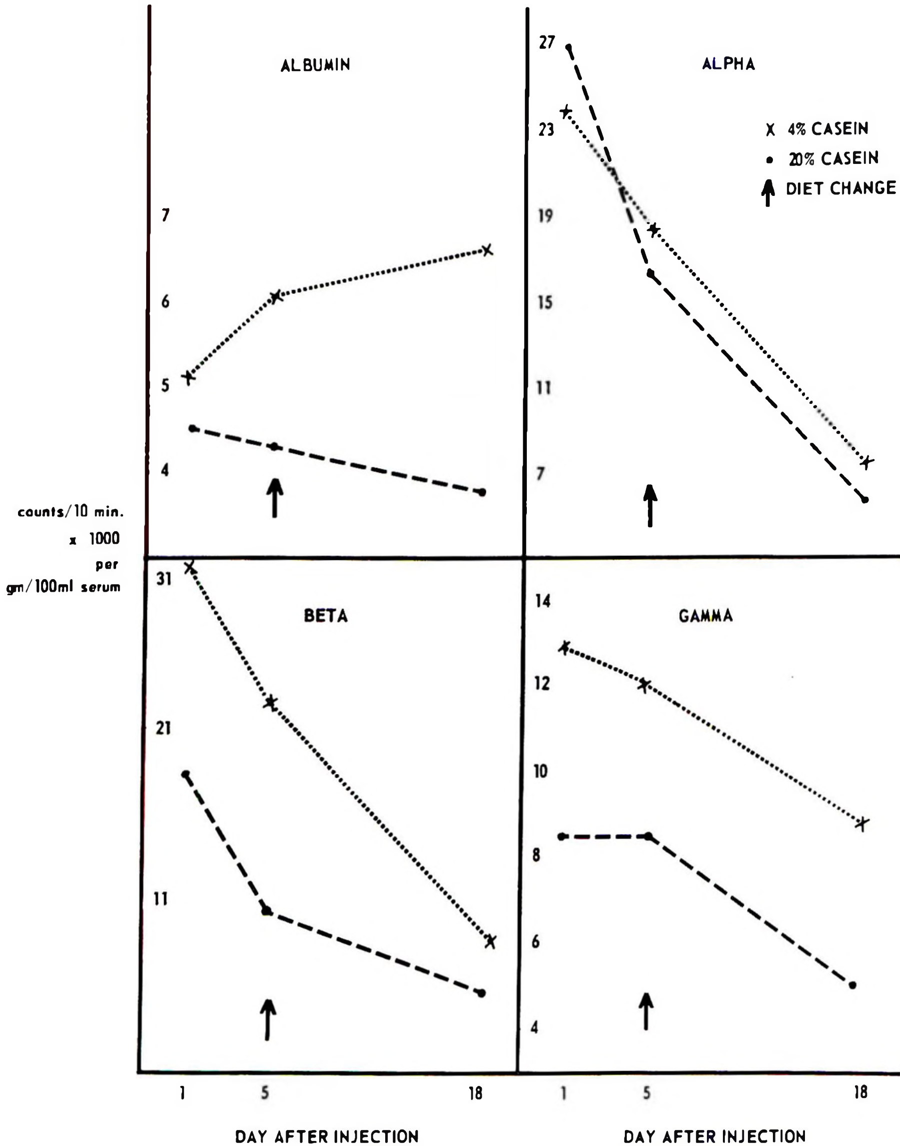

Fig. 3 Change in the average specific activity of S^{35} in serum protein fractions at 5 and 18 days after intravenous injection of S^{35}-L-methionine. The diet was changed from 4% casein to 20% casein in the 4% casein group 5 days after injection.

The second injection of S^{35} after 5 weeks of refeeding demonstrated no significant differences between groups A and B.

S^{35} uptake by serum proteins. The uptake of S^{35}-methionine by the serum proteins measured 5 times during an 8-hour period in the protein-depleted and control rabbits is illustrated in figure 4. The activity of the S^{35} measured in the precipitated total serum protein was calculated per milligram of serum nitrogen. The S^{35} label was taken up with greater avidity by the serum proteins of the protein depleted rabbits in contrast with the control animals. After a 5-week period of refeeding, a second injection of the S^{35} was taken up at a

TABLE 5

Average urinary excretion of S^{35} radioactivity at the end of dietary protein depletion and during repletion expressed as counts per unit of urinary nitrogen and as total counts excreted

Urine pools [1]	Study group	Counts [2]/mg N		Total counts [2]	
		Injection no. 1 (depletion)	Injection no. 2 (repletion)	Injection no. 1 (depletion)	Injection no. 2 (repletion)
1	A	17.4	8.2	5,374	8,719
	B	9.7	9.2	12,781	9,445
2	A	6.2	5.3	2,807	6,427
	B	2.5	5.7	2,835	6,321
3	A	5.0	3.7	2,483	3,765
	B	4.1	4.3	3,777	3,742
4	A	2.4	— [3]	1,420	—
	B	2.1	—	2,455	—
5	A	2.2	—	1,435	—
	B	2.1	—	2,263	—

[1] Consecutive 3-day pools.
[2] Expressed as actual counts/1000.
[3] Value not determined.

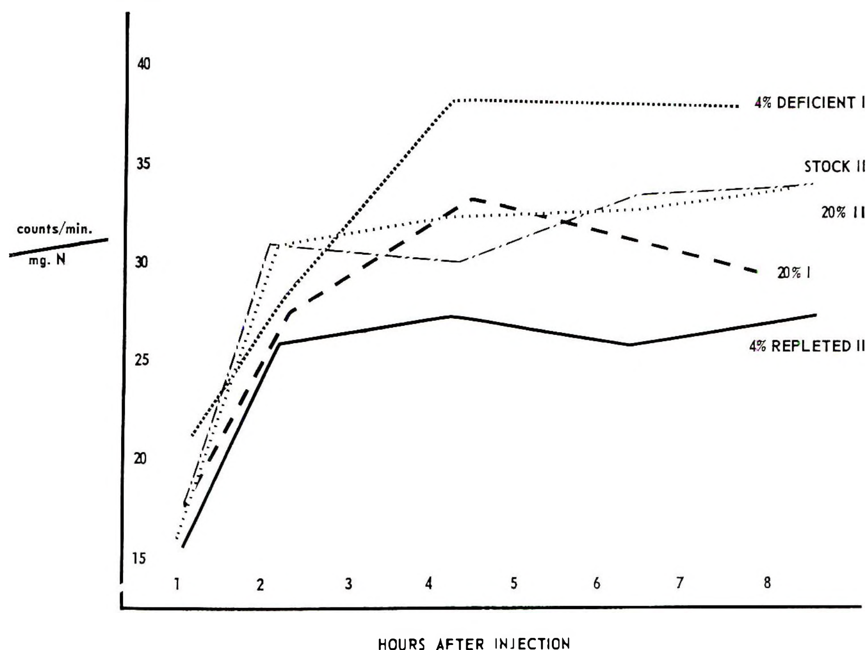

Fig. 4 Change in the average specific activity of S^{35} in total serum protein during 8 hours after intravenous injection of S^{35}-L-methionine. Curves marked with 1 bar were obtained during dietary depletion of the 4% group and curves marked with 2 bars were obtained after 34 days of dietary repletion of the same group.

significantly lower level by the rehabilitated rabbits. The uptake of the S^{35} did not change in the 2 study periods for the animals fed the 20% casein ration and followed a pattern very similar to that of the animals fed the stock diet.

DISCUSSION

Previous workers have shown that serum protein fractions, studied by chemical separations and electrophoretic techniques, have not been consistently altered in protein deficiency states (3, 17, 18). In fact

numerous inconsistencies are found in the literature with respect to changes in total serum protein concentration, serum protein fractions and the total amounts of circulating protein, as well as blood volume changes. This disagreement in the literature is more understandable when it is realized that: 1) subjects were at different stages of deficiency; 2) responses may be species specific; 3) different methods of serum protein separation were used; 4) certain adaptive mechanisms have been activated according to both inherited tendencies and environmental conditions. However explainable, this does not decrease the need for improved methodology to quantify protein nutriture.

Yuile and co-workers (19) demonstrated in protein deficiency that more protein was lost from the extravascular compartment than from the circulating plasma. Hoffenberg and co-workers (18) produced a mild protein deficiency in human beings over a relatively short duration. They observed no change in the albumin concentration or the total circulating albumin. Consistent with the observations of Yuile and co-workers, the greatest measurable decrease was calculated to occur in the extravascular pool. In this study, rabbits depleted in protein for a 5-week period showed no significant change in the blood volume, serum protein fractions or total circulating proteins. This gives further confirmation to the work of Whipple (20) which emphasized the preferential maintenance of intravascular proteins.

In 1945, two groups of investigators (21, 22) reported a decrease in serum albumin and certain globulins in the protein-depleted dog and the maintenance or an actual increase of the α-globulins. Succeeding field and laboratory studies did not confirm this observation (3). However, the use of the tagged amino acid, S^{35}-methionine, gives support to these observations. At the time the experiment reported herein was in progress, Wannamacher and co-workers (11) were studying serum protein synthesis during the development of protein deficiency in the dog. The dogs were injected with S^{35}-methionine after 4 and 8 weeks consumption of a protein free diet. Blood samples, obtained 6 hours after the injection, were fractionated and counted for radioactivity. With depletion, the α_1-globulins increased in percentage of activity, whereas the albumin, α_2- and β-globulins decreased. There was no change in the activity of the γ-globulins. The percentage of activity reported in these fractions is in agreement with the observations of the current experiment although the sample for study, fractionation and counting procedures differed. Wannamacher et al. (11) used a paper electrophoretic technique and a strip counter which recorded the activity of the intact pattern. In the present study a serum sample of 0.4 ml was separated on cellulose acetate support to obtain sufficient activity for liquid scintillation counting of the serum activity over a 30-day period. This methodology did not separate the α-globulins into their component parts, but the α-globulins, considered as one group, did maintain their activity in the protein-depleted rabbits. This is consistent with the observations of Wannemacher et al. (11) and earlier reports of others (21, 22). Recent work by Weimer (23) with rats depleted in protein specifically indicates that the α_2-globulins are maintained in conditions of severe nutritional stress in this species. The physiological function of this fraction remains unclarified.

When an adequate protein source was made available to the depleted rabbits the incorporation of the radioactive label by the serum fractions became similar to the control animals. Activity in the α-fraction decreased at the rate noted for the control rabbits directly after injection. Observation at a closer interval seems warranted to titrate the sensitivity of this response at different caloric and protein levels.

From the data here reported on both control and experimental animals, it was observed that after the original injection a higher concentration of the isotope was noted in the globulin than the albumin fraction. This could be related to equilibration phenomena or some peculiarity of transport. Following the label for a 30-day period in the serum protein fractions, it was observed that the activity tended to reverse itself from day 1 to day 18 at which time the activity was more nearly proportional to the actual protein concentrations in the fractions. The half-life of the albumin

versus the globulin components or a specific trend in the reutilization of the label, or both, must be considered in the interpretation of these data. The specific activity calculation, particularly for the albumin fraction, strongly suggests that albumin was preferentially being synthesized in the protein depleted rabbits. Work to confirm this observation seems indicated.

The urinary excretion patterns of S^{35} were definitive of protein deficiency. The control rabbits excreted more S^{35} during the observation period than the depleted animals. When the excreted S^{35} was related to the amount of urinary nitrogen excreted, the depleted animals excreted more S^{35}/mg of nitrogen than the control animals. This indicated conservation of the labeled amino acid by the depleted animals either through reutilization or reduced catabolism or both.

Garrow (6, 7) observed a higher rate of uptake of S^{35} methionine by the serum proteins of malnourished children and protein-depleted dogs. The present study also revealed higher S^{35} activity/mg of serum nitrogen in the protein-depleted rabbits in contrast with the normal control animals. This greater uptake of S^{35} by the rabbits marginally depleted in protein in contrast with the control rabbits demonstrated the sensitivity of this technique when other changes in serum protein were not apparent. These observations are consistent with the work of Garrow (6, 7) in more severe protein malnutrition. Bronsky and co-workers (24), however, were unable to demonstrate any change in the pattern of uptake of S^{35} by the serum proteins of a severely depleted patient after nutritional rehabilitation although the serum protein concentration of the patient had increased. After a 5-week period of refeeding, the rehabilitated rabbits had a reduced uptake in serum proteins, whereas the control animals showed no change in their pattern of uptake for the isotope. Explanation for this difference is not clear.

By the criteria of change in body weight, red cell indexes and nitrogen losses as determined by metabolic balances, there is an indication that a mild-to-moderate protein deficiency was produced by the 4% casein diet. Studies on the uptake of S^{35}-methionine by the serum proteins, the excretion pattern of the S^{35} label and the incorporation of the isotope into the serum protein fractions indicated that alteration in the metabolic processes of the rabbit had occurred and that this alteration was not detected by changes in total serum protein concentration or by changes in the serum protein fracitons. Continued investigation of the use of tagged amino acids for determination of nutritional status appears warranted.

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Excretion of Histidine and Histidine Derivatives by Human Subjects Ingesting Protein from Different Sources [1]

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ABSTRACT The 1-methylhistidine, histidine, 3-methylhistidine, anserine (β-alanyl-1-methylhistidine), and carnosine (β-alanylhistidine) content of urine from 5 subjects was determined by ion-exchange chromatography. Controlled diets containing 2 levels of protein were fed; one series contained protein from animal flesh; the other contained no flesh protein. More of the histidine derivatives were excreted by subjects eating the flesh protein diets than by those eating the non-flesh diets. The former subjects also responded to increased protein intake with increased excretion of the derivatives; the latter did not. 1-Methylhistidine excretion tended to parallel intake. Both of the dipeptides, anserine and carnosine, were present in the urine of all the subjects whether or not they were present in the diets; thus their source could be either exogenous or endogenous, or both. 3-Methylhistidine was not present in any of the foods analyzed (beef, chicken, milk, bread) but was present in the urine of all of the subjects. Its source is not known.

Hubbard and associates (1) reported that many patients with a certain dermatological disorder had abnormal ratios of 1-methylhistidine to histidine and 3-methylhistidine to histidine in the urine. Block and co-workers (2, 3) fed free L-histidine to these patients and to control subjects but no consistent effect on the excretion of the methylated histidines was noted. Earlier, Datta and Harris (4) had reported that urinary 1-methylhistidine was of dietary origin; and Stein et al. (5) concluded that its excretion roughly paralleled the amount of protein ingested by human subjects.

In the present investigation the effect on urinary content of 1-methylhistidine, histidine, 3-methylhistidine, anserine (β-alanyl-1-methylhistidine), and carnosine (β-alanylhistidine) was studied in human subjects, when diets containing 2 levels of protein were fed; one series of diets contained protein from animal flesh; the other series contained no flesh protein.

EXPERIMENTAL

Subjects. Five healthy adults, 4 male and 1 female, with normal liver and kidney function served as subjects. They ranged in age from 21 to 24 years; their weights, which remained constant throughout the study, were 83, 78, 62, 60, and 78 kg, respectively, for subjects F, K, P, Ke, and L. Throughout the study, they lived in a metabolic ward.

Diets. The major sources of protein and the amounts each contributed to the daily protein intake of the subjects are shown in table 1. Subjects F, K, and P were fed a constant diet in which 21 g of the total protein intake came from 100 g of beef during period 1 which lasted 10 to 11 days. In period 2, the intake of beef was increased to 400 g, and contributed 83 g of protein daily for 7 to 8 days. In period 3, white meat of chicken was substituted on an "iso-protein" basis for the beef for 3, 3, and 1 days, respectively, for subjects F, K, and P. Beef or chicken, plus milk and bread, accounted for between 65 and 90% of the total protein intake of the 3 subjects during the various diet periods (table 1).

Subjects Ke and L received no flesh protein; 50 g of their total protein intake of 54 g came from milk and bread during period 1 which lasted 6 to 7 days; milk and bread intake was doubled for the 7 days of period 2 (table 1).

Collection and analysis of urine. Twenty-four-hour urine collections were made dur-

Received for publication November 9, 1964.
[1] Supported in part by U. S. Public Health Service Grants GM-04413-09, AM-06825-02, and 5M01-FR-42-04.
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TABLE 1
Sources and amounts of protein fed to subjects

Source	Period 1				Period 2				Period 3		
	Subjects				Subjects				Subjects		
	F	K	P	Ke and L	F	K	P	Ke and L	F	K	P
Beef	21 [1]	21	21		83	83	83				
Chicken									84	84	84
Milk	14	9	6	42	32	25	7	84	32	21	4
Bread	16	11	11	8	16	11	11	16	16	11	11
Other	21	22	17	4	23	20	17	4	23	24	20
Total intake	72	63	55	54	154	139	118	104	155	140	119

[1] Grams of protein/day. U.S.D.A. Handbook no. 8, Washington, D.C., June 1950.

ing the last 3 days of periods 1 and 2, and during all the days of period 3. Phenol and HCl were used as preservatives, and the samples kept frozen at −20° until analysis was performed. They were prepared for analysis by the method of Stein (6), and analyzed for 1-methylhistidine, histidine, 3-methylhistidine, anserine and carnosine by ion-exchange chromatography (7).

Preparation and analysis of food samples. Beef and chicken. Ground beef or minced white meat of chicken was frozen and ground in a Wiley mill (40-gauge screen) at the temperature of liquid nitrogen. The powdered material obtained was lyophilized, defatted by extraction with diethylether-absolute ethanol mixture (3:1) for 24 hours in a Soxhlet extractor, and stored *in vacuo* until used. The powder was prepared for analysis of free amino acids (those contained in the non-protein fraction) by the procedure of Tallan and co-workers (8). For analysis of total amino acids (those contained in both the non-protein and protein fraction) a weighed portion of the powder was placed in 6 N HCl in a Pyrex tube, the tube sealed, and heated for 24 hours at 120°. HCl was removed under vacuum, the residue dissolved in a small amount of water, and transferred quantitatively to a volumetric flask with buffer (pH 2.2) (7).

Milk. If necessary, milk was defatted by centrifugation, and then lyophilized. The powder was prepared for analysis of free and total amino acids as above.

Bread. Bread was dried in an oven at 75° for 24 hours and ground to a fine powder. The powder was prepared for analysis of free and total amino acids as above.

The 1-methylhistidine, histidine, 3-methylhistidine, anserine, and carnosine content of the above preparations were determined by ion exchange chromatography (7).

RESULTS

Analysis of foods. Table 2 shows the free and total content of 1-methylhistidine, histidine, 3-methylhistidine, anserine, and carnosine of beef, chicken, milk, and bread in micromoles per gram of protein, and in grams per 100 g of food.

Free 1-methylhistidine, histidine, anserine, and carnosine were present in the non-protein extract from beef. The dipeptides, anserine and carnosine, were particularly abundant, 12.6 and 76.9 μmoles/g of beef protein, respectively. Approximately all of the 1-methylhistidine in beef was present as part of anserine (12.6 μmoles); this amount plus the 0.5 μmoles existing as the free amino acid account for 13.1 μmoles of the total 13.3 μmoles of 1-methylhistidine/g of protein in hydrolyzed beef. Histidine present as part of the dipeptide carnosine accounted for 76.9 μmoles or approximately 41% of the total amount of this amino acid present in beef after acid hydrolysis (table 2).

The non-protein extract from white meat of chicken contained 3.3, 85.8, and 58.8 μmoles of free histidine, anserine, and carnosine/g of protein, respectively. The total 1-methylhistidine present after acid hydrolysis of the chicken meat was 28.6 μmoles more than could be accounted for

TABLE 2

Free and total content of 1-methylhistidine, histidine, 3-methylhistidine, anserine, and carnosine of beef, chicken, milk, and bread

Compound	Beef		Chicken		Milk		Bread	
	Free	Total	Free	Total	Free	Total	Free	Total
1-Methylhistidine, µmoles/g [1]	0.5	13.3	0	114.4	0	0	3.8	0
1-Methylhistidine, g/100 g [2]	13×10^{-5}	0.036	0	0.400	0	0	54×10^{-5}	0
Histidine, µmoles/g	1.2	189.6	3.3	192.6	0	132.7	0	144.6
Histidine, g/100 g	27×10^{-5}	0.472	106×10^{-5}	0.617	0	0.072	0	0.191
3-Methylhistidine, µmoles/g	0	0	0	0	0	0	0	0
3-Methylhistidine, g/100 g	0	0	0	0	0	0	0	0
Anserine, µmoles/g	12.6	0	85.8	0	0	0	0	0
Anserine, g/100 g	470×10^{-5}	0	4129×10^{-5}	0	0	0	0	0
Carnosine, µmoles/g	76.9	0	58.8	0	0	0	0	0
Carnosine, g/100 g	2783×10^{-5}	0	2740×10^{-5}	0	0	0	0	0

[1] Micromoles/gram of protein.

[2] Grams/100 g of beef (16% protein); chicken (20.6% protein); milk (3.5% protein); bread (8.5% protein).

by liberation of this amino acid from anserine. Approximately 31% of 58.8 µmoles of the total histidine present could be accounted for by the histidine moiety of carnosine.

No free amino acids or dipeptides were present in milk. After hydrolysis, histidine was present.

Bread contained 3.8 µmoles of free 1-methylhistidine/g of protein; after hydrolysis, only histidine was observed.

3-Methylhistidine was not present in any of the foods analyzed (table 2).

Excretion of histidine and histidine derivatives. Table 3 shows the daily intake of 1-methylhistidine, histidine, 3-methylhistidine, anserine, and carnosine; and the excretion of these compounds expressed as milligrams per 24 hours and as percentage of intake by the 5 subjects.

The excretion of 1-methylhistidine by subjects F, K, and P tended to parallel intake during periods 1 and 2 when beef was the main source of the amino acid. The percentage of the ingested 1-methylhistidine excreted ranged from 75 to 88. During period 3, the intake of the methylated amino acid was increased approximately ninefold over that of period 2 without altering the total intake of protein; the amount of 1-methylhistidine excreted increased but not proportionately to its intake. Between 56 and 69% of the amount ingested appeared in the urine. Subject P, who received the chicken in period 3 for one day only, showed a delay in his output of 1-methylhistidine, e.g., the excretion remained high for several days after the period ended and he was returned to the beef diet of period 2. This delay in excretion could account for the lower percentage of 1-methylhistidine excreted during period 3. Subjects Ke and L, who received no flesh protein in either diet period 1 or 2, excreted approximately the same amounts of 1-methylhistidine during the 2 periods, although the intake during period 2 was double that of period 1.

The 5 subjects excreted between 4 and 10% of the histidine ingested. As the intake increased, the amount of histidine excreted increased but not in proportion of the intake, e.g., subject F ingested 1.265 and 3.543 g of histidine in periods 1 and 2, respectively, but his excretion increased

TABLE 3

Daily intake and excretion of 1-methylhistidine, histidine, 3-methylhistidine, anserine, and carnosine by human subjects

Subject	Diet period	1-Methylhistidine			Histidine			3-Methylhistidine		Anserine			Carnosine		
		Intake	Excretion		Intake	Excretion		Intake	Excretion	Intake	Excretion		Intake	Excretion	
		g	*mg*	%	*g*	*mg*	%	*g*	*mg*	*g*	*mg*	%	*g*	*mg*	%
F	1	0.057	45.1 ± 7.6 [1]	79	1.265	101.3 ± 28.5	8	0	49.9 ± 3.0	0.063	8.6	14	0.362	30.6 ± 23.3	8
	2	0.198	174.8 ± 7.1	88	3.543	154.9 ± 2.4	4	0	68.4 ± 7.2	0.252	58.3	23	1.448	86.8 ± 15.1	6
	3	1.703	1060.9 ± 153.9	62	3.657	166.4 ± 5.8	4	0	81.6 ± 6.2	1.692	225.0 ± 39.6	13	1.096	79.6 ± 6.2	7
K	1	0.054	43.0 ± 6.1	80	1.164	70.5 ± 19.8	6	0	48.7 ± 8.9	0.063	16.1 [2]	24	0.362	23.3 ± 16.3	6
	2	0.194	160.1 ± 5.3	82	3.321	114.3 ± 25.0	4	0	88.4 ± 9.8	0.252	26.8 [2]	11	1.448	89.4 [2]	6
	3	1.607	1117.2 ± 66.5	69	3.263	140.1 ± 22.4	4	0	nc [3]	1.692	nc		1.096	93.5 ± 14.9	9
P	1	0.054	42.8 ± 0.4	79	1.121	114.2 ± 27.0	10	0	47.5 ± 2.1	0.063	9.8 ± 0.7	15	0.362	15.9 [2]	4
	2	0.194	146.1 ± 4.2	75	2.961	173.1 ± 14.6	6	0	83.4 ± 5.8	0.252	nc		1.448	106.5 ± 11.8	7
	3	1.607	893.5 [2]	56	2.903	201.1 [2]	7	0	216.8 [2]	1.692	nc		1.096	109.9 [2]	10
Ke	1	0.005	3.2 ± 0.8	64	1.055	101.8 ± 6.5	9	0	25.9 ± 1.0	0	3.9 ± 3.7		0	11.6 ± 3.9	
	2	0.010	3.4 ± 0.6	34	2.110	138.4 ± 26.5	6	0	23.7 ± 2.3	0	11.8 ± 1.7		0	14.6 ± 1.2	
L	1	0.005	4.2 ± 2.2	84	1.055	70.2 ± 3.2	7	0	40.6 ± 2.0	0	12.4 ± 3.6		0	13.1 ± 4.7	
	2	0.010	4.7 ± 1.2	47	2.110	87.3 ± 10.7	4	0	39.0 ± 3.2	0	15.7 ± 8.9		0	14.4 ± 3.6	

[1] Mean ± SD excretion for 3 consecutive 24-hour periods.
[2] Excretion value for one 24-hour period.
[3] Not calculated for technical reasons.

only from 101.3 to 154.9 mg. The response of the subjects to increased intakes of histidine was the same whether they were eating diets containing flesh protein (subjects F, K, and P), or diets devoid of flesh protein (subjects Ke and L).

3-Methylhistidine was present in the urine of all subjects, and ranged in amounts from 23.7 to 216.8 mg/24 hours, although the apparent intake was zero. Subjects F, K, and P showed an increase in excretion of 3-methylhistidine from period 1 to period 2 when the protein intake was doubled; subjects F and P showed a further increase when chicken was substituted on an "iso-protein" basis for beef in period 3. Excretion of 3-methylhistidine by subjects Ke and L, who received non-flesh protein diets, remained essentially constant when the protein intake of period 1 was doubled (period 2).

Anserine was present in the urine of all 5 subjects, ranging in amounts from 3.9 to 225.0 mg/24 hours. The amounts excreted by subjects F and K increased when the intake of the dipeptide increased. Subject F excreted large quantities, 225.0 mg, during period 3 when chicken, a good source of anserine, was fed. Unfortunately, values for subjects K and P could not be calculated for this latter period. Subjects Ke and L, although ingesting diets in which no anserine could be detected, excreted the dipeptide (table 3).

Carnosine excretion by the 5 subjects ranged between 11.6 and 109.9 mg/24 hours. Excretion of the dipeptide tended to parallel its intake by subjects F and K; subject P, however, showed a progressive increase in the percentage of ingested carnosine excreted during periods 1, 2, and 3. Carnosine content of the urine of subjects Ke and L remained essentially the same regardless of protein intake. None of the dipeptide could be detected in the latter diets (table 3).

DISCUSSION

The source of dietary protein influences the amounts of 1-methylhistidine, 3-methylhistidine, anserine, and carnosine present in the urine of man. Subjects ingesting protein from animal flesh (beef and chicken) excrete more of the histidine derivatives than those eating non-flesh protein (bread and milk). Increasing the intake of flesh protein results in increased amounts of urinary 1-methylhistidine, 3-methylhistidine, anserine, and carnosine; increasing the intake of non-flesh protein does not. Histidine excretion is an exception to this; increased intakes of histidine result in increased excretion of the amino acid regardless of the source of the dietary protein.

The difference between the results found with the 2 sources of protein cannot always be explained by differences in their content of histidine and histidine derivatives. 3-Methylhistidine was not detected in any of the diets fed regardless of protein source, but it was present in the urine of all the subjects. Its excretion increased when increased amounts of flesh protein were fed, but remained constant when increased amounts of non-flesh protein were given.

3-Methylhistidine is a normal constituent of urine (9) but little is known of its source, and nothing of its function in the body. Block and co-workers (2) showed that L-histidine (free base) fed in a single 10-g dose decreased excretion of 3-methylhistidine by subjects receiving a controlled diet. Smaller amounts of histidine fed over a 3- to 7-day period had an erratic effect on excretion; 2 subjects showed a slight increase, one a decrease. Under the conditions of their study, urinary 3-methylhistidine did not appear to arise from ingestion of free histidine, per se. Tallan et al. (8), using ion-exchange chromatography, observed less than 3.2 mg of 3-methylhistidine in picric acid, protein-free extracts of 100 g of cat gastrocnemius muscle. In the present study, the analytical technique used (8) revealed no 3-methylhistidine in either extracts or acid hydrolysates of chick pectoral muscle and beef gluteal muscle. The metabolic inactivity of 3-methylhistidine is illustrated by the work of Cowgill and Freeburg (10) who showed that the radioactivity in the muscles of chicks, rabbits, rats, and frogs injected with 3-$C^{14}H_3$-histidine was present as the original injected amino acid and was not incorporated into a dipeptide or protein.

The 1-methylhistidine content of urine of man is dependent on his intake of flesh protein, and consequently of anserine, ac-

cording to Datta and Harris (4). The results of the present study confirm this observation. Stein et al. (5) reported that 1-methylhistidine excretion reflects the level of protein in the diet. In the present study, this was true when the protein in the diet was beef. If, however, protein intake was kept constant and chicken substituted on an "iso-protein" basis for beef, urinary 1-methylhistidine was substantially increased, presumably because of the large amount of anserine present in chicken muscle.

It has been reported that many patients with psoriasis vulgaris (1); hyperparathyroidism, argininosuccinic aciduria and Fanconi syndrome with cystinosis (11); and some women in the follicular phase of the menstrual cycle (12) excreted more 1-methylhistidine than histidine. The present study indicates that diet rather than abnormal metabolism may cause this effect in the diseases and the physiological state mentioned. More 1-methylhistidine than histidine was present in the urine of all the subjects (F, K, and P) when chicken was fed, and when 400 g of beef were ingested by subjects K and P.

1-Methylhistidine excretion remained constant for all practical purposes when diets devoid of anserine were fed (subjects Ke and L). Bread, the only source of 1-methylhistidine in these diets, contains 5 mg of the free methylated amino acid/100 g, but doubling the intake of bread did not alter the output of the amino acid by the subjects. 1-Methylhistidine has a high rate of renal clearance (11) and increased intakes would be expected to cause increased amounts in the urine if absorbed normally, unless it was metabolized or incorporated into some body tissue. Further substantiation of the effect of anserine intake on the excretion of 1-methylhistidine is evident if the percentage of the 1-methylhistidine moiety of ingested anserine excreted is compared with the percentage of the total 1-methylhistidine intake excreted. For example, subject F excreted 102, 99, and 90% of the 1-methylhistidine moiety of the dipeptide, anserine, ingested during diet periods 1, 2, and 3, respectively; if calculated on the basis of the percentage of total 1-methylhistidine ingested, the values were 79, 88, and 62. The percentage range of excretion is less, calculated by the former method than by the latter method; this was true for all of the 3 subjects, F, K, and P, eating flesh protein diets.

Datta and Harris (4), from the results of their studies with subjects fed rabbit meat (which contains a large amount of anserine), suggested that the dipeptide may be absorbed intact from the gut and excreted unchanged in the urine. Both the methylated and non-methylated dipeptides of β-alanine and histidine were present in urine from subjects in the present study, regardless of whether the dipeptides were present in the foods eaten. Thus it appears that urinary anserine and carnosine may arise either directly from absorption of the dipeptides through the gut or indirectly from formation of the dipeptides in the tissues of the body, or from a combination of the two.

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Effect of Dietary Fat on Plasma and Liver Lipids of Propylthiouracil-treated Rats

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ABSTRACT The administration of 0.03% propylthiouracil to rats resulted in an increase in plasma cholesterol and phospholipid levels. No change was observed in liver cholesterol and total lipids. The induced hypercholesterolemia was not lowered by feeding a supplement of 20% cottonseed oil. However, further increased concentrations of plasma cholesterol and phospholipid were observed in the hypothyroid rats maintained with a diet containing 20% hydrogenated coconut oil. In the normal animals, the plasma cholesterol concentrations were augmented by the addition of dietary fat. The liver cholesterol and total lipids increased in all animals when their diets were mixed with unsaturated fatty acids.

The degree of unsaturation of fatty acids has been shown to exert little or no effect on the serum cholesterol levels in rats (1). In man, on the other hand, the level of serum cholesterol can be lowered by the substitution of vegetable or unsaturated oil (2.3). The discrepancy between 2 species may be related to the relatively low values of serum cholesterol (4) and high resistance to the development of atherosclerosis in rats (5). The purpose of the present work was to investigate whether a dietary supplement of unsaturated fatty acids would alter the hypercholesterolemia which was induced by the administration of propylthiouracil.

MATERIALS AND METHODS

The 36 Sprague-Dawley rats used in this study weighed approximately 150 g. The animals were divided into 3 equal groups to which a basal ration of ground commercial laboratory chow [2] was fed. One group received 20% cottonseed oil, another 20% hydrogenated coconut fat, and the third, which served as a control, 36% sucrose. Furthermore, one-half of the rats in each group had 0.03% propylthiouracil added to their diets. The normal and propylthiouracil-treated animals were pair-fed among themselves with 3 different diets according to a caloric basis. Their body weights were measured weekly. At the end of 7 weeks, the rats were anesthetized with Na pentobarbital (40 mg/ kg in 1.5% solution). Blood samples were taken by cardiac puncture or from the dorsal aorta. A 2% solution of Mepesulfate [3] was used as an anticoagulant.

The weight of whole liver was determined. Total lipids in plasma and liver were extraced with a 2:1 chloroform-methanol mixture and the lipid extract was purified (6). Plasma and liver total cholesterol concentrations were determined according to Abell (7). Phospholipid phosphorus was analyzed by King's method (8). The liver total lipids were measured gravimetrically.

All values were analyzed statistically. The null hypothesis was rejected at a 5% level and a P value of 0.05 or less was considered significant.

RESULTS AND DISCUSSION

The results of the experiment are summarized in table 1. The rats receiving 0.03% propylthiouracil showed a distinct retardation of growth which became apparent after 3 weeks. The thyroid glands of these animals were hyperemic at the time of killing.

Although a significant increase in plasma cholesterol and phospholipid was demonstrated in the hypothyroid rats, the expectation that cottonseed oil might lower the elevated plasma lipid levels was not

Received for publication November 14, 1964.

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

[3] Mepesulfate (Roche) is the sodium salt of sulfated polygalacturonic acid methyl ester methyl glycoside.

TABLE 1
Effect of propylthiouracil and dietary fat on rat plasma and liver lipids [1]

Group	Wt gain	Plasma		Liver			
		Cholesterol	Phospholipid	Weight		Cholesterol	Total lipids
	g	*mg/100 ml*	*mg/100 ml*	*g*	*g/100 g body wt*	*mg/g wet tissue*	*mg/g wet tissue*
Sugar	188	81.4 ± 14.6	159.4 ± 16.4	12.2	3.7	2.6 ± 0.31	54.3 ± 5.6
Sugar + PTU [2]	51**	128.6 ± 9.3**	183.8 ± 17.9**	6.5**	3.1	2.8 ± 0.38	52.9 ± 5.5
Cottonseed	198	100.0 ± 9.2*	165.1 ± 20.2	11.7	3.3	5.9 ± 0.76*	86.3 ± 14.8*
Cottonseed + PTU	54**	121.9 ± 20.5**	175.3 ± 14.3**	6.1**	3.1	5.0 ± 1.5*	91.8 ± 23.7*
Coconut	179	106.3 ± 13.2*	181.5 ± 12.2*	10.5	3.2	2.9 ± 0.44	75.4 ± 9.6*
Coconut + PTU	51**	145.7 ± 13.9**	217.2 ± 28.8**	6.4**	3.2	3.2 ± 0.41	83.0 ± 12.0*

[1] All values are means ± SD: $S = \sqrt{\frac{\Sigma X^2 - (\Sigma X)^2/N}{N-1}}$

[2] PTU indicates 0.03% propylthiouracil.

* Statistically significant when compared with the sugar-fed animals.

** Statistically significant when compared with the corresponding group without PTU.

fulfilled. However, in the propylthiouracil-treated animals, the addition of 20% hydrogenated coconut fat in the diet increased the phospholipid level ($P < 0.05$) and the severity of hypercholesterolemia ($P < 0.05$) when the values were compared with those of the sugar-fed animals. In the normal rats, increased plasma cholesterol was observed by the addition of either cottonseed or hydrogenated coconut oil into the diet ($P < 0.05, < 0.02$). This observation indicates that the plasma cholesterol level in normal rats cannot be lowered by the supplement of unsaturated fatty acids. On the contrary, there is a tendency for plasma cholesterol level to increase when an excess amount ($\geqq 20\%$) of any dietary fat is substituted.

The administration of propylthiouracil to the rats resulted in a decreased liver weight ($P < 0.001$). This reduction is proportional to the decreased body weight. The levels of liver cholesterol and total lipids were not significantly changed by the administered drug. However, the supplement of 20% cottonseed oil increased both liver cholesterol ($P < 0.01$) and total lipids ($P < 0.01$) in the normal and propylthiouracil-treated rats. The addition of 20% hydrogenated coconut oil only increased the liver total lipids ($P < 0.001$). The accumulation of liver cholesterol might be due to an increased formation of cholesterol ester. The incorporated fatty acid of the ester may be derived from the unsaturated linoleic acid of the cottonseed oil (9, 10).

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Use of a Re-entrant Ileal Fistula to Study Carbohydrate Utilization by the Young Bovine [1]

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ABSTRACT Four calves were fitted at 3 weeks of age with re-entrant ileal fistulae located about 13 cm orad to the ileo-cecal junction. The calves were fed milk, or milk with sucrose or starch added, at various ages from 1 to 4.5 months. The diet was supplemented with vitamins, trace minerals, and an antibiotic. Polyethylene glycol (PEG) was added as a marker. Ileal and fecal samples were taken at zero, 3, 6, 9, and 12 hours after the morning feeding. The utilization of lactose orad to the fistula was high, and digestion in the entire digestive trace was essentially complete. The average apparent digestion of sucrose by the 4 calves was 84%. The recovery of sucrose at the fistula varied with the time of sampling, and was usually highest at 3 and 6 hours after feeding. Limited observations indicated that 87% of the digesta that passed through the fistula in the 12-hour period passed during the 6-hour interim from 1.5 to 7.5 hours after feeding. Based on these data, sucrose digestion orad to the fistula averaged 41% for the 4 calves. Digestion of starch orad to the fistula could not be determined, since the starch did not pass at the same rate as the PEG.

When young calves consume liquid feed, the material by-passes the rumen and goes directly into the abomasum (true stomach) via the esophageal groove and sulcus omasi. Initially, it was assumed that digestion in young calves was similar to that in non-ruminants, but recent research has shown that this is not always the case. In studies with carbohydrates, which were fed so as to avoid rumen fermentation, it was shown that although glucose and lactose were very well utilized, the use of maltose was dependent on the age of the animal (1–8). The degree of utilization of sucrose and starch remains unclear. When blood reducing sugar was used as a measurement of response, starch was poorly utilized and sucrose was not utilized at any age (1, 2, 4, 7). However, conventional digestion trial studies indicated that sucrose and starch were utilized to an appreciable exent (5, 8–10). Other data have been published which indicated that the disappearance of sucrose and starch was due to the action of microorganisms in the posterior portion of the intestinal tract (1, 5).

The objective of the present investigation was to determine, by means of a re-entrant ileal fistula technique, how much of the apparent utilization of certain carbohydrates was due to true digestion and how much was due to microbial breakdown in the intestines.

EXPERIMENTAL

Animals. Four male dairy calves, ranging in size at birth from 38 to 46 kg, were used. The animals remained with their dams for 3 days after birth, then were housed in individual metal and masonry pens. Wood shavings were used to bed calf 5180 at all times, and calf 6062 for the first 6 weeks. With these exceptions, the calves were maintained on heavy metal screens.

When the calves were 3 weeks old, a re-entrant ileal fistula was established by the method described by Markowitz (11) for establishment of a Crocker-Markowitz intestinal fistula. The rubber cannulae were the type described by Dougherty (12); their sizes are shown in table 1. Externally, the fistulae were located slightly ventral to the right paralumbar fossa and, internally, were orad to the ileo-cecal junction the following distances: (in cm) 15

Received for publication September 19, 1964.

[1] Journal Paper no. J 4937 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project no. 1324.

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TABLE 1

Size of cannulae

Animal no.	Base			Barrel		External washer	
	Thickness	Length	Width	I. D.	O. D.	Thickness	O. D.
	cm	*cm*	*cm*	*cm*	*cm*	*cm*	*cm*
5180	0.8	8	2.9	1	1.6	1.6	5.7
6062, 7046, 7051	0.8	8	3.3	1.3	2.1	1.6	5.7

in calf no. 5180; 4 in no. 6062; 12.5 in no. 7046; 15 in no. 7051. The exposed ends of the cannulae were connected in calf no. 5180 by a U-shaped Plexiglas tube. In the other 3 animals the connection was made by inserting a right angle Plexiglas elbow into each cannula and forming a union with plastic tubing.

Treatments. The claves did not consume dry feed at any time. Whole milk was fed by nipple pail in two equal feedings at 12-hour intervals at the rate of 10% of body weight/day. The milk was supplemented with a premix which provided each calf approximately 50 mg chlortetracyline, 960 IU vitamin D_2, and 10,000 IU vitamin A/day. Each calf was also fed 4 g/day of a trace mineral mix [3] containing manganese sulfate, iron sulfate, iron oxide, copper sulfate, potassium iodide, cobalt carbonate and zinc oxide, and which was added to the milk. The utilization of sucrose, lactose and starch was evaluated in separate trials. The number of trials with each carbohydrate and the ages of the calves at the time the trials were conducted are shown in table 2.

During the sucrose trials, milk was fed at the usual rate. Sucrose[4] and polyethylene glycol, average molecular weight 4000, were added to the milk at the rate of 10 g and 1 g, respectively, for each 454 g of milk. Starch trials were conducted in the same manner except that starch [5] was substituted for sucrose. During the lactose trials, no carbohydrate was added to the milk; polyethylene glycol was added at a rate calculated to give a ratio of lactose to polyethylene glycol of 20 to 1. The actual ratio fed was determined by chemical analysis.

Most of the trials consisted of a 3-day adjustment period and a 4-day collection period. Longer periods were not deemed advisable due to the stresses imposed by sucrose feeding and by frequent manipulation of the cannulae.

TABLE 2

Age of calves at beginning of trials

Trial	Animal no.			
	5180	6062	7046	7051
	days	*days*	*days*	*days*
Sucrose 1	29	35	31	31
Sucrose 2	85	85	80	80
Sucrose 3	—	128	—	127
Lactose	—	49	50	50
Starch	—	56	65	65

Collection and analysis of samples. Ileal and fecal samples were taken at zero, 3, 6, 9, and 12 hours after the morning feeding. Ileal samples were obtained by disconnecting the exterior connecting tube and removing digesta in the tube or a portion of that which passed through within 20 minutes. An equal volume of warm Ringer's solution was placed in the tube to replace the sample removed. Fecal samples were obtained by mechanical stimulation. The pH of all samples was determined immediately; the samples were then frozen for subsequent analyses.

In one sucrose trial with each of 3 calves, the ileal sample was collected, when possible, in 2 ways. Digesta in or near the exterior tube at sampling time were collected and designated as fraction 1. Another sample, designated as fraction 2, was collected from digesta passing from the proximal cannula from 1 to 20 minutes after sampling time. Trials conducted in this manner were no. 6062 — sucrose 3; no. 7046 — sucrose 2; and no. 7051 —

[3] Analysis of the trace mineral mix was: (in per cent) manganese, 12.5; iron, 15; copper, 2; cobalt, 1.5; zinc, 4.0; and iodine, 0.2.

[4] Table grade, obtained from local food store.

[5] Amioca (amylopectin), National Starch Products, Inc., Plainfield, New Jersey.

sucrose 2. (Trials are designated by calf number, carbohydrate fed, and sequence, respectively.)

During the last 2 days of trial 7051 — sucrose 3, all of the digesta passing from the ileum were collected between the morning and the evening feedings. The digesta were collected in a ballon placed over the proximal cannula. At frequent intervals the contents of the balloon were transferred to a container in an ice chest. At the end of each hour, an aliquot was taken for analysis and an equal amount of Ringer's solution was added to the remainder. The mixture was warmed and introduced into the distal cannula during the next hour at approximately the rate at which it had been collected.

Sucrose and reducing sugar in samples from sucrose trials were determined by the method of Huber et al. (5). Samples from lactose trials were analyzed by removing proteins by the method of Nelson (13) and determining total carbohydrate by the method of Montgomery (14). Carbohydrate in samples from starch trials was determined by the method of Montgomery (14) after hydrolysis with hydrochloric acid and precipitation of proteins with trichloroacetic acid.

Analysis for polyethylene glycol in feces and in milk was conducted according to the method of Hydén (15). The same method was used for ileal samples in the first 2 trials conducted. In the second and subsequent trials, Smith's (16) modification of Hydén's method (15) was used. Similar results were obtained in the second trial when both methods were used.

Statistical analysis. Statistical significance was determined by a complete least squares analysis. Further statistical treatment was according to Snedecor (17).

RESULTS

Growth and development of all calves were satisfactory and the fistulae remained functional for 11 to 41 weeks. Stoppage of the exterior connection sometimes occurred, particularly when calves were bedded with wood shavings, some of which they consumed. However, the stoppage was reduced greatly when the calves were maintained on screens.

Ileal samples. Sucrose and polyethylene glycol content of ileal samples from all sucrose trials is shown as the ratio of sucrose to polyethylene glycol (fig. 1). Since the sucrose-to-polyethylene glycol ratio as fed was 10, a straight line at a ratio of 10 would indicate complete recovery of sucrose. Recovery was usually highest at 3 and 6, and lowest at zero and 12 hours after feeding. Figure 2 shows the results of the trial undertaken to determine the flow rate of ileal digesta. Approximately 87% of the digesta that passed through the fistula in the 12-hour period passed during the 6-hour interim from 1.5 to 7.5 hours after feeding. The correlation coefficient of the sucrose-to-polyethylene glycol ratio and ileal digesta flow ($r = 0.94$) was significant ($P < 0.01$). Because of the different amounts of digesta represented by the samples taken 3 and 6 hours after feeding as compared with the other samples, weighted average digestion coefficients (based on the amount of digesta each of the 5 samples represented) were calculated. In the trials in which 2 types of samples were taken, an average of the two was used. Table 3 shows the coefficients of digestion orad to the large intestine for sucrose and lactose trials.

It was apparent from the starch-to-polyethylene glycol ratios, which often greatly exceeded 10, that the starch used in this study and polyethylene glycol did not pass at the same rate; therefore digestion coefficients could not be calculated.

The reducing activity of ileal digesta in sucrose and lactose trials is shown in figure 3. In lactose trials, this activity could result from lactose, glucose, and galactose, the last two from hydrolyzed lactose. In sucrose trials this activity could result also from glucose and fructose derived from hydrolysis of sucrose. Although no lactose trial was conducted with calf no. 5180, the absence of an appreciable amount of reducing activity in the sucrose trials indicated a high utilization of lactose orad to the large intestine by this animal as well. The trend, particularly with certain trials, was for the reducing activity to be directly correlated with sucrose digestion. However, with the data from all trials combined, the reducing activity of digesta from

sucrose-fed calves was not significantly higher than that from lactose-fed calves.

There were no significant differences in ileal pH values among the different calves or due to the various carbohydrates fed. The ileal pH values for samples collected in trial 5180 — sucrose 2 (the only trial in which pH was measured with this calf) were more alkaline than in other trials. There was a trend, especially with calf 7046, for the ileal pH of fraction 1 samples to be lower than that for fraction 2.

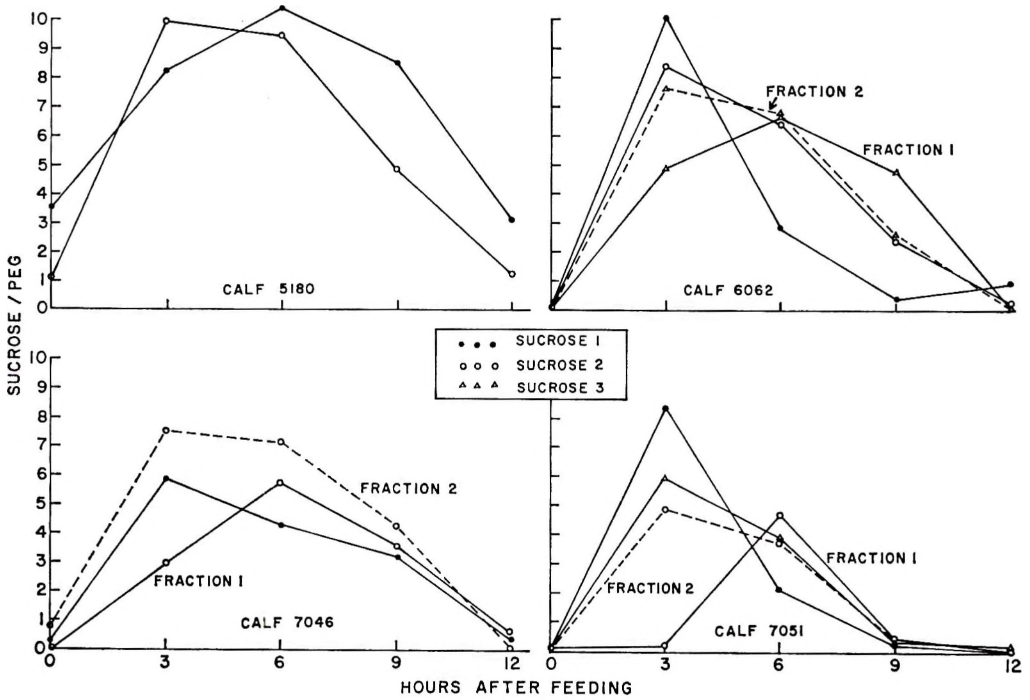

Fig. 1 Changes in ileal sucrose-to-polyethylene glycol (PEG) ratio.

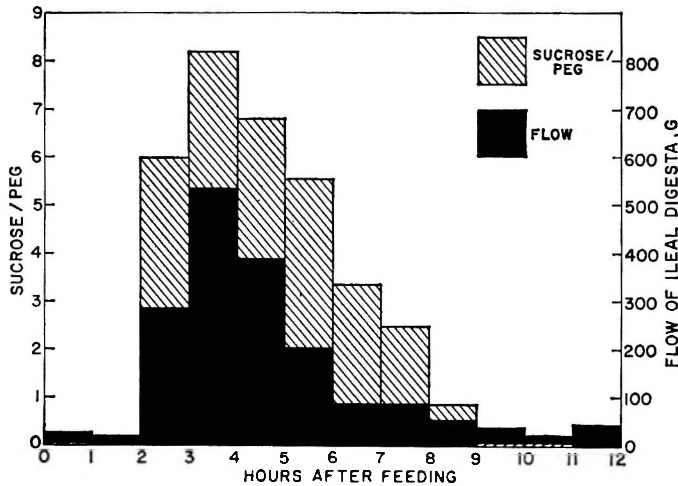

Fig. 2 Comparison of sucrose-to-polyethylene glycol (PEG) ratio with ileal digesta flow, trial 7051-sucrose 3.

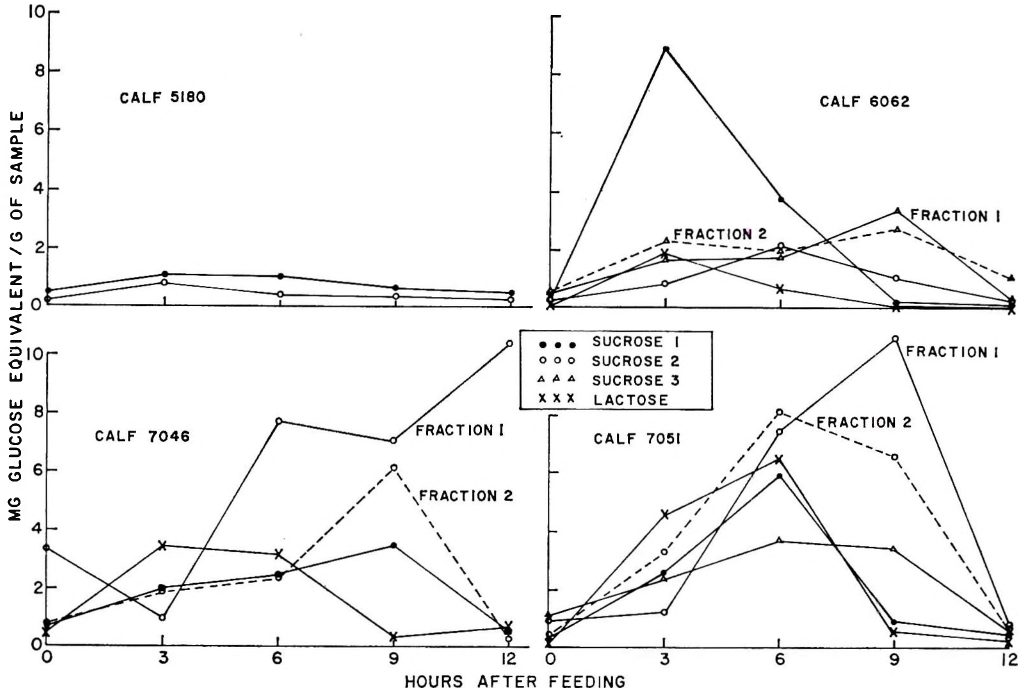

Fig. 3 Reducing activity of ileal samples.

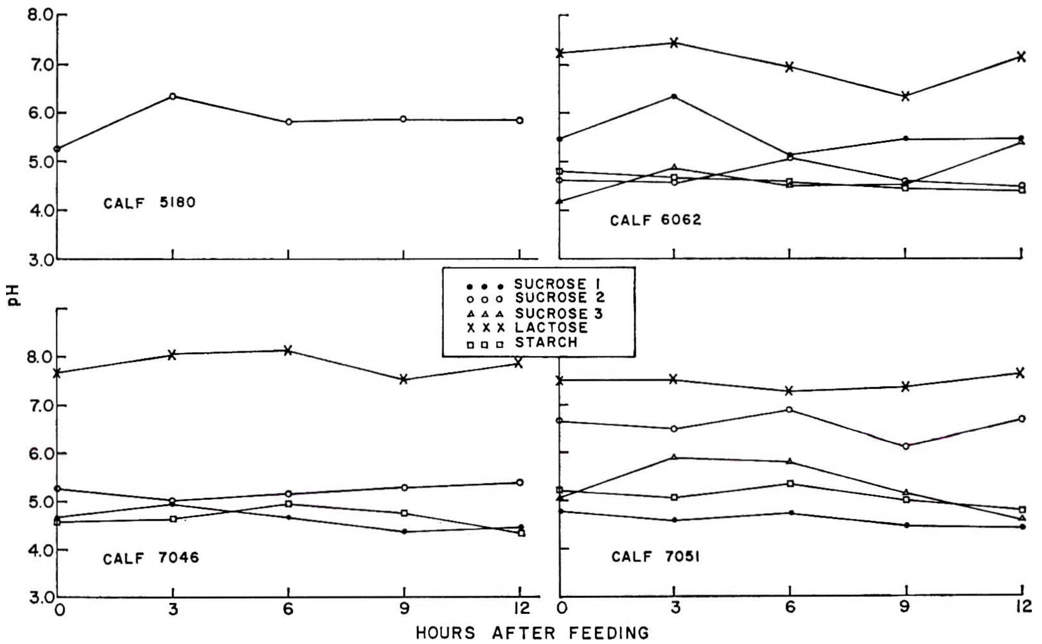

Fig. 4 The pH of fecal samples.

Fecal samples. Digestion coefficients for sucrose and lactose trials are presented in table 4. The variation in sucrose-to-polyethylene glycol ratios observed in ileal samples was not observed in fecal samples; therefore in the calculation of digestion coefficients equal consideration was given to all samples. Lactose digestion in the entire digestive tract by the 3 animals used was essentially complete. Evidence has already been presented which indicates high utilization of lactose by calf 5180 also. The digestion of sucrose between the ileum and rectum is shown in table 5.

The fecal pH values for lactose trials (fig. 4) were significantly higher ($P < 0.01$) than those for sucrose trials. A low fecal pH was usually associated with a high reducing activity level. Calves had lower fecal pH ($P < 0.05$) when fed starch than when fed sucrose.

Fecal dry matter content was much higher in the starch and the lactose trials than in the sucrose trials. No apparent

TABLE 3

Coefficients for digestion orad to fistulae [1]

Trial	Animal no.				Average
	5180	6062	7046	7051	
Sucrose 1	12.4	43.0	54.3	54.2	41.0
Sucrose 2	13.1	34.7	47.2	71.2	41.6
Sucrose 3		41.7		56.8	49.2
Average	12.8	39.8	50.8	60.7	
Lactose		94.6	96.9	88.4	

[1] Sucrose means not underlined by a common line are significantly different ($P < 0.01$) from each other.

TABLE 4

Digestion coefficients, sucrose and lactose trials [1]

Trial	Animal no.				Average
	5180	6062	7046	7051	
Sucrose 1	55.7	88.5	79.6	98.7	80.6
Sucrose 2	66.7	87.6	99.0	100	88.3
Sucrose 3		88.3		99.8	94.0
Average	61.2	88.1	89.3	99.5	
Lactose		99.4	99.8	99.7	99.6

[1] Sucrose means not underlined by a common line are significantly different ($P < 0.01$) from each other.

TABLE 5

Apparent digestion between ileum and rectum

Trial	Animal no.				Average
	5180	6062	7046	7051	
	%	%	%	%	%
Sucrose 1	43.3	45.5	25.3	44.5	39.6
Sucrose 2	53.6	52.9	51.8	28.8	46.8
Sucrose 3		46.6		43.0	44.8
Average	48.4	48.3	38.6	38.8	

difference was noted in dry matter content among the various ages at which sucrose trials were conducted. There were no significant differences among calves with respect to fecal dry matter content.

DISCUSSION

These data confirm other reports of the high degree of utilization of lactose by young calves, and show that much of this utilization occurs orad to the large intestine. The reason for this efficient use undoubtedly is the liberal production of large amounts of lactase by the young calf (1, 18).

Conversely, calves apparently do not secrete sucrase (1, 18) and evidence has been published indicating that the breakdown of sucrose in the alimentary tract is due to the action of microorganisms in the large intestine. In this study, an average of 43.5% of the sucrose fed was hydrolyzed in the large intestine, an organ which apparently does not secrete enzymes. Because of the wide experimental variation encountered it was not possible to definitely establish that the levels of reducing activity in ileal digesta from sucrose-fed calves were significantly higher than from lactose-fed calves. However, it appears that, in certain trials, reducing activity may have been present in the lumen of the intestine, which resulted from the hydrolysis of sucrose (a non-reducing sugar). This has been substantiated by the observation that reducing activity increased rapidly when sucrose was incubated in vitro with intestinal microorganisms.[6] Since disaccharides are hydrolyzed by mammalian enzymes in the mucosal cells of the small intestine (19, 20), reducing activity would not be expected to occur in the lumen of the intestine if hydrolysis resulted only from mammalian enzymes.

It was realized that some of the collected digesta might have been returned from the large intestine by reverse peristalsis. Therefore, in some trials, samples were collected in 2 ways, one of which (fraction 2) was designed to preclude the collection of digesta which had been exposed to fermentation in the large intestine. Evidence that the breakdown of sucrose actually began in the small intestine is shown by the sucrose-to-polyethylene glycol ratios and reducing activity levels of fraction 2 samples (figs. 1 and 3). There were consistent differences between fractions 1 and 2 only with calf 7046. The arrangement of cannulae in this animal was such that digesta usually moved from the distal cannula only when displaced by more digesta from the proximal cannula. For this reason digesta were present in the distal cannula and subject to fermentation in this area for a longer period of time than with other calves.

The difference in ileal sucrose-to-polyethylene glycol ratios at various times after feeding appears to be due to the time of exposure to digestive secretions (secreted by the calf or by microorganisms) in the abomasum and small intestine. Since the samples taken at zero hours after feeding were taken just after initiation of feeding, it appears highly unlikely that material would travel the entire length of the small intestine during the time required to collect the sample. Therefore, samples taken at zero and 12 hours after feeding represented material fed at least 12 hours earlier and samples taken at 9 hours after feeding represented material which was in the digestive tract at least 9 hours.

Studies with calves have invariably shown no increase in blood reducing sugar due to the ingestion of sucrose. It appears that monosaccharides in the lumen of the small intestine (resulting from the hydrolysis of sucrose) would be absorbed, resulting in an increase in blood reducing sugar. Dahlqvist and Borgstrom (19) have shown that in the human small intestine there are areas where certain disaccharides are absorbed and subsequqently hydrolyzed. Perhaps in the small intestine of the calf there also exists an area where monosaccharides are efficiently absorbed, and in all probability this area is orad to the location where sucrose is first hydrolyzed. Larsen et al. (6) reported that glucose was more readily absorbed from the jejunum than from the ileum, the cecum, or the rumen of the calf. The fact that sucrose is hydrolyzed in vivo without a resulting increase in blood reducing sugar or an excessive accumulation of monosaccharides

[6] Morrill, J. L., and J. L. Noordsy, unpublished data, 1964.

indicates that the invert sugar is further metabolized, possibly to volatile fatty acids.

The pH of ileal digesta and of feces is believed to be an indication of the degree of fermentation within the material. The extent of fermentation would be related to the amount of substrate available, and to the ease with which the substrate was fermented. Thus the lactose-fed calves may have had high fecal pH values because essentially all the readily available carbohydrate had been digested. In one sucrose trial (7051 — sucrose 2) most of the sucrose, and also the reducing sugar, was gone and the fecal pH values were higher than in any other sucrose or starch trial.

This is the first known report on the degree of utilization of carbohydrates in the gastrointestinal tract posterior to the reticulo-rumen and orad to the large intestine in young calves. Henschel et al. (21) introduced carbohydrates into the duodenum of 4- to 6-month-old steers to measure digestion in the small intestine. They reported average sucrose digestion of 38% in the small intestine, as compared with the average of 41% observed in the present study. The average lactose digestion of 86%, reported by the British workers, is lower than the 93% average reported herein. What effect, if any, the abomasum may have had on disaccharide digestion in the present study is not known.

The efficient utilization of lactose and various monosaccharides by young calves is well established, but further research is needed to determine the extent to which other carbohydrates are utilized. Possible utilization of carbohydrates via fermentation to volatile fatty acids in the intestinal tract and their subsequent absorption have received little attention, although volatile fatty acids are known to be produced in (22) and absorbed from (23) the large intestine of ruminants. This study emphasizes the importance of further research to determine the rate of flow of digesta in the intestines.

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