Pancreatic Pathology in Manganese-deficient Guinea Pigs[1,2]

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ABSTRACT A high incidence of stillbirths and poor viability of neonatal guinea pigs has occurred in offspring born to females maintained throughout pregnancy with a diet deficient in manganese. At autopsy these animals were observed to have gross abnormalities of the pancreas. Guinea pigs, presumably less severely affected, which were continued with the manganese-deficient dietary regimen to young adult life, had previously been found to have diabetic-like responses to administered glucose. Former work also has demonstrated that supplementation of such young adult animals with dietary manganese for 2 months resulted in normal utilization of glucose. Histological examination of tissues from animals of these various groups forms the basis of the study. The most seriously defective newborn young killed for examination of tissues showed aplasia or marked hypoplasia of all cellular components of the pancreas. Where hypoplasia occurred, islet population was reduced but islet size was increased. Islets contained fewer and less intensely granulated beta cells when compared with pancreatic islets in the tissues of control newborn guinea pigs. Young adult manganese-deficient guinea pigs also had decreased numbers of pancreatic islets which were enlarged in size and contained less intensely granulated beta cells and increased numbers of alpha cells. Following dietary supplementation with manganese, increased numbers of islets were found to be present. These islets contained more beta cells which were more heavily granulated than those present in the islets of the pancreas of deficient guinea pigs.

The investigations reported in this paper were undertaken to (a) examine changes in pancreatic morphology which accompany reduced glucose utilization in young adult guinea pigs congenitally deficient in manganese (1); and (b) to determine the effect of this maternal dietary deficiency on the prenatal development of the pancreas, as evidenced by the condition of this gland in the neonatal guinea pig.

Earlier work had shown that newborn young of manganese-deficient females had reduced amounts of acid mucopolysaccharides in rib and epiphyseal cartilage (2), and in the matrix of the otolithic membrane of the ear (3). Deficient young were also found to have abnormal electrocardiographic patterns.[3]

Many young born to females fed the deficient diet were stillborn, others, nearly moribund at birth, died within the first few postpartum days. Animals that survived to adult life withstood stress very poorly, had decreased resistance to infection, suffered from ataxia and gross body tremors and had a late-developing Parkinsonian-like twitch. Supplementation with dietary manganese was found to correct the abnormal utilization of glucose, the electrocardiographic abnormalities, and the persistent twitching in young adult animals.

Postmortem examination of young born to manganese-deficient females had shown that many neonatal animals had complete aplasia or marked hypoplasia of the pancreas. The present paper reports the histological structure of the pancreas in neonatal and young adult manganese-deficient guinea pigs.

MATERIALS AND METHODS

Diets containing less than 3 ppm manganese (deficient diet) or 125 ppm manganese (control diet) were fed to female guinea pigs before breeding and throughout pregnancy (2). At autopsy the pancreas and adjacent visceral organs were removed from representative neonatal guinea pigs born to females fed the deficient and control diets. All young included in this study

Received for publication July 5, 1967.

[1] Supported in part by Public Health Service Research Grant no. HD-00429-10 from the National Institute of Child Health and Human Development.

[2] Presented in part at the 51st annual meeting of the Federation of American Societies for Experimental Biology, Chicago, 1967.

[3] Parker, H. R., G. J. Everson, R. Shrader and L. S. Hurley 1964 Electrocardiological changes in offspring of normal and manganese-deficient guinea pigs. Federation Proc., 23: 292 (abstract).

were born alive and tissues were taken from viable animals immediately after killing. Ten neonatal deficient animals and 8 young born to females fed the control diet were studied in detail.

Young adult animals born to females fed the 2 dietary rations were continued with the same diet regimen postpartum. Twenty-three manganese-deficient animals were selected from those having the most marked deficiency symptoms. Using paired deficient and control animals, evaluation of pancreatic endocrine function was made by means of oral and intravascular glucose tolerance tests (1). Approximately one-third of the animals were killed at the completion of the 4-hour testing period and tissues were removed for histological examination. Four animals were allowed a 4-day period in which to recover from the effects of the glucose load before being killed. The remaining 13 deficient animals were given the control diet for 2 months. Glucose tolerance tests were then repeated (1). The animals were killed at the termination of the second test and the pancreatic tissues were taken for histological study.

All tissues were fixed in calcium formol. Neonatal tissues were processed "in toto" following examination with the dissecting microscope for initial gross evaluation of gland volume. Pancreatic tissues from adult animals were examined sub-grossly at 70 × magnification and were cut to provide three or more blocks representing

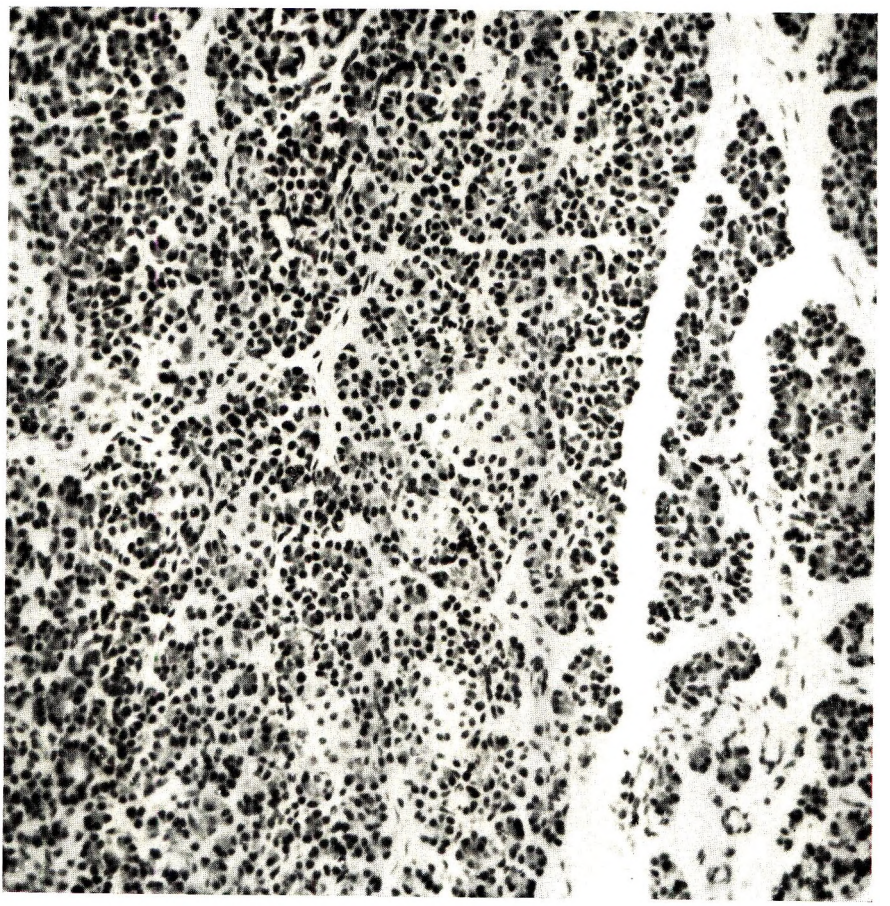

Fig. 1 Morphology of pancreas in newborn control guinea pig illustrating the number and size of the islets and the degree of acinar tissue development. H & E. ×192.

the head, body and tail of the organ. All tissue blocks were dehydrated in ethanol, followed by clearing in xylol and embedding in paraplast. A few samples of tissue were placed in gum sucrose overnight and sectioned with a cryostat at 4 μ. These frozen section preparations were stained for pancreatic beta cell granules using Jenning's technique (4). All paraffin embedded tissues were routinely sectioned serially at 7 μ. Slides were stained with hematoxylin and eosin, Goldner's stain (5) or with Lazarus' modification of Schiff's trichrome stain (6) for examination of pancreatic morphology. Blocks of tissue were serially sectioned by cutting 50 sections at a thickness of 7 μ followed by 20 sections cut at 4 μ. This pattern was repeated throughout the entire block to provide representative material for examination with special stains. The routine stains previously described were applied to the 7-μ sections. The thinner sections were stained using Scott's modification of Gomori's aldehyde fuchsin technique (7), Schiebler and Schliessler's procedure using pseudoisocyanin (8) and Hellman's silver method for alpha$_1$ and alpha$_2$ cells (9).

Measurements of islet area were made using the method of Segida (10). Using an ocular micrometer, length and width measurements were made on all islets encountered in 5 sections from the head of the pancreas from each animal. Each measured section was separated from the next by serial sections amounting to 0.1

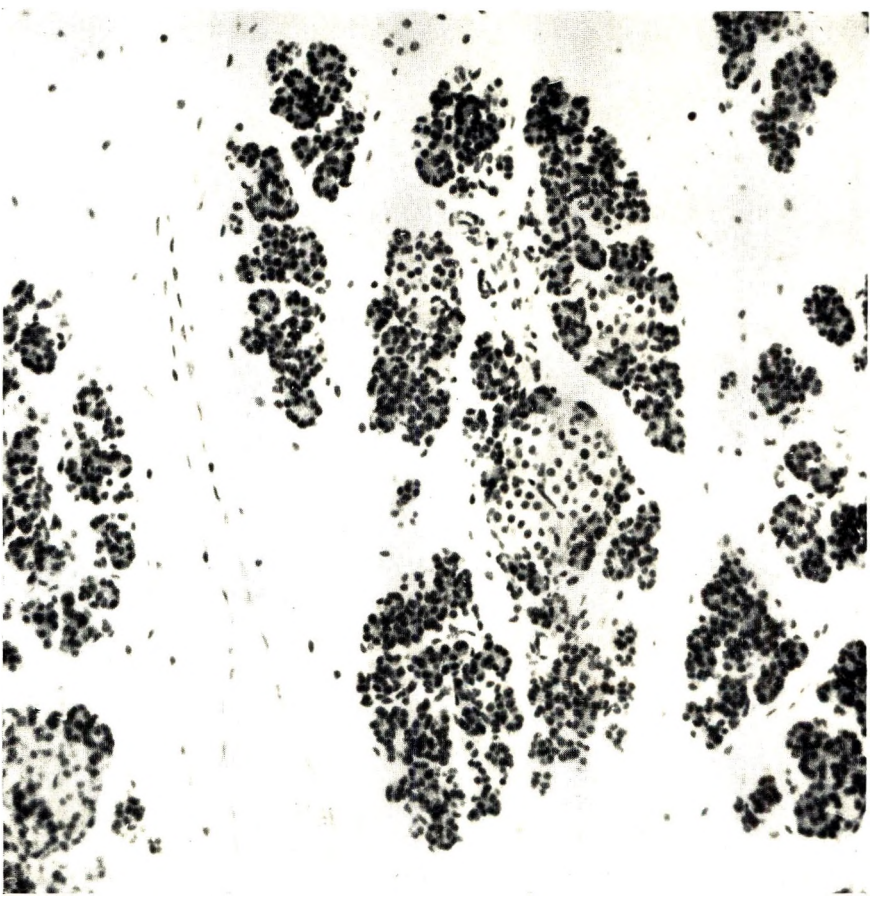

Fig. 2 The hypoplastic acinar tissue and enlarged islets characteristic of the pancreas of severely affected manganese-deficient newborn guinea pigs. H & E. ×192.

mm of tissue. The sections on which islet measurements were made were projected using a camera lucida and the outlines of islets and of sections were drawn at a magnification of 7 ×. The area of the outlined tissues was measured with the aid of a planimeter. The total area of insular tissue was calculated by adding the area of the individual islets. The percentage of islet tissue in the pancreatic gland was calculated using the formula $\frac{s \times 100}{S}$, where s equals the total islet area and S is the area of the sections (11). These measurements were made on 5 sections from the head of the pancreas from 3 different animals in each of the 3 groups of guinea pigs.

The random sampling method of Vranic (12) was used to obtain the cell distribution figures upon which alpha/beta cell ratio calculations were based. Sampling was carried out on 5 sections for each animal and a total of 1500 cells were counted in each section. The area occupied by alpha and beta cells was compared under 125 × magnification through the use of an ocular grid. Fields consisting solely of alpha cells or of beta cells were centered in the grid and the number of cell nuclei lying within a single grid square was counted. One hundred such fields were counted in each

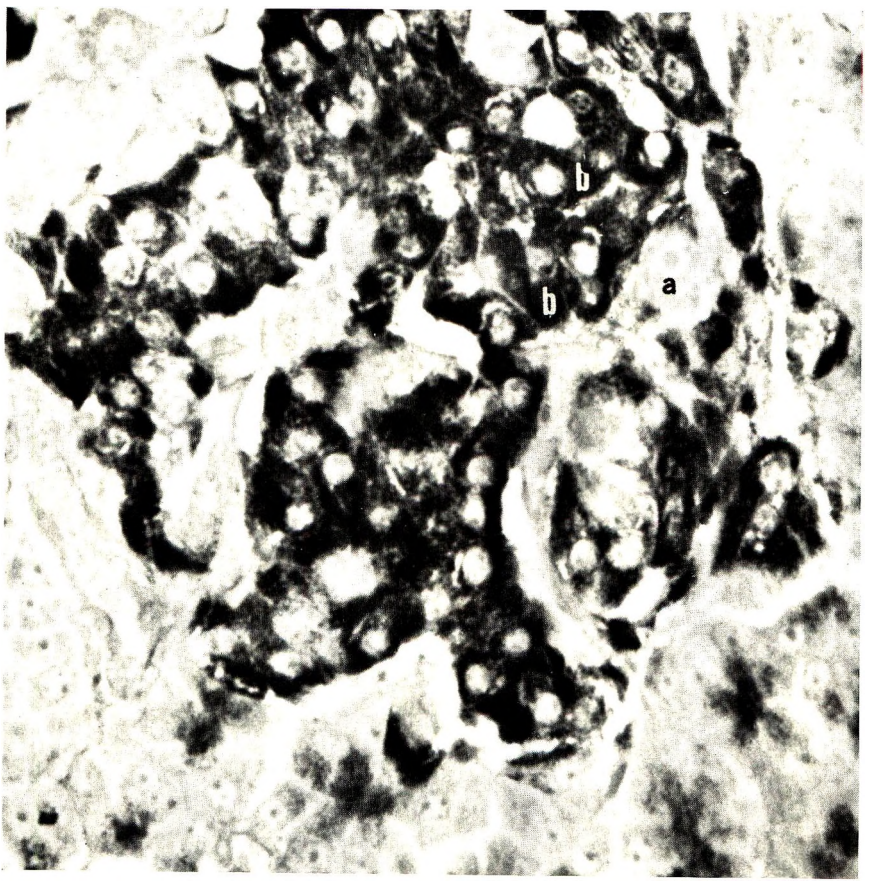

Fig. 3 High power view of islet stained with aldehyde fuchsin. Tissue from young adult control animal killed immediately upon completion of glucose tolerance test. The alpha and beta cells are labeled. Note relative number of each cell type and density of deeply stained beta granules. Aldehyde fuchsin. ×860.

of the sections upon which islet measurements were made. Student's *t* test was applied to all data where determinations of the significance of differences were made (13).

RESULTS

Neonatal guinea pigs

The pancreatic tissue of newborn control guinea pigs appeared to be morphologically normal (14, 15). Figure 1 illustrates the well-developed acini and numerous islets of various sizes which are characteristic of these glands. In sections stained with Lazarus' granule stain, and in those treated with aldehyde fuchsin, the alpha and beta cells were heavily granulated. *D* cells were occasionally observed in the islets, and occasionally intra-acinar alpha or beta cells were encountered.

Of the 10 manganese-deficient guinea pigs included in this study, 3 animals which appeared to have minimal symptoms of manganese deficiency had pancreatic tissues indistinguishable grossly or microscopically from those found in control animals. The remaining 7 animals had marked symptoms of manganese deficiency (2). In some cases these animals were judged to be moribund. Three of these severely affected animals were found to have no discernible pancreatic tissue. Microscopic examination of omental membranes and adjacent visceral organs failed to reveal

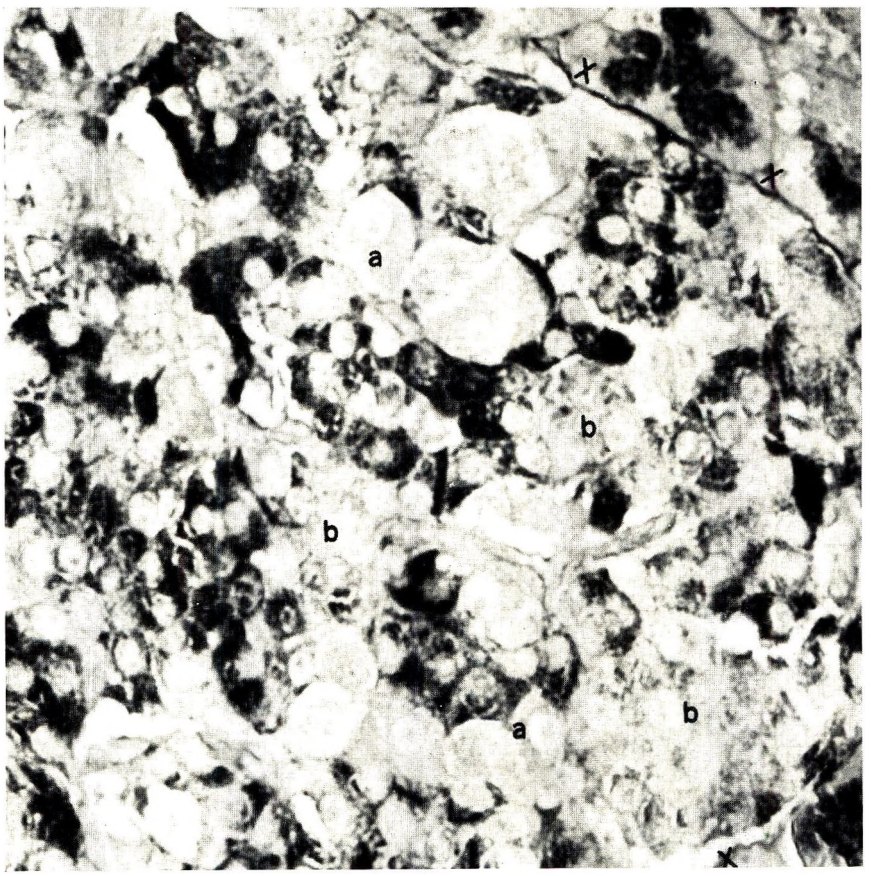

Fig. 4 Aldhyde fuchsin stained islet of young adult manganese-deficient guinea pig, killed 4 days after completion of glucose tolerance test. Islet margins indicated by *x* marks. The alpha and beta cells are labeled. Compare number of each cell type and density of beta cell granules with control shown in figure 3. Note that 4 days after glucose loading beta granulation was still reduced in amount. Aldehyde fuchsin. ×860.

any abnormalities of these tissues or the presence of any aberrant pancreatic rudiments.

The remaining 4 manganese-deficient newborn guinea pigs had marked hypoplasia of the pancreas. A typical section of the pancreas from one of these animals is shown in figure 2. The striking hypoplasia of the ductile and acinar elements of the exocrine pancreas is immediately apparent. The ducts were found to be very primitive and showed only tertiary branching. The ductile epithelium was simple low cuboidal in type, and no areas of stratification were observed. The acini appeared mostly as primary clusters surrounding the ductile ramifications from which they originated. The cells were small, poor in cytoplasm, and contained small densely granulated areas at the apical portion of the cell. The perinuclear cytoplasmic basophilia normally observed at the wide end of the cell was lacking. The islets in these pancreatic tissues appeared to be decreased in number and enlarged in size in comparison with those present in normal newborn animals (compare figs. 1 and 2). The usual intra-acinar alpha and beta cells could not be identified. No bizarre forms of islet cell orientation were observed, and vascular patterns within the islets appeared to be normal. Large numbers of granulated alpha cells were present in all of the enlarged islets. The beta cells, which presented an apparently normal appearance in sections stained with hematoxylin and eosin, were

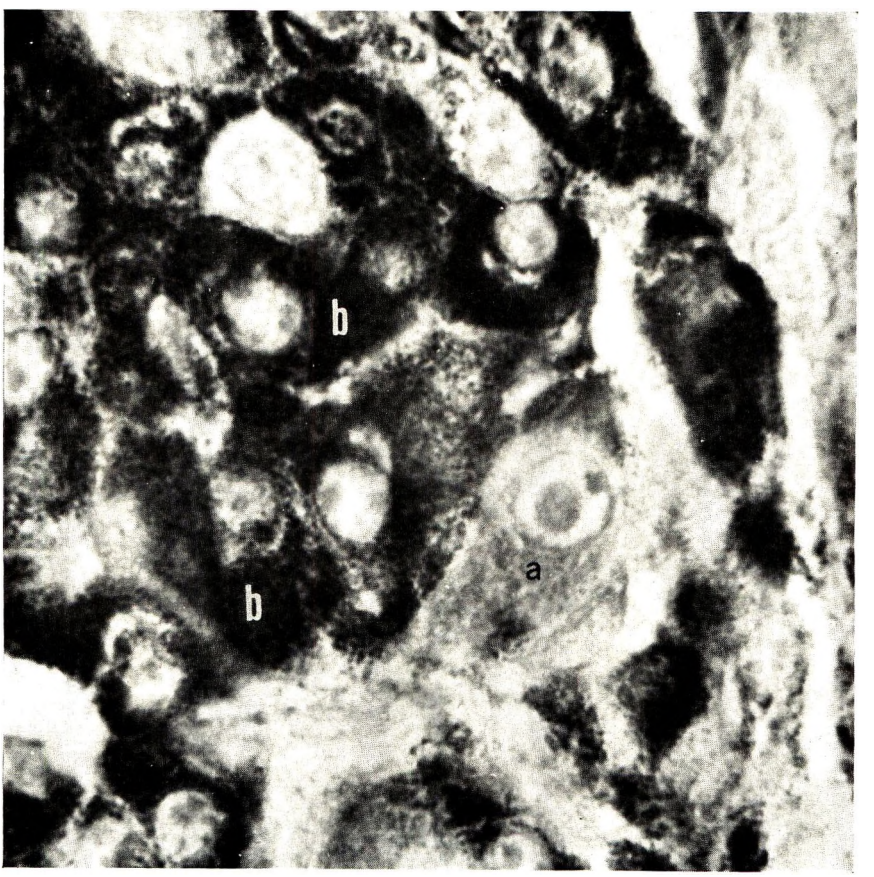

Fig. 5 Oil immersion view of same islet shown in figure 3 with the same cells labeled. Density of beta granules is shown 4 hours after administration of a glucose load. Aldehyde fuchsin. ×1890.

less readily stained with either aldehyde fuchsin or pseudoisocyanin. Only sparse granulation was present in these beta cells and many appeared completely degranulated. Normal numbers of *C* and *D* cells were present in these islets. Increased numbers of neutrophils and eosinophils were observed in the edematous embryonal type connective tissue of the pancreas of the deficient animals. This connective tissue showed a paucity of fibrous elements and contained immature fibroblasts of the stellate type.

Quantitative observations

Comparisons of total pancreatic mass were made using tissues from control and manganese-deficient newborn animals. The volume of the pancreas in control guinea pigs ranged between 6.1×10^{-4}mm^3 and 7.4×10^{-4}mm^3. In the deficient guinea pigs the 3 groups discussed above had (a) no pancreatic tissue, (b) normal amounts of pancreatic tissue, or (c) very much reduced amounts of pancreatic tissue (range 6.6×10^{-6}mm^3 to 4.13×10^{-6}mm^3). Islet volumes calculated by the methods of Maclean and Ogilvie (16) indicate that in newborn control guinea pigs the islet tissue occupies approximately 10% of the gland volume, whereas in the deficient animals showing islet hypertrophy, the islet volume is 5.6 to 6.2% of the gland mass (table 1). Random alpha and beta cell counts indicate that in the neonatal deficient guinea pigs which have hypertrophied islets, the alpha cells were increased and the beta cells were decreased in number. A summary of the comparative data is presented in table 1.

Adult guinea pigs

Manganese-deficient animals

Pancreatic tissues from young adult manganese-deficient guinea pigs contained morphologically normal exocrine and hypertrophied islet tissues. These animals had been found to have diabetic-like glucose tolerance curves (1). The enlarged islets had extremely irregular outlines and appeared at times to be continuous with the acinar tissues. Cellular organization, within the islets, resembled the normal pattern except that large clumps of alpha cells were prominent in all parts of the islets; beta cells appeared to be larger than normal and to be somewhat degranulated; alpha cells stained typically with granule stains. No evidence of hydropic change, vacuolation, nuclear pyknosis, or of "Körnchen" formation was seen in any of the islet tissues. Few *D* or *C* cells were observed, but argyrophilic cells were scattered throughout the islets. No areas of leucocytic infiltration or inflammatory foci were seen, nor were fibrotic changes present in acinar, ductile or insular tissues; beta cells in tissues of deficient guinea pigs that had been killed immediately upon completion of glucose tolerance testing (1) contained fewer granules than the cells in

TABLE 1

Area measurements, islet and cell counts in pancreatic tissue

Measurements	Adult			Newborn	
	Control	Manganese-deficient	Supplemented	Control	Manganese-deficient
Gland area, cm^2	1.21	0.41	0.93	0.26	0.04
Islet area, cm^2	4.06×10^{-2}*	2.33×10^{-2}	2.9×10^{-2}**	2.9×10^{-3}	2.5×10^{-3}
Islet area, % of gland area	3.3	5.7	3.0	1.1	6.2
No. of islets	1647	635	1387	431	213
No. of islets < 300 μ	3	324	316	—	—
alpha/beta cell ratio	1:4	1:1.7	1:2.5	1:4	1:3
alpha/beta area ratio	1:9	1:8	1:9	1:11	1:9
Area of islets occupied by beta cells, %	32.6	17.3	21.7	26.6	25.2
Area of islets occupied by alpha cells, %	67.4	82.7	78.3	73.4	74.8

* $P = < 0.001$, difference between adult control and manganese-deficient animals.
** $P = < 0.01$, difference between adult manganese-deficient and supplemented animals.

the islets of deficient animals killed 4 days after being tested. Despite the 4-day "recovery" period, the beta cell granulation was not as dense as in control or supplemented animals examined immediately after completion of the glucose test (1) (see figs. 3–6).

Supplemented animals

Low power photographs of typical sections of pancreatic tissue from control and supplemented adult guinea pigs are shown in figures 7 and 8. Both groups of animals had previously been found to respond normally to glucose loading (1).

The islets in the pancreas of the supplemented guinea pigs varied greatly in size. The majority were either extremely large, resembling those present in the deficient animals, or were found to be very small clusters of three to ten cells These small islets consisted almost entirely of beta cells (fig. 8). The large islets in these tissues were sharply defined and lacked the confluency with the acinar tissue seen in the deficient animals.

Mitotic figures were frequently observed within the cells in the smallest islets. The alpha cells in the large islets of the pancreas from supplemented animals appeared to be as numerous and to have the same distribution as those observed in the deficient animals. No evidence of degenerative change of any type was observed in the islets of these glands; beta granulation ap-

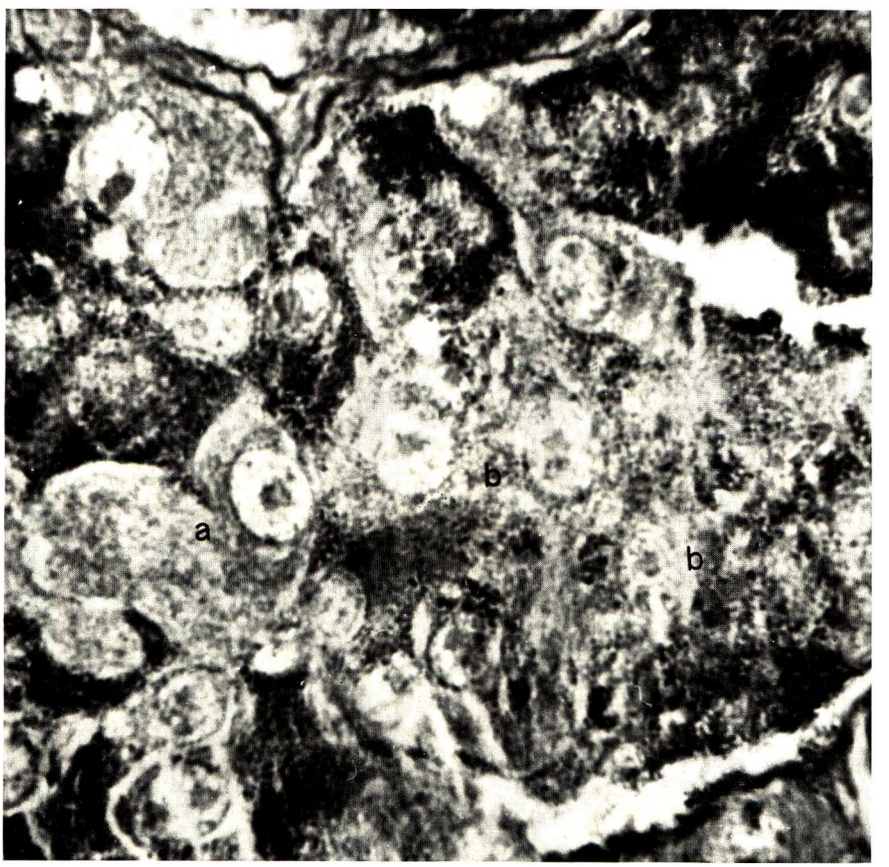

Fig. 6. Oil immersion view of islet cells shown in figure 4 with the same cells labeled. Density of beta granules 4 days after administration of glucose load is illustrated. Aldehyde fuchsin. ×1890.

peared to be comparable to that in the cells of the control tissues.

The acinar and ductile elements in the 2 glands appeared to be normal. The presence of adipose tissue in both the inter- and intralobular connective tissue in these glands is in contrast with the absence of fat cells in the more compactly arranged parenchyma of the deficient pancreas (fig. 9).

Quantitative observations

The numerical distribution of islets with different diameters present in equal numbers of tissue sections from deficient, supplemented and control guinea pigs is shown graphically in figure 10. The large number of small and medium-size islets and the extremely small numbers of large islets found in the pancreas of control animals is illustrated in this figure. This type of curve is typical of normal animals in a number of species (9). The presence of many excessively large islets and a marked decrease in the number of small islets is characteristic of the pancreas of the manganese-deficient adult animals (fig. 9). Supplemented animals similarly had excessively large islets and in addition had numerous smaller islets consisting for the most part of beta cells (fig. 8). The islet population for the supplemented animals resembled the pattern of the deficient animals more closely than that of the controls.

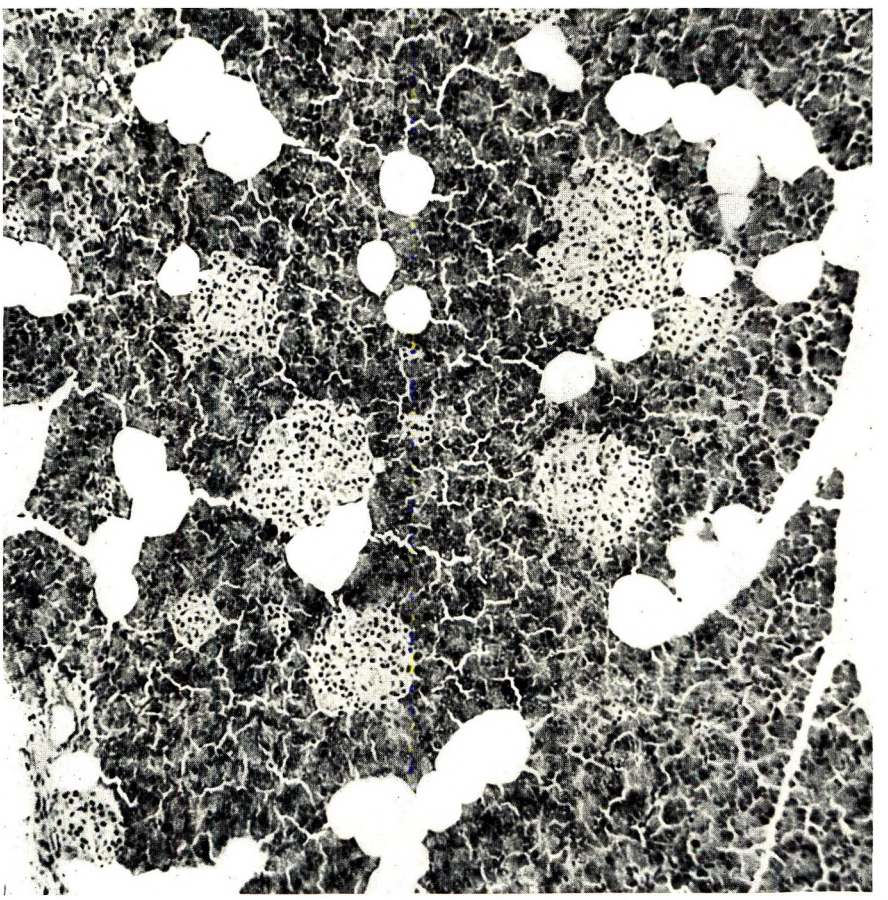

Fig. 7 Pancreatic tissue from adult control guinea pig. Note number and size of islets and presence of fat. H & E. ×135.

A comparison of gland and islet area measurements on tissues from the manganese-deficient, supplemented and control adult animals is given in table 1. The cross-section area of the pancreas was reduced in both supplemented and to a somewhat greater extent in the manganese-deficient animals. In these same categories of animals, islet size was increased. The data in table 1 indicate that these large islet masses occupied a disproportionately large part of the reduced area of pancreatic tissue. In both control and supplemented animals, increased numbers of small islets and the presence of more fat than observed for deficient animals must be taken into account to understand the lower proportion of total gland mass occupied by islet tissue; alpha/beta cell ratios were found to be increased in both manganese-deficient and supplemented animals. Supplementation with manganese appeared, however, to have restored beta cell size to normal and to have increased the proportionate numbers of beta cells present in the total islet mass.

DISCUSSION

The abnormalities observed in the pancreatic tissues of the young of female guinea pigs maintained throughout pregnancy with a diet deficient in manganese

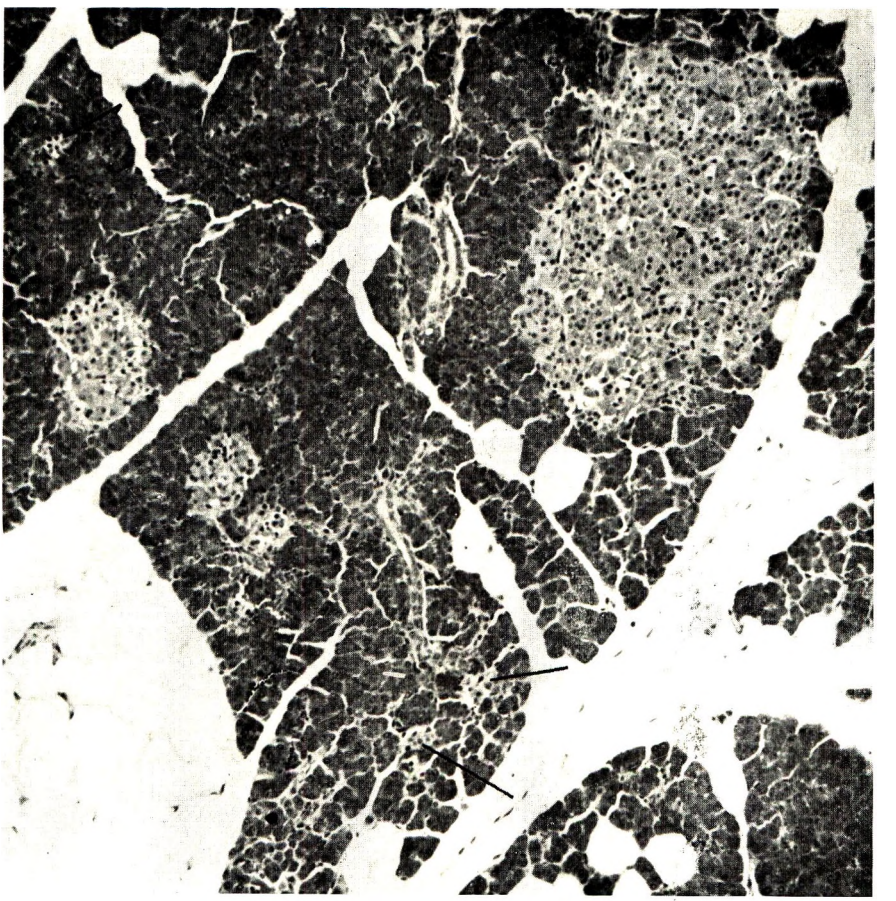

Fig. 8 Pancreatic tissue from supplemented adult guinea pig. Note large islet similar to those in manganese-deficient animals and several minute islets (marked with arrows). The fat content of the inter-acinar connective tissue is similar in amount to that present in the controls. H & E. ×135.

do not appear to conform to any single syndrome previously reported in the literature. The changes in the acinar tissues resemble those present in human infants with congenital pancreatic hypoplasia (17). In these rare cases of obscure etiology, however, the endocrine pancreas appeared to consist only of normal-appearing beta cells. Manganese-deficient guinea pigs have hypertrophied islet tissue consisting of degranulated beta cells and increased numbers of alpha cells. Such insular hypertrophy and beta cell degranulation are characteristic of the pancreas of infants born to diabetic mothers (18). Maternal hyperglycemia has been found to be causally related to insular hypertrophy and beta cell degranulation in the young of animals given diabetogenic drugs (19). The exocrine tissues in the pancreas of these young have not been found to be abnormal. Ethionine has been reported to induce changes in exocrine, but not endocrine tissues of the pancreas of adult rats (20–22) but no description of its effect on this organ in the newborn has been found.

It is evident that in the absence of adequate manganese there is a reduction of pancreatic tissue formation involving both acinar and islet cells, there may be specific inhibition of beta and acinar development, beta cell granularity is decreased and insulin production may be diminished. The role of manganese in these diverse cellular

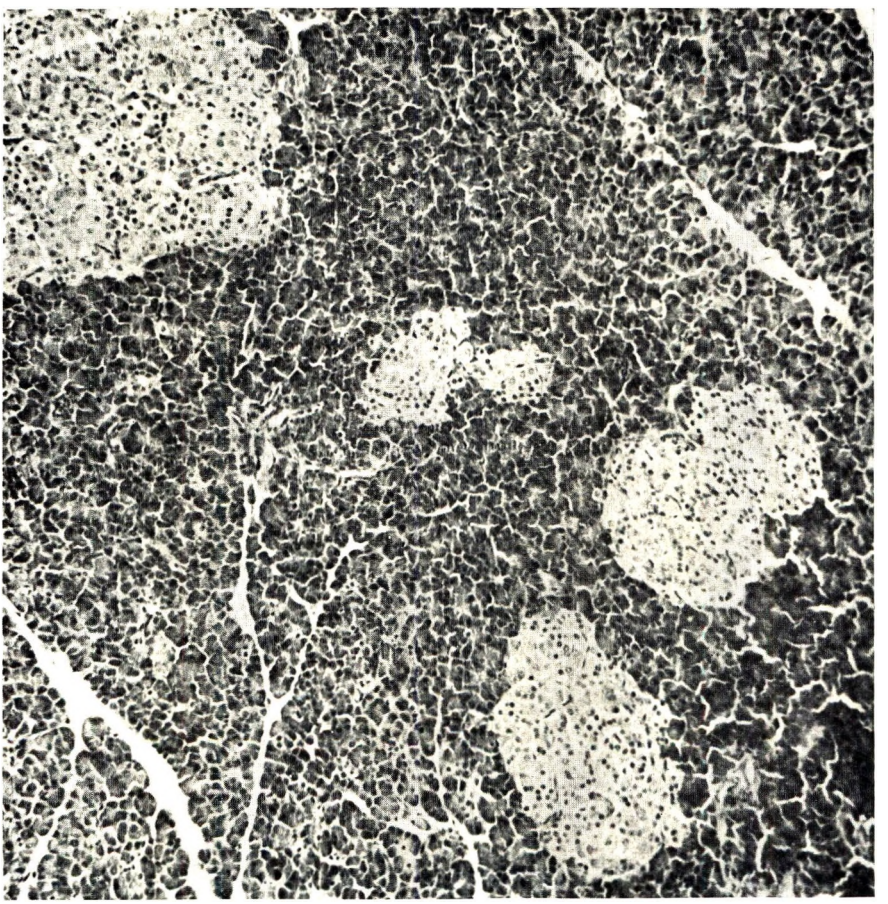

Fig. 9 Pancreatic tissue of a manganese-deficient adult guinea pig. Note compact parenchyma, normal acinar morphology and lack of fat cells. Islets show normal configuration but are enlarged in size. H & E. ×135.

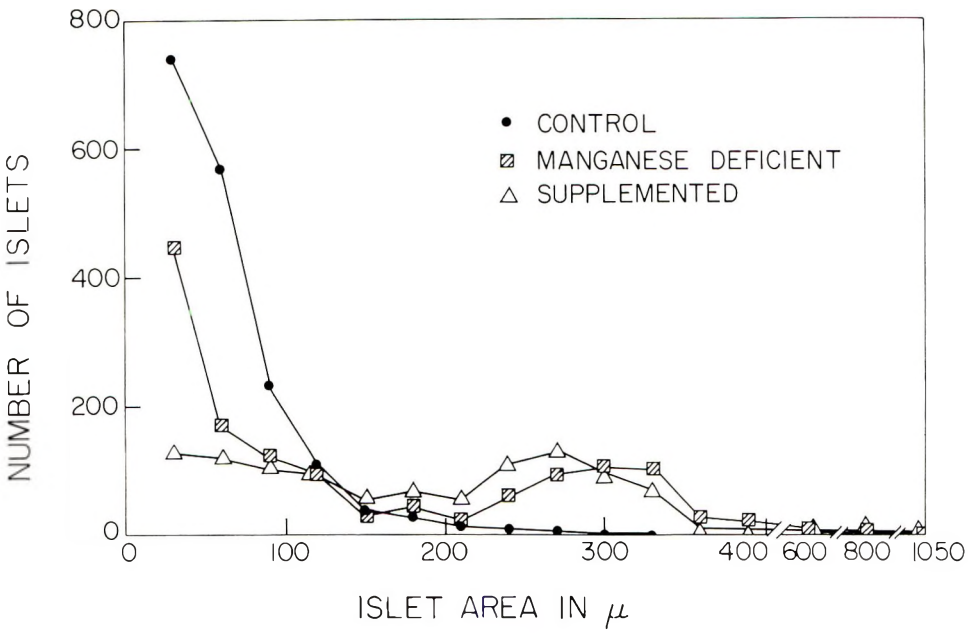

Fig. 10 The numerical distribution of islets of various sizes in pancreatic tissues from manganese-deficient, supplemented and control adult guinea pigs.

activities is further emphasized by the finding that supplementation of these deficient animals was found to produce an increase in the granule content of the beta cells. and a return to normal of the glucose tolerance curve. The gland mass of the supplemented animals also increased and the characteristic diffuse arrangement of the parenchyma was restored.

Further investigation is needed to determine whether these are direct effects of manganese on pancreatic tissue or whether the effects observed reflect alterations of extrapancreatic metabolic systems.

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Metabolism of Radioactive Cesium (^{134}Cs and ^{137}Cs) and Potassium by Dairy Cattle as Influenced by High and Low Forage Diets[1,2]

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ABSTRACT A feeding trial was conducted with 10 lactating Holstein cows to determine the excretion patterns of fallout ^{137}Cs and potassium in milk, urine and feces as affected by a high hay (80%) or high grain (80%) ration. It was found that ^{137}Cs was excreted principally via the feces as compared with potassium which was largely excreted in the urine. Tracer doses of soluble ^{134}Cs were transferred to milk and urine to a greater extent than fallout ^{137}Cs. A marked decrease in absorption was noted when the dose was administered with a high hay ration as compared with a high grain ration, which was apparently due to adsorption by fibrous components of the ration. The mean transfer coefficients of fallout ^{137}Cs to milk (pCi per liter/total daily intake in pCi) were 0.48 for the high hay and 1.21 for the high grain ration. Tracer doses of ^{134}Cs showed transfer coefficients of 0.92 and 1.36 when added to the same rations. Differences in absorption of potassium and ^{137}Cs as judged by transfer to milk and urine could be explained on the basis of a lower absorption of ^{137}Cs due to its physical form in fallout contaminated forages.

Previous work in this laboratory indicated that the transfer to milk of fallout cesium-137 found in dairy cattle feeds was much less efficient than the transfers reported from studies with tracer doses of ^{137}Cs (1–3). The data also showed a less efficient transfer to milk with diets consisting predominantly of hay as compared with rations containing a high percentage of grain.

The reduction in transfer resulting from feeding ^{137}Cs in the form of fallout material in natural feeds and from high hay as compared with high grain rations were both ascribed in an earlier publication to the adsorption of the radionuclide by the indigestible crude fiber fraction of the ration (4). This postulate was based largely on the experiments of Mraz and his associates (5, 6) which demonstrated reduced absorption for ^{137}Cs as a result of feeding fibrous material to laboratory animals.

The present study was designed to investigate, under controlled conditions, the relation between the crude fiber content of the ration and the metabolism of fallout ^{137}Cs in dairy cattle, and to add to the knowledge on the relative metabolism of cesium and potassium. To test the hypothesis that the chemical or physical state of fallout ^{137}Cs was different from orally administered radioactive cesium salts, the uptake and excretion patterns of soluble $^{134}CsCl$ were also studied and compared with those of fallout ^{137}Cs. Potassium determinations were included because of the similarity of the metabolism of potassium to the metabolism of cesium, and because with the technique of gamma-ray spectrometry ^{137}Cs and ^{40}K (which represents 0.0119% of stable potassium) can be determined concurrently. Information on potassium is also of interest because the influence of dietary potassium on the metabolism of cesium has not been clarified in the bovine.

METHODS

Eight high-producing Holstein cows were used in a double reversal experiment, four fed a high hay and four a high grain diet. The high hay diet was 80.6% hay and 19.4% grain. The high grain diet was 22.1% hay, 70.0% grain and 7.9% soybean oil meal. The protein intake was thus the same for each ration. The experimental

Received for publication October 21, 1967.

[1] Published with the approval of the Director of the Colorado Agricultural Experiment Station as Scientific Series Paper no. 1235.

[2] Published as AEC Report no. COO-1171-53.

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periods lasted 10 days. The time allotted between experimental periods ranged from 13 to 24 days depending on how readily a constant intake of the new diet could be established. During the experimental periods, excreta and milk were composited for 2 days for each cow. Feces were collected in pans beneath the collection stalls and urine was collected in polyethylene bottles by means of a tube connected to a rubber collector fitted around the vulva of the cows. Feed and refusals were weighed and sampled for gamma-ray counting analysis and for chemical analysis.

The hay in this study was baled first-cutting 1964 alfalfa and was fed as long hay. The concentrate consisted of no. 2 flaked yellow corn (cooked for approximately 12 minutes at 93°, rolled and dried to approximately 15% moisture) and solvent-processed 46% protein soybean oil meal. The composition of the feeds is shown in table 1. The trials were initiated in November, 1964, and terminated in February, 1965.

For comparison with results of tracer experiments, doses of ^{134}CsCl were administered either by stomach tube or as adsorbed on grain pellets to 2 cows, one fed the high hay and the other the high grain diet. The doses were given daily for 6 to 12 days. The experiment was repeated by reversing the treatment for each cow. Doses of 0.1 μCi per day of ^{134}Cs were administered to cows fed the hay diet in order to observe both ^{134}Cs and fallout ^{137}Cs. At comparable activities both can be determined simultaneously by gamma-ray spectrometry. Excreta were collected and analyzed for sufficient periods after cessation of the dose to determine the components of the biological excretion function.

Milk samples were counted in a 5.47-liter "Marinelli Hat" beaker on a 20-cm by 10-cm NaI(TI) scintillation crystal. The detector was housed in a low background chamber of 0.9 m by 1.2 m inside dimensions, and with 12.7-cm thick steel walls. The output of the detector was to a 400-channel pulse-height analyzer.

Urine samples were counted in 2, one-liter polyethylene bottles lying flat on the 20-cm by 10-cm crystal. Feces samples were air-dried to equilibrium and ground to a constant density and counted as 900 or 1350 g compacted in 4.54-liter cardboard containers directly on the scintillation crystal. Hay, grain and other feed samples were counted in the same geometry as the feces samples. The gamma-ray spectra were analyzed by a matrix inversion, spectrum-stripping procedure written for an IBM 1620 computer (7).

All counting geometries were calibrated by counting standardized activities of the radionuclides added to material of the proper density. Water, with tracer radioactive cesium was used for milk and urine standards, and sawdust with tracer radioactive cesium was used for feces and hay standards. Grain with tracer radioactive cesium was used for grain standards. ^{137}Cs activities were from a secondary laboratory standard solution that had previously been cross-calibrated by several methods and also intercompared with other counting laboratories.

Potassium concentrations were determined by measurement of the radioactive isotope ^{40}K. Since the half-life of ^{40}K is 1.3×10^9 years, its abundance in natural potassium (0.119%) is essentially constant, and standards were prepared by adding a known amount of oven-dried KCl to the samples. The counting times for ^{40}K and fallout ^{137}Cs determinations were 2 hours for milk and feed, and 1 hour for urine and feces. Samples that contained

TABLE 1

Average composition of feeds

	^{137}Cs	K	Moisture	Protein	Ether extract	Crude fiber	Acid detergent fiber (9)	N-free extract	Ash
	pCi/kg	*g/kg*	%	%	%	%	%	%	%
Alfalfa hay	3494	23.5	9.0	15.1	1.1	31.5	39.6	34.9	8.4
Corn	110	3.9	16.00	8.7	3.4	1.8	4.8	68.3	1.8
Soybean oil meal	851	25.3	6.8	45.5	.9	5.9	16.6	34.1	6.8

tracer ^{134}Cs were counted for shorter times, the time being inversely proportional to the added activity. Chemical analyses of feed were performed according to standard methods of AOAC (8) and the acid detergent fiber determinations according to the methods of Van Soest (9).

RESULTS

It was observed that in addition to the difference in fiber intake between the 2 rations, there was a great difference in K concentration as well. The K intake with the high hay ration was higher than that with the high grain ration by a factor of 2.6. The intake of ^{137}Cs with the high hay ration was 3.9 times as great as the ^{137}Cs intake with the high grain ration.

The percentage of fallout ^{137}Cs and K found in milk, urine and feces for individual cows fed the 2 rations is presented in table 2. The percentage of ^{137}Cs secreted in milk was less than that for potassium with both diets ($P = < 0.01$). Urine was the major excretory route for K, and ^{137}Cs was excreted principally in the feces. The fecal-to-urine ratios were 4.6 and 3.5 for ^{137}Cs with the high hay and high grain rations, respectively. The same ratios for excretion of K were 0.1 and 0.3. A greater fraction of the K intake was secreted in milk and feces with the high grain ration which resulted from a lower K intake than from the high hay ration.

The passage of ^{137}Cs from feed to milk has been described as the transfer coefficient defined as the percentage of daily intake secreted per liter of milk (4). Stewart[4] has shown that the percentage of ^{137}Cs intake found in milk is a linear function of the volume of milk produced. Thus, the transfer to milk based on the transfer coefficient is more nearly constant than the percentage based on daily output in milk. The mean transfer coefficient observed for the high hay diet was 0.48 as compared with 1.21 for the high grain diet. These values were significantly different at the 1% confidence level.

[4] Stewart, H. F. 1964 Factors influencing the levels of cesium-137 secreted in milk. Ph.D. Thesis. Colorado State University, Fort Collins.

TABLE 2

Distribution of ^{137}Cs and K excretion in milk, urine and feces of individual cows

Cow no.	Feed-milk transfer coefficient [1] for ^{137}Cs	^{137}Cs intake excreted in			K intake excreted in		
		Milk	Urine	Feces	Milk	Urine	Feces
		%	%	%	%	%	%
		High hay ration					
1	0.50 ± 0.012 [2]	8.6	15.9	75.5	9.2	82.2	8.6
2	0.44 ± 0.046	3.0	19.0	77.9	4.9	86.9	8.2
3	0.44 ± 0.060	2.5	17.7	79.8	3.0	88.0	9.0
4	0.48 ± 0.048	6.6	14.7	78.7	9.4	81.8	8.7
5	0.36 ± 0.042	5.3	18.0	76.7	6.0	87.1	6.9
6	0.44 ± 0.012	3.2	17.2	79.6	5.2	84.9	9.9
7	0.58 ± 0.022	9.1	13.8	77.1	11.9	77.9	10.3
8	0.64 ± 0.014	5.4	18.6	76.0	6.2	86.1	7.8
Mean	0.48 ± 0.032	5.4	16.7	77.6	6.9	84.3	8.6
		High grain ration					
1	1.21 ± 0.068	16.4	17.5	66.1	29.3	53.3	17.4
2	1.04 ± 0.028	6.3	29.5	64.2	12.6	67.1	20.3
3	1.40 ± 0.222	4.3	25.7	70.0	7.3	72.0	20.7
4	1.21 ± 0.064	9.2	18.5	72.3	21.2	59.5	19.3
5	0.88 ± 0.104	11.1	17.4	71.5	22.3	57.9	19.8
6	1.28 ± 0.028	5.1	20.8	74.1	13.7	76.3	10.0
7	1.28 ± 0.148	18.8	12.1	69.1	31.4	40.4	28.2
8	1.54 ± 0.058	12.5	19.6	67.9	17.6	65.8	16.6
Mean	1.21 ± 0.090	10.4	20.1	69.4	19.4	61.5	19.0

[1] Transfer coefficient = % of ^{137}Cs intake/liter of milk.
[2] SD of mean of 10 observations for each cow.

TABLE 3

Comparison of output of ^{137}Cs and K in milk, urine and feces from cows receiving high hay and high grain diets

	High hay diet	High grain diet	Ratio
Milk, kg/day	11.7	11.4	1.0
Urine, kg/day	15.6	8.4	1.9
Fecal H_2O, kg/day	17.2	6.2	2.8
^{137}Cs intake, pCi/day	35,250	9,045	3.9
^{137}Cs, pCi/kg milk	177.7	97.5	2.1
^{137}Cs, pCi/kg urine	393.4	247.3	1.6
^{137}Cs, pCi/kg fecal H_2O	1,690	1,181	1.4
K intake, g/day	249	94	2.6
K, g/kg milk	1.66	1.58	1.0
K, g/kg urine	15.0	6.6	2.3
K, g/kg fecal H_2O	1.29	2.75	0.5

TABLE 4

Milk secretion of orally administered ^{134}Cs [1] and fallout ^{137}Cs from high hay or high grain rations

	Milk transfer coefficient		Daily intake secreted in milk	
	^{134}Cs	^{137}Cs	^{134}Cs	^{137}Cs
	%	%	%	%
High hay ration	0.92	0.48	12.8	4.4
High grain ration	1.36	1.21	18.8	9.3

[1] The cows were given doses daily for up to 12 days; all ^{134}Cs values corrected for equilibrium by integration of the excretion functions from $t = 0$ to $t = \infty$.

Additional insight into the comparative metabolism of ^{137}Cs and K as a function of diet can be obtained from the data presented in table 3. The urine output was considerably greater with the hay ration and the increase in fecal water was even greater as compared with the high grain ration. However the concentration of both ^{137}Cs and K in urine and fecal water was lower than would be anticipated from the ratio of ^{137}Cs and K in the 2 diets. The K concentration in fecal water was greater on the high grain diet (table 3). The K concentration in milk was same for both treatments and appears to be nearly constant for all cows (10).

The results of the double tracer experiment comparing simultaneous milk secretion of fallout ^{137}Cs and orally administered ^{134}Cs are summarized in table 4. There was, in the case of both ^{137}Cs and ^{134}Cs, a greater transfer of the dose to milk when administered with the grain ration as compared with the hay ration. The transfer coefficient for ^{134}Cs with the grain diet was 1.5 times that for the hay diet, whereas for fallout ^{137}Cs this same ratio was 2.5 in the feeding trial (table 1). Since the milk production of the 2 cows used was essentially the same for both tracer experiments, the percentage intake secreted per day for 2 treatments is comparable (table 4).

Milk secretion of ^{134}Cs following cessation of the dose was analyzed to determine the components of the biological retention function. The function was found to be adequately described by the sum of three exponential terms. Since 75% of the total excretion consisted of 1- and 3-day half-times, it indicated that sufficient time was allowed during the change-over periods to reach near-equilibrium conditions.

DISCUSSION

The excretion pattern of fallout ^{137}Cs differed from that observed for K in the diet. K was excreted principally via the urine for both diets. This is consistent with the assumption that there was nearly complete absorption of K from the ration. The urine-to-fecal ratios for fallout ^{137}Cs, however, were considerably less than one. This

TABLE 5

Distribution of ^{137}Cs and K in milk, urine and feces

Source of cesium	Milk		Urine		Feces		Reference
	Cs	K	Cs	K	Cs	K	
			% of intake				
Tracer ^{137}Cs	9.6	—	30.0	—	40.0	—	Hood and Comar (1)
Tracer ^{134}Cs	10.5	—	30.0	—	32.7	—	Cragle (2)
Tracer ^{137}Cs	7.9	8.0	25.5	86.6	54.3	7.8	Sansom (3)
Fallout ^{137}Cs	7.4	11.9	13.5	62.4	81.5	18.9	Stewart et al. (4)
None	—	12.0	—	75.0	—	13.0	Ward (18)
None	—	24.0	—	66.0	—	10.0	Forbes (17)
Fallout ^{137}Cs	13.0	9.4	—	—	—	—	Kahn et al. (12)
Fallout ^{137}Cs hay diet	5.4	6.9	16.7	84.3	77.6	8.6	Present study
Grain diet	10.4	19.4	20.1	61.5	69.4	19.0	

is in sharp contrast with the results observed in rats in which urine-to-fecal ratios of cesium were approximately 9:1 (1, 11).

A comparison of our mean values with results of other studies is presented in table 5. Hood and Comar (1) and Cragle (2) both found a ratio of radioactive cesium in feces to urine of about one for the cow. Sansom (3) found a ratio of 2:1, whereas our data with fallout ^{137}Cs indicated ratios of 3.5 with the high grain ration and 4.4 with the high hay ration.

Comparing values from the 2 rations, there was more transfer of fallout ^{137}Cs to milk from the high grain diet. Both transfer coefficients are lower than those reported previously for tracer studies. Cragle (2) found the transfer coefficient for tracer ^{134}Cs to be about 1.7% per liter and Sansom (3) 0.84% per liter. In the only other study of the transfer of fallout ^{137}Cs to milk, Kahn et al. (12) found 0.70% per liter for both pasture and feedlot conditions.

Lower transfer coefficients with the hay diet indicate that a smaller fraction of the fallout ^{137}Cs found in the hay component as compared with the grain is available for absorption. From a comparison of the milk transfer coefficients resulting from the administration of two cesium isotopes in two different diets it is possible to deduce information on the availability of the radionuclide to dairy cattle. The transfer coefficients for cesium from the high grain diet were about the same whether in the form of the soluble tracer dose of ^{134}Cs or as fallout ^{137}Cs from the ration. This can be interpreted to mean that soluble $^{134}CsCl$ and the ^{137}Cs contained in grain are equally available. However, the high hay diet reduced the transfer coefficient 30%. This reduction can be interpreted as a fiber effect, probably due to adsorption of ^{134}Cs to undigested fiber particles. The transfer coefficient for fallout ^{137}Cs from the hay diet was reduced relative to the high grain diet by 50% in the tracer study (table 4) and by 60% in the feeding trial (table 2). Availability of the fallout ^{137}Cs apparently was considerably below what could be explained by a fiber effect.

This is interpreted to mean that a fraction of fallout ^{137}Cs in forages is less available than that found in grain. It appears that this may be due to claybound ^{137}Cs which adheres to the outside of forage plants. That found in grains by comparison gets there only by translocation from exterior parts of the plant and thus must be soluble. The reduction in transfer coefficient due to factors concerned with alfalfa hay other than fiber are thus the difference between 50% and 30% as indicated by the tracer study or 60% and 30% for the feeding trial. The latter case indicates an equal importance for the fiber effect and of other factors; perhaps irreversible ion exchange by plant surface clay minerals.[5] The ability of clay minerals, such as Illite or Montmorillonite, to bind ^{137}Cs ions is well-known. Experiments in this laboratory showed that when alfalfa plants were washed with water, the clay fraction in the wash residue contained up to 30% of the total fallout ^{137}Cs.

[5] Johnson, J. E. 1965 Metabolism of fallout ^{137}Cs in bovine. Ph.D. Thesis. Colorado State University, Fort Collins.

The effects of the great difference in K intake between the 2 rations cannot be completely differentiated from the effects of the high fiber content. As shown in table 1, both K intake and fiber intake were considerably higher with the hay ration. There was a greater transfer of K to milk than of fallout ^{137}Cs. This is the reverse of findings by Kahn et al. (12) and Cragle (2), both of whom reported a greater transfer of cesium to milk than K.

The large difference in urine output (15.6 vs. 8.4 kg/day, table 3) between cows on the 2 treaments was interpreted as being due to differences in potassium intake (13) and the consequent necessity to void larger amounts of the potassium salt. Blaxter and Rook (14) studied rations which differed in potassium intake and observed a similar increase in urine volume with increased potassium intake.

The water loss in feces was also considerably higher for the roughage ration (17.2 vs. 6.2 kg/day) even though the average water content of feces was slightly higher for the high grain ration. Rook and Balch (15) found a potassium concentration of 2 g per kg of fecal water which compares favorably with the values from this experiment of 1.1 and 2.4 g per kg for the 2 types of rations studied. The volume of water excreted in feces was 2.8 times greater with the hay diet. It was expected that K and ^{137}Cs loss in the feces would be a function of the quantity of fecal water because the 2 cations should be in equilibrium with plasma water. It might be anticipated, however, that the higher concentrations of Ca and Mg in fecal water with the hay diet (table 6) would reduce the concentration of both K and ^{137}Cs by osmotic effects. The K concentration was in fact lower in fecal water with the hay ration but the ^{137}Cs concentration was higher.

TABLE 6

Concentration of calcium, magnesium, sodium and potassium in fecal water from a high hay or high grain ration

Cation	High hay ration	High grain ration
	g/kg H_2O	*g/kg H_2O*
Ca	32.6	18.8
Mg	7.4	0.5
Na	4.0	0.6
K	1.3	2.8

It appears that only a fraction of the fallout ^{137}Cs measured in a forage sample is available to the cow for absorption from the rumen or the lower gastrointestinal tract. Nearly all of the cesium salts that would be expected to occur in the primary fallout particle are soluble (16), yet the transfer coefficient was a factor of nearly 2 less for the fallout form as for soluble $^{134}CsCl$ administered with an identical high hay ration. An appreciable fraction of the portion that is soluble is apparently adsorbed on the fibrous materials in the ration. This is apparent from the ratio of the milk transfer coefficient for the tracer doses given with the 2 rations. The transfer coefficient was nearly 50% higher when given with the high grain ration. It is concluded, therefore, that primary fallout deposition is present in forage samples in 2 forms, one bound by clay residues on the plant surface and another soluble fraction absorbed into the plant tissues. The former fraction is completely unavailable to the cow and of the latter, a fraction is adsorbed by fiber in the rumen and hence also unavailable.

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Measurement of Available Lysine in Heated and Unheated Foodstuffs by Chemical and Biological Methods[1]

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ABSTRACT The availability of lysine in meat, and in egg albumin before and after heat treatment in the presence of glucose, was determined by rat growth assay and by the fluorodinitrobenzene (FDNB) method. A 25% gluten diet was used as a basal diet to which graded levels of lysine or the protein under test were added. There was a high correlation between lysine consumed and weight gain of rats fed the standard diets for 2 weeks. Autoclaving of egg albumin to which 1 to 2% glucose had been added decreased the availability of lysine as measured by the FDNB method. However, the rat-growth assay gave a far lower value. This lowering was found not to be due to the formation of toxic compounds nor to loss of methionine. Similar results were obtained using autoclaved beef. Digestibility measurements of autoclaved egg albumin gave values similar to the value obtained by rat-growth assay. Fecal analysis showed that not only was lysine excretion increased but also excretion of other amino acids as well. The FDNB method does not appear suitable for the estimation of availability of lysine in heat-treated foods. It appears from fecal excretion studies that some lysine in heat-treated proteins measured as available by the FDNB method may be excreted as part of the undigestible residue in the feces.

The primary function of dietary protein is to furnish amino acids for the synthesis of tissue proteins. A measure of the efficiency with which the amino acids of highly digestible proteins can be utilized is readily obtained by calculating chemical score from an amino acid analysis of the protein (1). However, particularly in heat-processed proteins, some amino acids may be unavailable owing to interactions among themselves or with other components of the food or feed, or to alteration of the amino acid molecule itself (2). Lysine and methionine are the amino acids most subject to such changes.

Lysine is the indispensable amino acid that is most limiting in many foods of plant origin, especially in cereal grains, which represent the major source of protein for most of the poor countries (3); consequently, particular attention has been given to determination of availability of lysine. Carpenter (4) developed a method based on the reaction of the ϵ-amino group of lysine with fluorodinitrobenzene (FDNB) for measuring the availability of lysine. Many biological methods have also been used. Enzymatic methods have been developed by Mauron et al. (5) and Sheffner et al. (6). Schweigert and Guthneck (7) and Guthneck et al. (8) developed a method based on the growth of the protein-depleted adult rat. Kuiken and Lyman (9) and Kuiken (10) measured the amount of ingested lysine excreted in the feces of rats fed different foodstuffs. Gupta et al. (11) used the rate of growth of the young rat over a 2-week period as the basis for measuring lysine availability. Ousterhaut et al. (12) estimated the availability of various essential amino acids in proteins from the growth of chicks fed a basal diet containing crystalline forms of all of the essential amino acids except the one being assayed, with the protein under test as the only source of this amino acid.

Microbiological assay methods have been devised and used for the estimation of available lysine in foods. Ford (13) compared different test microorganisms as a means of assessing the availability of methionine, tryptophan and lysine. Scott and Smith (14) used the microorganism, *Tetrahymena pyriformis*, and found that microbiological assays of meat meals gen-

Received for publication September 22, 1967.
[1] Supported in part by a grant from the National Live Stock and Meat Board, Chicago.

erally gave lower values than the FDNB method. They thought other factors might have influenced the assay besides the unavailability of amino acids.

Several of these methods have been used to study the effect of heat treatment on the nutritive value of proteins. Moderate heat treatment has been shown to improve the food value of certain plant proteins, mainly those of legumes (15), whereas severe heat treatment usually lowers the nutritive value. Observations in vitro have shown that the rates of enzymatic release of lysine from proteins decreased when the proteins were heated dry (16, 5). Autoclaving at 121° (250° F) produced a marked decrease in the utilization of the lysine by animals for growth (17).

Carpenter et al. (18) measured the amount of lysine present in different fish proteins using standard chromatographic methods and the FDNB method. Values obtained with the FDNB method were lower than those obtained by the chromatographic method; however, values obtained through biological assays were higher than the values obtained by chemical methods.

The present studies were undertaken in an attempt to assess the reliability of the growth rate of rats as a means of estimating the amount of available lysine in foods and feeds and to study some of the factors affecting estimates of available lysine in heat-treated proteins.

EXPERIMENTAL

Chemical estimation of lysine and other amino acids. The FDNB method as developed by Carpenter (4) was used as a chemical procedure to determine the amount of lysine available in proteins under test.

In some experiments lysine, as well as other amino acids in proteins or fecal materials, was determined after hydrolysis of the material with 6 N hydrochloric acid under nitrogen in a sealed tube in an oven at 105° for 18 hours. The amino acid composition of the hydrolysate was determined using a Technicon amino acid analyzer.

Heat treatment of proteins. Samples of egg albumin were first autoclaved for different time-intervals at 121°. Other samples were prepared to contain graded levels of glucose and were then autoclaved for 12 hours. Egg albumin autoclaved with 2% glucose was used in the later studies.

Fresh lean beefsteak obtained commercially was autoclaved for different time-intervals in the same way as described above. The samples were freeze-dried and then extracted with diethyl ether for 24 hours. Samples were aerated and ground in a ball mill.

Growth experiments. Male weanling rats (Holtzman and Sprague Dawley strains) weighing 50 to 55 g were used throughout these investigations. The rats were kept for 2 days in individual suspended screen-bottom cages and fed a basal diet containing wheat gluten. The wheat gluten contained 11.3% butanol-extractable material, and this was taken into account in preparing the basal diet which contained wheat gluten, 25%; fat, 5%; vitamin mixture, 5%; salt mixture (23), 5%; and was made to 100% with a 2:1 starch-dextrin mixture.

The diets used as standards for determining available lysine were prepared by adding graded levels of lysine hydrochloride to the basal diet. Additions to the basal diet were made at the expense of the dietary carbohydrate. Groups of 6 animals each were fed ad libitum for 2 weeks. Rats were weighed every 2 days and food intake was recorded either for the group or for individual rats.

Digestibility measurement. Groups of 3 animals each were fed 5% of the test protein in the 25% wheat gluten basal diet for 8 days. The rats were then transferred to stainless steel metabolic cages where feces were collected for 4 days, during which food consumption was recorded daily. A control group received 4% of whole egg protein. Nitrogen in feed and feces was then determined. Corrections for metabolic fecal nitrogen were based on total body weight (19). As all diets were low in fiber, no adjustment was made for differences in diet composition.

RESULTS

The amount of available lysine in the different proteins as determined by the FDNB method is shown in table 1. Al-

TABLE 1

Available lysine in proteins as determined by the fluorodinitrobenzene (FDNB) method

Protein	Lysine
	%
Egg albumin	6.24
Wheat gluten	1.54
Meat	9.10

TABLE 2

Effect on the availability of lysine of heating egg albumin-glucose mixtures in an autoclave (121°) for 12 hours

Glucose added	Available lysine	Original lysine available [1]
%	%	%
0	6.24	—
1.0	4.91	78
1.5	3.99	64
2.0	3.73	60

[1] Determined by the fluorodinitrobenzene (FDNB) method.

TABLE 3

Amino acid analysis of egg albumin heated for 12 hours at 121° with 2% of D-glucose

Amino acid	Untreated sample	Heated in autoclave	Recovery after heating
	%	%	%
Val	5.99	4.99	83.3
Met	3.04	0.601	19.8
Ile	4.58	3.67	80.1
Leu	7.6	6.07	79.9
Phe	5.19	4.72	90.9
Lys	6.59	4.53	69.7
His	2.20	1.49	67.7
Arg	5.74	4.02	70.0

TABLE 4

Effect on "availability" of lysine of heating beef muscle in an autoclave (121°)

Time of autoclaving	Available lysine [1]	Original lysine available
	%	%
Untreated	9.10	100.0
30 minutes	8.87	96.5
3 hours	8.76	96.3
12 hours	7.60	83.5

[1] Determined by the fluorodinitrobenzene (FDNB) method.

though very low in lysine, 25% of wheat gluten provides all of the other indispensable amino acids in amounts that should satisfy the requirements of the rat. For this reason, wheat gluten was used as the protein source in the basal diet.

Heating egg white in an autoclave (121°) for 2 hours did not reduce its content of available lysine but when it was heated for 12.5 hours, the loss of available lysine was about 13%. When samples containing different amounts of glucose were similarly heated for 12 hours, the loss of available lysine increased as the amount of glucose added was increased (table 2). The sample that contained 2% of glucose was analyzed for amino acids and not only was the value for lysine low, but also the values for methionine, histidine and arginine (table 3). Beef muscle heated in an autoclave for different periods of time lost from 3.5 to 16.5% of its available lysine, as determined by the FDNB method (table 4).

Reliability of standard curve for growth method. The results of 3 experiments in each of which 6 groups of rats received 6 different levels of lysine are shown in table 5. The slope and the intercept of the sample regression line were calculated for each experiment (20). The standard deviation of the regression coefficient and the significance of the regression and the correlation coefficients were calculated. A summary of the values obtained is tabulated in table 6. The 18 individual group values were used to calculate the average standard curve for the 3 experiments and the confidence belt was calculated. This is illustrated in figure 1.

The same procedure was applied to test the relationship between protein efficiency ratio (PER). and lysine consumed. The correlation coefficients for the 3 sets of experiments were 0.985, 0.978 and 0.991, respectively.

Measurements of biologically available lysine. It was necessary at the outset to compare the value for the availability of lysine in wheat gluten as determined biologically with the value obtained by the FDNB method. Two diets were used in which wheat gluten furnished 2 different amounts of lysine. Food intake, weight

TABLE 5

Effect of lysine content of diet on food consumption, weight gain and protein efficiency ratio (PER)

Set no.	Lys level	Food consumed	Lys consumed	Wt gain	PER
	%	g/2 wk	g/2 wk	g/2 wk	
1	0.385	111.3	0.429	21.6 ± 2.19 [1]	0.88
	0.485	145.7	0.707	43.3 ± 1.78	1.35
	0.585	152.4	0.892	50.1 ± 2.25	1.48
	0.685	151.2	1.036	65.4 ± 3.04	1.96
	0.785	141.2	1.108	63.3 ± 2.15	1.77
	0.885	137.8	1.219	68.2 ± 2.98	2.19
2	0.385	127.6	0.491	25.7 ± 1.7	0.92
	0.435	144.1	0.627	35.7 ± 1.02	1.12
	0.535	163.2	0.873	55.6 ± 3.22	1.54
	0.635	180.3	1.145	71.1 ± 3.39	1.62
	0.735	176.7	1.299	86.7 ± 1.88	1.99
	0.835	174.4	1.456	85.3 ± 1.41	1.98
3	0.385	107.9	0.415	20.8 ± 1.39	0.88
	0.435	120.7	0.525	25.6 ± 1.57	0.96
	0.535	142.8	0.764	42.7 ± 2.94	1.35
	0.635	170.2	1.081	61.0 ± 1.61	1.61
	0.735	169.8	1.248	77.0 ± 3.48	2.02
	0.835	168.2	1.404	85.3 ± 3.71	2.25

[1] Average for 6 rats ± SE of mean.

TABLE 6

Statistical analyses of regression lines relating weight gain to lysine consumed

	Regression coefficient (b)	$t = b/Sb^*$	r
Set 1	59.98	11.14	0.984
Set 2	65.74	12.92	0.988
Set 3	66.96	32.36	0.998
Pooled sets	66.0	27.92	0.990

* t = test of significance of b. All values, $P < 0.001$.

gain, weight gain computed from the standard curve and the amount of lysine estimated by the FDNB method are shown in table 7. The correspondence of the actual and exepected values for weight gain indicates that lysine of untreated wheat gluten is highly available.

From 2 to 7% of the different proteins examined were incorporated in the basal diet for the determination of lysine availability by the growth method. Lysine availability was calculated using the following equations:

(1)

$$\% \text{ lysine available} = \frac{\text{total Lys by rat assay}}{\text{total Lys by chemical assay}} \times 100.$$

This gives the availability of the total dietary lysine.

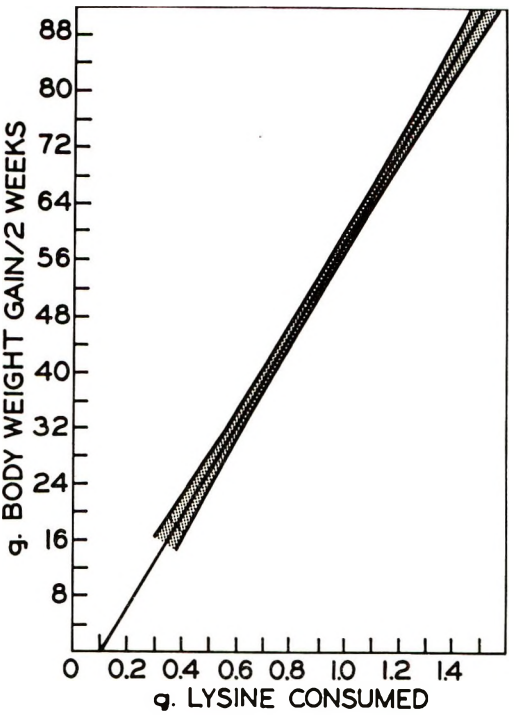

Fig. 1 Standard curve for the pooled values from 3 experiments relating weight gain to lysine consumed. The shaded zone represents the confidence belt.

(2)

$$\% \text{ lysine available} = \frac{\text{total Lys by rat assay} - \text{gluten Lys consumed}}{\text{total Lys by chemical assay} - \text{gluten Lys consumed}} \times 100.$$

This gives the available lysine in the test sample only. The values obtained for autoclaved egg albumin by the second equations were extremely low (table 8). Those for autoclaved meat were below the values obtained by the FDNB method. The low values for the autoclaved products were at first thought to result from the presence of a toxic material formed during autoclaving of the protein which might have affected the body weight gain of the animals.

Groups of 6 animals each were fed a basal diet containing 18% casein supplemented with 0.2% L-methionine and the same diet with 2, 4 or 9% autoclaved egg albumin added. No depression in body weight gain was observed over the 2-week feeding period (72.5 g to 74.9 g) and feed efficiencies ranged from 45.7 to 51.7 (g weight gain/100 g food consumed). Therefore, it seemed unlikely that any toxic substance was formed during autoclaving.

Using values for the amino acid composition of egg albumin, a 25% gluten diet was prepared to contain amino acids in the amounts that would be supplied by 5% of unautoclaved egg albumin but with the amount of lysine that would be supplied by 5% of autoclaved egg albumin as determined by the FDNB method. Body weight gain of the group that recevied the amino acid mixture coincided with the value pre-

TABLE 7

Availability of lysine in wheat gluten

Lysine level (FDNB) [1]	Food consumed	Lysine consumed	Avg wt gain	
			Found	Expected [2]
%	g	g	g	g
0.485	133.0	0.645	36.3	36.0
0.635	134.9	0.857	45.6	49.2

[1] Indicates fluorodinitrobenzene method.
[2] Computed from the standard curve of grams weight gain vs. grams lysine consumed.

TABLE 8

Availability of lysine in autoclaved meat and in egg albumin autoclaved with glucose

Protein level	Wt gain	Lysine consumed	% available lysine using equation no.[1]	
			1	2
%	g/2 wk	g/2 wk		
Unautoclaved egg albumin				
1.6	52.8	0.98	100.1	100.2
4.8	53.3	0.93	97.6	94.0
5.0	57.1	0.99	98.0	94.6
Autoclaved egg albumin				
5	25.4	0.785	62.7	16.8
5	29.5	0.884	66.5	25.6
5	29.3	0.902	61.5	13.4
7	31.9	1.004	59.0	20.2
7	33.6	1.124	55.0	15.2
7	32.2	1.102	54.6	13.5
Unautoclaved meat				
2	46.0	0.784	102.7	106.3
4	63.5	1.161	93.6	91.7
Autoclaved meat				
2	32.5	0.687	87.3	61.4
4	55.5	1.159	81.5	65.5

[1] See text for details about equations; equation no. 1 gives a value for the total diet and equation no. 2 for the supplement.

TABLE 9

Effect of addition of methionine on availability of lysine of autoclaved proteins

Protein level	Wt gain	Lysine consumed	% available lysine using equation no.[1]	
			1	2
%	*g/2 wk*	*g/2 wk*		
Autoclaved egg albumin				
5	26	0.759	66.3	32.6
5 + 0.2% L-Met	25	0.723	67.5	27.2
Autoclaved meat				
2	34.3	0.699	90.1	62.5
2 + 0.2% L-Met	34.0	0.710	88.9	56.1

[1] See text for details about the equations and footnote as for table 8.

dicted from the standard curve (g weight gain/2 weeks vs. g lysine consumed). This shows that values for untreated samples determined by the FDNB method coincide with values obtained by the growth method. However, it also indicates that values for autoclaved materials obtained by the FDNB method do not agree with those obtained biologically.

In another experiment, methionine was added to diets containing the autoclaved egg albumin and beef. The availability of lysine was calculated by the previously mentioned methods and the results (table 9) show that the addition of methionine was without effect.

Digestibility of egg albumin. The digestibility values were calculated for the autoclaved and nonautoclaved samples:

$$\text{True digestibility} = \frac{\text{N intake} - (\text{fecal N of test group} - \text{metabolic fecal N}) \times 100}{\text{N intake}}$$

This equation measures the digestibility of the total dietary protein. Therefore, to calculate the digestibility of the protein supplement, the following equation was used:

$$\text{Gluten N consumed} \times \frac{100}{100} + \text{test protein N consumed} \times Y = \text{total dietary protein N} \times \text{observed digestibility}$$

where Y is the digestibility value of the test protein (assuming 100% digestibility of gluten). Results obtained (table 10) show that the digestibility of the autoclaved egg albumin was greatly decreased.

Amino acid analysis of the feces showed that not only was lysine excretion greatly increased but also excretion of other essential amino acids (table 11).

TABLE 10

Digestibility of autoclaved and unautoclaved egg albumin [1]

Protein	Observed true digestibility of total dietary protein	Calculated [2] digestibility of test protein
	%	%
Unautoclaved	99.2	94.4
Autoclaved	87.6	18.1

[1] Average of 3 experiments.
[2] See text for the method of calculation.

TABLE 11

Fecal excretion of amino acids by groups of rats fed 5% autoclaved and 5% nonautoclaved egg albumin samples [1]

Amino acid	Fed treated sample (1)	Fed untreated sample (2)	1/2
	mg/100 g food consumed		
Val	343	84	4.1
Ile	261	69	3.8
Leu	419	99	4.2
Phe	288	58	5.0
Lys	309	94	3.3
His	102	26	3.9
Arg	231	64	3.6

[1] Amino acids recovered in pooled feces from 4 rats in 4-day period.

DISCUSSION

Gupta et al. (11) used a basal diet containing 20% of wheat gluten supplemented with some essential amino acids for measurement of availability of lysine by the growth method. Munaver and Harper (20) suggested that a 30% wheat gluten diet would satisfy the accepted amino acid requirements of the rat except for lysine. We found that a 25% wheat gluten diet sup-

plemented with lysine to give a lysine content of 0.835%, supported a rate of weight gain that compared well with that obtained with an 18% casein diet supplemented with 0.2% L-methionine. Calhoun et al. (21) also found a wheat gluten diet to be suitable for the assessment of lysine availability by the growth method.

Table 4 shows that addition of lysine to the basal diet stimulated food intake; also that body weight gain increased with increasing lysine consumption. However, total food intake increased with increasing dietary lysine content only up to about 0.6% and after that it remained more or less constant, but growth rate continued to respond to increasing dietary lysine content. A high degree of correlation between lysine intake and growth was obtained. This is in agreement with the results obtained by Calhoun et al. (21) and de Muelenaere et al. (22). Calhoun et al. (21) suggested the use of the relationship between the increase in carcass nitrogen and the amount of available lysine consumed. Our results show that the standard curve for the growth method can be used, with confidence, as a measure of available lysine in untreated proteins. A high correlation was also obtained between protein efficiency ratio and lysine intake.

De Muelenaere et al. (22) suggested that an increase in the protein content of the diet may tend to decrease the value for availability of lysine as determined by the growth method. However, values obtained for the untreated protein samples show that this does not occur when a 25% gluten diet is used and 5% of the protein supplement is included. Also the possibility that an amino acid imbalance might have depressed the growth of rats under such conditions (23) appears unlikely as the amino acid mixture simulating egg albumin in its composition with lysine equivalent to that in heated albumin did not depress growth.

The amino acid composition of a protein can be altered by severe heat treatment and even more if it is heated in the presence of free aldehyde groups of glucose (24). This is shown clearly in tables 2, 3 and 4. However, values for availability of lysine can vary with the method of assay used (21, 22). Available lysine in autoclaved egg albumin as determined by the FDNB method was about 56%, whereas that determined by the growth method was about 20%. Similar but smaller differences were observed for autoclaved beef muscle. The reason for such differences is presumably that the presence of bound sugars interfered with enzymatic hydrolysis in the intestine, in such a way that adjacent amino acids were not hydrolyzed (2). This is indicated by our observation that about 80% of the autoclaved egg albumin-lysine was excreted in feces (calculated on the assumption that lysine of wheat gluten was completely available).

In a comparison of a chick assay method with the FDNB method for determining the availability of lysine in foods, Carpenter and associates (18) used a nonpurified diet to which were added different protein supplements. The FDNB method gave values that were lower than the values obtained by the chick assay.

Evans and Butts (25) autoclaved a mixture of 8 g soybean and 2 g sucrose for 4 hours at 120°. On acid hydrolysis of the product, they found that 47% of lysine content was lost. However, enzymatic hydrolysis showed a loss of 84%.

Recently, Stott and Smith (14) compared values for lysine available in some foods as determined microbiologically with those obtained by the FDNB method. They found that values obtained microbiologically were much lower than those obtained by the FDNB method and in some instances the microbiological values were about 60% of those given by the chemical method. Ford (13) mentioned that the availability of lysine in a leaf protein concentrate was low using a microbiological assay procedure, whereas the FDNB method gave higher values. However, microbiological assay results were consistent with those obtained with a biological assay using the rat (26). Of interest is that when enzymatic digestion with pepsin or higher concentrations of papain was more extensive, higher values were obtained (13). This indicated that the protein as a whole was digested slowly. Our results show similar differences between the rat assay and the FDNB determination of available lysine in heat-treated proteins (FDNB method gave a

value of 60% available lysine in autoclaved egg albumin and the rat assay gave about 20% available). Also, when availability was calculated using the equation:

$$\text{Total availability of Lys in diet} = \frac{\text{Lys intake} - (\text{Lys excreted by test animals} - \text{Lys excreted by controls}) \times 100}{\text{Lys intake}}$$

the value obtained was 65%. This compares well with the average value of 62% obtained using equation 1 (table 8).

Our results show a high correlation between the values obtained by the FDNB method and the growth assay procedure for untreated egg albumin, beef protein and casein. The discrepancy occurred only with the autoclaved protein preparations. The FDNB method as recommended by Carpenter would not take rate of digestion into account nor the possibility that, after digestion of a severely processed "food," many of the lysine moieties reacting as "available" might remain bound in indigestible peptide residues and thus be unavailable to the rat.

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Development of a Semipurified Diet for the Adult Pocket Mouse (Perognathus)[1]

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ABSTRACT A semipurified diet was developed for the pocket mouse (*Perognathus longimembris* and *P. penicillatus*), small desert rodents that do not drink water. The key difference between this diet and a standard semipurified mouse diet is the mineral composition. The ratio of K/Na and Mg/Ca is high and the inorganic phosphates are replaced by the calcium, magnesium and sodium salts of glycerophosphates. The adequacy of this diet has been shown by the maintenance of over 100 pocket mice for 6 months without weight loss, with a normal behavioral pattern and in apparent good health. Carcass composition and the size and microscopic appearance of organs were the same for animals fed this diet as compared with animals fed their customary mixed seed diet.

The pocket mouse (*Perognathus*) is one of the smallest known mammals, and is typical of a group of desert rodents that do not drink water. Because of their small size, their ability to survive for long periods of time with dry food (1), their resistance to radiation (2) and their ability to hibernate and estivate (3), these animals are of great interest to the physiologist and biologist. They may be suitable tools for the study of mammalian physiology in long space flights, for understanding many aspects of the control mechanism of hibernation and for studies of electrolyte and water economy.

Because this animal produces only small amounts of excreta, it is exceptionally clean, is easy to handle in a laboratory, and requires minimal care. Most laboratories (1, 4) maintain these animals and related desert rodents with mixtures of dry seeds. Apparently no semipurified diet has been developed for them, and no report has come to our attention regarding their nutritional requirements. The development of a semipurified diet adequate for reproduction, growth and maintenance of the pocket mouse appears necessary before most of the studies outlined above can be undertaken. Recently, Zeman (5) developed a semipurified diet for the Mongolian gerbil (*Meriones unguiculatus*), but our studies indicate that the nutritional requirements of the pocket mouse differ from those of that rodent.

In the present report we describe a diet adequate for maintenance of adult pocket mice, together with certain unusual mineral requirements of the animal, found during development of the diet.

METHODS AND RESULTS

Male and female adult pocket mice of 2 subspecies were used in our experiments, all trapped in the Sonora desert region of the United States: *Perognathus penicillatus*[2] (weight at maturity, 15–25 g; trapped in the high desert around Tucson, Arizona, elevation 1,000 m) and *P. longimembris*[3] (weight at maturity, 7–11 g; trapped in Antelope Valley, California, elevation 1,000 m).

The mice were kept in the laboratory for at least 30 days after arrival, on a mixture of seeds that had proved satisfactory for their maintenance (1, 4). The mixture is made of equal parts of sunflower seeds, millet, canary seeds (canary grass, *Phalaris canariensis*) and oats. Some fresh carrots and lettuce were added twice a week.

The animals were housed either in 4-liter clear glass jars[4] with perforated tops, or in plastic cages[5] with screen tops. The bottom of each jar or cage was covered with 3 to 5 cm of washed sand[6] and a small amber jar was provided for a nest.

Received for publication September 18, 1967.
[1] Supported in part by NASA Grant NGE 05-003-118.
[2] Supplied by The Pet Corral, 446 Oracle Road, Tucson, Arizona.
[3] Supplied by Ernest Carl, 38960 Yucca Tree Street, Palmdale, California.
[4] Scientific Products Company, Menlo Park, California.
[5] Del Monte Properties, Pebble Beach, California.
[6] ElMolino Mills, Alhambra, California.

TABLE 1

Composition of basal diet (modified from Bell (6))

	g/100 g diet		*mg/100 g diet*
Casein [1]	22.3	$ZnCO_3$	25.0
Cornstarch	44.3	$CuSO_4$ (anhydrous)	25.0
Sucrose	5.2	$MnSO_4$	25.0
Corn oil	9.0	KI	5.0
Fiber [2]	11.2	choline chloride	135.0
$CaHPO_4$ (anhydrous)	1.2	riboflavin	0.4
$CaCO_3$	2.5	thiamine hydrochloride	0.3
NaCl	1.3	niacin	0.3
KCl	0.4	pyridoxine hydrochloride	0.1
$MgSO_4 \cdot 7H_2O$	0.1	folic acid	2.5
Fe citrate	0.1	biotin	0.01
		inositol	0.1
		p-aminobenzoic acid	1.2
		pantothenic acid	0.9
		menadione	10.0
		dl-α-tocopheryl acetate	6.0
			μg/100 g diet
		vitamin B_{12}	0.5
			IU/100 g diet
		vitamin A acetate	50
		vitamin D [3]	20

[1] Vitamin-Free Casein, General Biochemicals, Chagrin Falls, Ohio.
[2] Solka Floc, Brown Company, 733 Third Avenue, New York.
[3] Viosterol (irradiated ergosterol in oil).

The provision of sand and nest proved to be essential for the well-being of the animals. Clean cages and sand were provided monthly. The animals have to be housed individually because they are very territorial, and do not accept other mice except during estrus of the female.

Lighting in the animal room was kept on a schedule similar to the outdoor day-and-night cycle, and temperatures were maintained between 21° and 23°.

All diets had to be pelleted. The pocket mouse does not handle powders or moist mixtures well. Pellets 1 cm to 3 cm long and approximately 1 cm wide proved satisfactory. They were made by pressing the wet mixture of ingredients through a large commercial Hobart Model M-80 meat grinder and drying on screens under an air fan. All diets were kept under refrigeration, in closed containers.

The first step in the development of the diet was the attempt to feed a semipurified, pelleted diet used for regular laboratory mice. Table 1 lists the composition of this basal diet, which is a modification of that described by Bell (6). This diet failed. The mice lost weight rapidly and died unless they were returned to the standard seed diet before the weight loss reached about 25% of their original body weight (table 2). Various modifications were tried, to find out which portion of the diet was causing the problem. Diets were prepared containing one of the following changes: increases of fat, protein or vitamin content, decreases of fiber or mineral content, replacement of casein by soy protein, of corn oil by sunflower oil, of cornstarch by glucose, or of sucrose by cornstarch. All of the modified diets failed.

TABLE 2

Typical weight loss for Perognathus penicillatus *and* P. longimembris *with basal diet*

Species	Weight after fed diet, days				
	1	7	14	21	28
			g		
P. penicillatus	20.0	16.0	13.8	13.8	died
P. longimembris	8.8	8.0	6.2	5.2	died

When, however, the basal diet was supplemented with fresh carrots ad libitum, the mice did well. We are assuming that the carrots served as a vehicle of water because carrots, extracted with petroleum ether and water and autoclaved to remove as many of the micronutrients as possible, served as well as untreated carrots. Lyophilized carrots were ineffective in improving the performance of the basal diet to any noticeable extent. The addition of 5 to 10% carrot powder to the basal diet was also

ineffective. Only very large amounts of carrot powder (approximately 50% of the diet) were effective. The composition of this diet was, however, so different from that of the original, that it can no longer be considered as a modification of the basal diet.

Since pocket mice thrive for long periods on sunflower seeds, it was decided to study which fraction of this seed was responsible for the success of the natural diet. Sunflower meal was fractionated into: 1) ether extract (lipids and oil-soluble ingredients); 2) protein-mineral-carbohydrate residue of ether extraction; 3) fraction 2 after heating to destroy heat-labile vitamins; 4) protein (fraction 2 extracted by means of diluted NaCl solution); and 5) ash. Each fraction was then used separately as the appropriate part of the basal diet (table 1) with the other ingredients left unchanged.

The diets containing the fat extract (fraction 1) instead of corn oil, and in which casein was replaced by the protein (fraction 4), failed. The residue of the fat extraction, both before and after heat treatment (fractions 2 and 3), produced a satisfactory diet when used in the basal diet. Ash (fraction 5), used at a level of 5% in place of the original salt mix, produced a diet that was more satisfactory than the basal diet. The animals did fairly well when fed this diet for two or three times as long as the basal diet, but after 7 to 10 weeks, this diet also failed. These results indicated that the mineral composition of the diet was responsible for its success or failure.

An analysis of the sunflower meal showed that the seeds have a high potassium and a low sodium content, and contain a large amount of magnesium and little calcium as compared with Bell's mouse diet. The total phosphorus is also high (table 3).

Salt mixes imitating this natural mineral composition were prepared, and used in the basal diet (table 4). Even though the correct K/Na and Mg/Ca ratios and concentrations, as well as a high percentage of phosphorus were used, weight losses and death occurred in the same way as with the basal diet.

Various modifications of the salt mixture described in table 4 were tried. The ratios and concentrations of calcium, magnesium, potassium and sodium were varied and different proportions of chlorides, sulfates and citrates were used. All these modifications failed.

TABLE 3

Mineral composition of sunflower meal

	Ash [1]	Whole meal
	g/100 g	
Na	0.2 [2]	0.01
K	17.2	0.8
Ca	2.8	0.1
Mg	10.1	0.5
Fe	0.1	0.005
Cl	—	0.1 [2]
SO_4	—	0.2
P_2O_5	—	1.9

[1] Ash approximately 5% of meal.
[2] The cations were determined in the ash, the anions were determined in the meal because of possible losses during ashing.

TABLE 4

Typical salt mix used to simulate the composition of sunflower meal [1]

	g/100 g diet
NaCl	0.03
KCl	0.20
K_2HPO_4	1.36
$MgSO_4$ (anhydrous)	0.65
$MgHPO_4$	3.1
$CaHPO_4$	0.45
Fe citrate	0.025

[1] The same trace minerals were used as in Bell's salt mix (table 1).

Finally success was achieved when the inorganic phosphates were replaced by organic phosphates in the form of glycerophosphates of calcium, magnesium and sodium.

Table 5 shows the composition of the diet that has proved successful in maintaining weight, good appearance and a normal behavioral pattern in adult *P. penicillatus* and *P. longimembris*. Over 100 animals have been maintained with this diet in our laboratory for over 6 months.

Two groups of animals, one fed the seed diet and one the semipurified diet, were autopsied after 2 months. The gross appearance of the organs was normal, and organ size was the same in the 2 groups (table 6). Microscopic examination showed that the kidney, liver and intestinal mucosa of animals in both groups were histologically essentially normal. The analyses of the carcasses showed that the animals

TABLE 5

Composition of semipurified diet for the Perognathus

	g/100 g diet
Soy protein [1]	22.0
Cornstarch	52.2
Corn oil	10.0
Fiber [2]	7.0
Gum arabic [3]	3.5
Vitamin mix [4]	2.0
Na glycerophosphate [3]	0.30
KCl	0.60
Ca glycerophosphate [3]	0.60
Mg glycerophosphate [3]	1.48
$MgSO_4$	0.30
Fe citrate	0.04
	mg/100 g diet
$ZnCO_3$	25.0
$CuSO_4$ (anhydrous)	25.0
$MnSO_4$	25.0
KI	5.0

[1] Soya Assay Protein, General Biochemicals, Inc., Chagrin Falls, Ohio.
[2] Solka Floc, Brown Company, 733 Third Avenue, New York.
[3] S. B. Penick and Company, 100 Church Street, New York.
[4] See table 1.

in these 2 groups did not differ in respect to moisture, protein, fat and ash content (table 6). A rather great variability of fat and moisture content within each group was noted, which was the result of a higher fat and lower moisture content in the females compared with the males.

DISCUSSION

Although the diet we have described appears to be adequate for the maintenance of the adult Perognathus, preliminary results indicate that it is not adequate to support growth without supplementation with lettuce and carrots. These supplements probably provide a source of water which appears to be necessary for the weanling Perognathus.

We have limited our studies to the use of glycerophosphates as they are easily available and the purpose of this research was to develop a practical diet. Other organic phosphates, such as the calcium and magnesium inositols, may or may not be equally effective.

We do not yet know why the adult Perognathus can thrive with a diet containing calcium and magnesium as glycerophosphates, but not with the same diet in which

TABLE 6

Organ weights and carcass composition of Perognathus penicillatus fed seeds or the semipurified diet [1]

Diet	Body wt at autopsy	Carcass [2] wt	Moisture [3]	Protein	Fat	Ash	Liver	Heart	Kidney	Lung
	g	*g*		*g/100 g carcass*			*g/100 g body wt*			
Seeds [4]	21.2 ± 1.1	12.8 ± 0.6	60.7 ± 4.1	19.3 ± 1.3	20.9 ± 4.5	3.3 ± 0.3	3.4 ± 0.4	0.5 ± 0.04	0.9 ± 0.08	0.6 ± 0.06
Semipurified [5]	22.4 ± 1.1	13.4 ± 0.7	57.2 ± 4.3	18.5 ± 1.3	19.9 ± 6.1	2.7 ± 0.2	3.1 ± 0.1	0.5 ± 0.03	0.9 ± 0.09	0.5 ± 0.03

[1] Four animals (2 males, 2 females) each fed the respective diet for 2 months (mean ± SE).
[2] Carcass represents body after removal of head, tail, and all viscera except perirenal fat pads.
[3] Moisture was determined by drying at 110° to constant weight; fat was determined by Soxhlet extraction of the dried carcass with petroleum ether for 24 hours and weighing of extracted crude fat; proteins and ash were determined on aliquots of the powdered dry, fat-free carcass by the Kjeldahl method of nitrogen determination and ashing at 800°, respectively.
[4] Equal parts of sunflower seeds, millet, canary seeds and oats.
[5] See table 5.

these minerals are present as inorganic salts. Nor is it clear which of the minerals —calcium, magnesium or phosphorus—is responsible for the difficulty.

The results obtained when carrots were added to the otherwise lethal basal diet suggest two possible alternate explanations. The minerals (or one of them) in the form of glycerophosphates may be more readily available to the animals in the absence of water than are inorganic salts, and the additional moisture in the carrots may increase the availability of the inorganic salts. Alternately, too much of the minerals may be absorbed when they are present in the inorganic form, and the mice may be able to utilize or excrete the excess only in the presence of additional water. Experiments are now in progress to study these problems.

ACKNOWLEDGMENTS

The authors thank Mrs. Benny Thomas and C. de Forest for their assistance with the care of the animals, preparation of diets and autopsy procedures, W. Yamanaka for the histological examination of the organs, and M. Light for the carcass analyses.

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Retinoic Acid: Some aspects of growth-promoting activity in the albino rat[1]

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ABSTRACT On the basis of previous metabolic work it seemed likely that the lower biological activity of retinoic acid is due to its rapid metabolism and excretion as compared with retinol. When retinoic acid dissolved in oil was given orally in small multiple doses (0.5 μg given at 6-hour intervals) to deficient rats, a growth effect resulted which was equivalent to that obtained with a single 2-μg dose of retinol. In contrast, giving retinol in small multiple doses had no additional effect on its apparent biological activity. The effectiveness of a single dose (8 μg) of retinoic acid was increased by 49% when it was administered in two 4-μg portions and by 66% when administered in four 2-μg portions. These results are consistent with the concept that retinoic acid has a lower biological activity than retinol because it is metabolically destroyed more rapidly. A single large dose of retinoic acid (200–500 μg) sustained growth of retinol-deficient rats at a reduced rate for 1 week. However, 3 hours after a 1.5-mg dose of retinoic acid only 2.1% of it was present in liver, 0.94% in intestine and 0.13% in kidney, suggesting that retinoic acid induces some changes or is itself changed into a form which persists for as long as 1 week. In contrast with other reports, vitamin C had no effect on the growth of retinol-deficient rats, which casts doubt on the suggestion that a defect in vitamin C biosynthesis in vitamin A deficiency contributes to the apparent lesions of vitamin A deficiency. Finally, the methyl esters of all-*trans* and 13-*cis* retinoic acid were bioassayed for growth in retinol-deficient rats. The 13-*cis* ester was equal in its growth-promoting activity to all-*trans* retinoic acid, whereas the all-*trans* ester was slightly higher in its biological activity.

In view of the possibility that retinoic acid might be the form of vitamin A required for some biochemical reactions important to growth (1–7), we have reexamined several aspects of the growth-promoting activity of this compound and some of its derivatives.

The rapid elimination of administered retinoic acid (6, 8) offers a possible explanation for its low (6–10%, in oil, orally) biological activity as compared with retinol. It is very likely that the biological activity is only a reflection of the amount of retinoic acid that remains available to the animal during the course of the elimination process. We have tested this hypothesis by providing retinol-deficient animals with small but frequent doses of retinoic acid and comparing its effect on growth with that obtained from retinol similarly administered.

MATERIALS AND METHODS

Animals. Male albino rats of the Holtzman strain were obtained at weaning and fed a retinol-deficient diet (9). After about 4 weeks they usually ceased growing and their weight remained constant for a few days. At this point (plateau stage) they were used for bioassay. Bioassay samples, containing a twofold excess of *dl*-α-tocopherol, were administered orally by stomach tube in 0.1 to 0.3 ml of cottonseed oil.[2] Ascorbate (ascorbic acid neutralized with 0.1 N NaOH) was administered intraperitoneally in 0.2 ml saline.

Chemicals. All-*trans* retinol, all-*trans* retinoic acid and its *cis*-isomers were commercial products[3]; 15-^{14}C- and 6,7-$^{14}C_2$-retinoic acids and the *cis*-isomers of retinoic acid were gifts[4]; and *dl*-α-tocopherol was obtained commercially.[5] Methyl esters of retinoic acid were prepared by methylation of all-*trans* retinoic acid with diazomethane and the various isomers were isolated and identified as described previously (10). Isolation of labeled retinoic acid from tissues was accomplished by methods de-

Received for publication October 9, 1967.

[1] Supported by a grant no. GB-3086 from the National Science Foundation. Published with the approval of the director of the Wisconsin Agricultural Experiment Station.

[2] Wesson Oil; Hunt-Wesson Foods, Fullerton, California.

[3] Obtained from Distillation Products Industries, Rochester, New York.

[4] Supplied by Hoffmann-LaRoche and Company, Basle, Switzerland and Nutley, New Jersey.

[5] Merck and Company, Rahway, New Jersey.

scribed previously (7, 11). The estimation of all vitamin A compounds was made by measuring their absorbancy at the required wave lengths in a Cary 15 recording spectrophotometer using published extinction coefficients (12).

RESULTS

Biological activity of various levels of retinoic acid. Various levels of retinoic acid were tested for their growth-promoting activity on retinol-deficient rats (table 1, group 1). Nearly normal growth rate resulted from a single daily dose of 47 μg of retinoic acid, administered by stomach tube. When some of the same levels of retinoic acid were administered to other groups of rats (groups 2–5) in subsequent studies, the response was not identical (table 1). However, maximal growth, approaching that of normal rats, was always obtained with the highest level of retinoic acid used (16–47 μg).

Biological activity of small, multiple doses of retinoic acid. Table 2 summarizes the results obtained by administering to deficient rats a single dose of the vitamin or administering the dose in small fractions at spaced intervals during 24 hours. Results from experiment 1 (table 2) demonstrate that the efficiency of a single dose of retinoic acid (8 μg) is increased by 49% if it administered in two 4-μg portions and increased by 66% if it is administered

TABLE 1

Growth responses of retinol-deficient rats to various daily doses of retinoic acid

Group	Supplement		No. of rats in exp.	Wt gain/day during supplementation [1]
		μg/day		*g*
1	Control (cottonseed oil)	—	4	−6.0 ± 0.44 [2]
	Retinoic acid	0.5	4	−5.9 ± 1.43
	Retinoic acid	0.9	4	−0.6 ± 0.41
	Retinoic acid	4.0	4	0.2 ± 0.32
	Retinoic acid	8.0	4	0.3 ± 0.10
	Retinoic acid	16.0	4	3.6 ± 0.13
	Retinoic acid	28	4	3.6 ± 0.32
	Retinoic acid	32	5	4.1 ± 0.33
	Retinoic acid	47	6	5.0 ± 0.37
	Retinol	0.8	4	0.2 ± 0.93
	Retinol	8.0	5	5.2 ± 0.94
Normal growth rate [3]			45	4.5 ± 0.04
2	Retinoic acid	2	11	1.86 ± 0.25
		4	10	3.20 ± 0.33
		8	7	3.20 ± 0.50
		16	9	3.44 ± 0.46
		32	9	4.47 ± 0.30
Normal growth rate [3]			62	5.28 ± 0.02
3 [4]	Retinoic acid	8	5	2.82 ± 0.13
		16	5	4.23 ± 0.45
	Retinol	3	5	2.23 ± 0.61
		6	4	4.57 ± 0.36
4 [4]	Retinoic acid	4	5	3.16 ± 0.32
		8	4	4.03 ± 0.25
	Retinol	2	5	2.81 ± 0.56
		4	4	5.70 ± 0.15
5 [4]	Retinoic acid	2	6	2.60 ± 0.08
		4	8	2.43 ± 0.21
		8	6	3.33 ± 0.31

[1] Duration of experiments was 14–21 days. All supplements were administered as a single daily dose by stomach tube.
[2] SEM.
[3] This definition applies to the growth rate measured during the depletion period, while the growth is still linear.
[4] Normal growth rate was comparable to that of group 2.

TABLE 2

Growth of retinol-deficient rats given multiple doses of retinoic acid

Exp. no.	Substance administered	Dose	No. of doses/day	Total/day [1]	No. of rats	Avg wt gain/day [2]
		μg		μg		g
1	Retinoic acid	2	4	8	5	4.67 ± 0.19 [3]
		4	2	8	6	4.21 ± 0.34
		8	1	8	5	2.82 ± 0.13
		16	1	16	5	4.23 ± 0.45
2 [4]	Retinoic acid	0.5	4	2	7	2.64 ± 0.22
		0.75	4	3	6	3.62 ± 0.28
		4	1	4	5	3.16 ± 0.32
		8	1	8	4	4.03 ± 0.25
	Retinol	0.5	4	2	6	2.59 ± 0.23
		2	1	2	5	2.81 ± 0.56
		4	1	4	4	5.70 ± 0.15

[1] The length of the experiments was 14 to 18 days.
[2] Growth rate during depletion period (normal growth rate) was approximately 5.3 g/day.
[3] SEM.
[4] Valid comparison of values in experiment 2 can be made with the single-dose experiments in group 2, table 1.

TABLE 3

Effect of vitamin C on growth rate of retinol-deficient rats [1]

Supplement	Wt loss [2]/day
	g
Saline (controls)	−5.07 ± 0.62 [3]
500 μg vitamin C [4] (ascorbic acid)	−6.90 ± 0.86

[1] Seven rats/group.
[2] Weight loss occurred at a constant rate and was measured for 13 days; a few days thereafter most of the animals died.
[3] SEM.
[4] Administered daily intraperitoneally as sodium ascorbate in 0.2 ml saline solution over a period of 13–16 days.

in four 2-μg portions. Furthermore, when administered in 4 doses, the 8-μg level produced a growth response comparable to that of a single dose of 16 μg of retinoic acid.

Experiment 2 (table 2) shows that when 2 μg of retinoic acid was given to the animal in four 0.5-μ fractions over a period of 14 to 18 days it had a biological activity that approached that of 2 μg of retinol. The administration of retinol in small multiple doses for the same period did not increase its biological activity.

Effect of vitamin C on growth of retinol-deficient rats. At the plateau stage of growth, retinol-deficient rats were divided into 2 groups, one of which received an intraperitoneal injection of 500 μg of neutralized vitamin C daily in 0.2 ml saline and the other, an injection of saline alone (control group). Both groups were fed the same amount of retinol-deficient diet during the experiment (13–16 days). The amount to be fed to both groups was based on the consumption by the control group of animals. The food consumption averaged

TABLE 4

Growth of retinol-deficient rats after a single dose of retinoic acid

Exp. no.	Retinoic acid given	No. of rats	Wt gain/day [1]		
			0–7 days	0–10 days	0–14 days
	μg		g	g	g
1	50 [2]	4	0.36 ± 0.45 [3]	−0.25 ± 0.10	
2	200 [2]	7	2.40 ± 0.44	0.87 ± 0.71	−2.15 ± 0.67
	500 [2]	8	2.30 ± 0.74	0.70 ± 0.80	−1.20 ± 0.72
3	500 [4]	7	2.63 ± 0.85	0.85 ± 1.05	−0.69 ± 1.00
1, 2 and 3	—	14		−5.30 ± 0.71	

[1] Growth rate during the depletion period (21 days) was 5.3 ± 0.02 g/day for rats in experiments no. 1 and no. 2 (determined for 62 rats); and 4.45 ± 0.28 g/day for rats in experiment no. 3 (determined for 8 rats).
[2] Orally, as acid in oil.
[3] SE of mean.
[4] Orally, as sodium salt in aqueous buffer.

about 5 to 9 g/day, the higher range being at the start of the experiment and decreasing as the animals became more deficient. The results compiled in table 3 demonstrate that vitamin C-treated rats lost weight at about the same rate as the rats that received only saline injections.

Effect of a single large dose of retinoic acid on the growth of retinol-deficient rats. The results of these experiments are summarized in table 4. With a supplement of a single dose of 50 μg of retinoic acid in oil (exp. 1) the rats had ceased to grow at 7 days and thereafter lost weight. With a 200-μg or 500-μg supplement (exp. 2), the animals grew for 7 days, but at a submaximal rate, then leveled off by 10 days and started to lose weight rapidly by day 13 of the experiment.

When a single dose of 500 μg of retinoic acid was administered orally as the sodium salt in aqueous buffer, the growth response of the animals was similar to that obtained with the free acid in oil (exp. 3).

Retinoic acid in tissues following the oral administration of retinoic acid. Various tissues, obtained 3 hours after the oral administration of 1.5 mg of 6,7-$^{14}C_2$- or 15-^{14}C-retinoic acid, were analyzed for their retinoic acid content by the methods described previously (7, 10). The results are summarized in table 5. Of the tissues analyzed, liver had the highest amount of unaltered retinoic acid after 3 hours (2.1% of administered dose).

*Biological activity of all-*trans *and 13-*cis *methyl esters of retinoic acid.* The methyl esters of retinoic acid were prepared as described in the Methods section. Different groups of retinol-deficient rats were tested for their growth response to the methyl esters at levels producing submaximal growth as determined with all-*trans* retinoic acid. The all-*trans* methyl ester of retinoic acid produced a slightly better growth response than either the 13-*cis* ester or the all-*trans* acid (table 6).

TABLE 5

Retinoic acid in various tissues 3 hours after the oral administration of a single dose of 1.5 mg of 6,7-$^{14}C_2$- or 15-^{14}C-retinoic acid (specific activity, 0.5 μCi/mg)

Tissue	No. of tissues examined	Retinoic acid found [1]	
		% of dose	*μg/tissue*
Liver	96	2.1	32
Small intestine	68	0.94	14
Kidney (pairs)	30	0.13	2

[1] Values have been corrected for 40% loss of retinoic acid during isolation procedure.

TABLE 6

Growth-promoting activity of isomers of methyl retinoate

Exp. no.	Supplement		No. of rats	Days on experiment	Wt gain/day
		μg/day			*g*
1	all-*trans* retinoic acid	15	4	9	3.72 ± 0.18 [1]
	all-*trans* methyl retinoate	15	4	9	3.61 ± 0.35
	13-*cis* methyl retinoate	15	4	9	3.89 ± 0.85
	Normal growth rate (growth rate during depletion period)	—	17	25	4.78 ± 0.10
2 [2]	all-*trans* retinoic acid	8	8	14	2.63 ± 0.13
	all-*trans* retinoic acid	32	8	14	2.62 ± 0.06
	all-*trans* methyl retinoate	8	8	14	2.95 ± 0.38
	all-*trans* methyl retinoate	32	5	14	4.34 ± 0.32
	13-*cis* methyl retinoate	8	6	14	2.61 ± 0.37
	13-*cis* methyl retinoate	32	6	14	2.66 ± 0.47
3 [2]	all-*trans* retinoic acid	4	8	14	2.43 ± 0.21
	all-*trans* retinoic acid	8	7	10	3.30 ± 0.31
	all-*trans* methyl retinoate	4	7	14	3.50 ± 0.34
	all-*trans* methyl retinoate	8	8	11	4.40 ± 0.33
	13-*cis* methyl retinoate	4	7	14	2.20 ± 0.06
	13-*cis* methyl retinoate	8	7	11	2.75 ± 0.28

[1] SEM.
[2] Normal growth rate was 5.38 g/day.

DISCUSSION

It is well-known that the biological activity of retinoic acid varies depending upon the mode of its administration (13, 14). In the present studies we chose to administer the supplements in oil by a stomach tube. The presence of a slight excess of α-tocopherol was adequate to prevent oxidative destruction of the bioassay samples for several weeks when stored at 10° and in the absence of light.

Growth assays, as performed generally, are based on daily or even less frequent intervals of administration of the bioassay compounds. However, retinoic acid cannot be stored in body tissues (14–16) and the elimination of it is rapid (8, 17). Consequently it was not unexpected to find that relatively large amounts were required for normal growth, especially when administered in single daily doses. A more frequent administration of retinoic acid should improve its availability for biological functions. In fact the provision of a 2-μg dose of retinoic acid in four 0.5-μg portions, given at 6-hour intervals, resulted in a growth rate approaching that obtained with a single dose of 2 μg of retinol (table 2). Maximal growth rate was obtained with 4 μg of retinol. Although not demonstrated in this experiment, we would expect 4 μg of retinoic acid to be adequate for maximal growth if the supplement were administered frequently in small portions. The administration of retinoic acid in two or more portions as compared with a single daily dose, increased the biological activity of 8 μg by 49 to 66% (table 2, exp. 1). Increased frequency of retinol administration did not alter its biological effect (table 2, exp. 2), an observation in accord with the well-known fact that retinol is stored in the body and released as the need arises.

The possibility that retinoic acid or the biochemical effects of it, could persist for a period after the administration of it, has received some attention recently. Malathi and co-workers (18) reported that when retinoic acid was administered to rats orally as the salt in an aqueous buffer, the growth-promoting activity of a single 500-μg dose lasted for 4 weeks. Subsequently, the same authors demonstrated that after a 5-mg dose of it, only small quantities of retinoic acid could be detected in stomach, intestine, blood or liver at 4 to 48 hours (17). Similarly, we found that 3 hours after the oral administration of a large dose of retinoic acid (1.5 mg, in oil) only a small amount of retinoic acid (0.1–2.0% of the dose) could be found in the liver, intestine and kidney of the rat. A 14-μg dose of retinoic acid was reported by Roberts and DeLuca (8) to be completely metabolized in 48 hours.

With respect to the long-term effect of retinoic acid, we found that a single 200- to 500-μg oral dose of retinoic acid in oil could sustain a decreased growth rate (45% of normal) for about a week. Thereafter weight loss was rapid and within 3 to 4 weeks all animals were dead. After a single 50-μg dose the weight of the rats remained on a plateau for the first week, then decreased as indicated above. When 500 μg of retinoic acid were administered orally as a salt in a buffer (18) the effect was similar to that obtained with retinoic acid administered in oil. The finding is in contrast with that reported by Malathi and co-workers (18). They found that a single oral dose of 500 μg of retinoic acid, administered as the salt in buffer, sustained a normal growth rate in retinol-deficient rats for 4 weeks, whereas retinoic acid administered in oil did not have as prolonged an effect. The reasons for the difference are not clear at this time. In either case, the effects of retinoic acid outlasted its detectable presence in the body. Also, a large single dose of retinoic acid produces a longer-lasting effect than a small dose. Thus we must conclude that there may be a mechanism of storage or preservation, although perhaps not one as understood in the ordinary sense. Possibly the larger dose of retinoic acid produces a more extensive biochemical change at the cellular level (than a small dose), enabling the animal to withstand the deprivation of the vitamin for a correspondingly longer period. One may also envisage that the availability of retinoic acid from any given dose might be determined by the efficiency of mechanisms for its distribution, reabsorp-

tion and elimination. All of these factors could be altered in the deficient animal and may not be comparable to the mechanisms operating during normal conditions.

In our studies we were unable to observe a beneficial effect of vitamin C during the course of retinol deficiency, contrary to what has been reported by Malathi and Ganguly (19). The daily supplement of 500 μg of ascorbate (vitamin C) did not alter the characteristics and progress of an ensuing retinol deficiency. During the experiment, the rate of weight loss was the same in both the control and ascorbate-supplemented groups of rats and within 2 weeks all rats were dead (table 3). In our experiments we began ascorbate supplementation at the time when cessation of growth indicated that retinol stores were depleted but the animals were still in good physical condition. This differs from the experiments reported by Malathi and Ganguly, in which ascorbate supplements were started at weaning. However, if retinol is expected to be involved in the biosynthesis of ascorbic acid (19), the most logical time to evidence this experimentally would be during a period of retinol absence. A possible explanation for the data of Malathi and Ganguly is that excess vitamin C administration actually prevented oxidative destruction of vitamin A stores, much as has been shown for other antioxidants (20, 21).

In our studies reported here, the all-*trans* methyl ester of retinoic acid appeared to have higher biological activity than the free acid or the 13-*cis* ester (table 6, exps. 2 and 3). Redfearn reported in 1960 (22) that 8 to 15 days after the daily administration of 1 mg dose of all-*trans* methyl retinoate, some of the ester was found stored in the body lipids. In our earlier studies we found 13-*cis* and all-*trans* retinoic acids to be equal in their growth-promoting activity in the rat (11). Although differences in biological activity between the 13-*cis* and all-*trans* isomers may be due to some storage or to a slower destruction of the all-*trans* ester, stereochemical specificity for vitamin A-active compounds in general body functions must remain a possibility.

Generally, we have noted that in the rat the dose-biological activity relationship for retinoic acid is not a simple one. Although we have consistently used animals from the same source, their response to vitamin A compounds has varied at different times, especially when low levels of substances were tested. Even the growth rate of rats during the depletion period varied for different groups of animals (table 1). These observations only serve to emphasize that growth bioassays for vitamin A must always include at least 2 levels of vitamin A standard and that completely valid growth comparisons can be made only within each group of animals.

The possibility that retinoic acid could be the biologically active form of vitamin A compounds for the growth-promoting function remains an unsettled question. If a small but continuous supply of exogenous retinoic acid were provided to a normal animal for a long period, it might be possible to observe an effect of it on the utilization of retinol stores. The finding that the biweekly administration of retinoic acid did not alter the rate of utilization of retinol (16) could be attributed to: 1) rapid elimination of retinoic acid and consequently a lack of available retinoic acid for most of the time; 2) the technical difficulty in detecting very small changes in retinol concentration. If the physiological level comprises 0.1 to 0.2 μg of retinol per day (23), a 2-week experiment would change the retinol stores only by 1 to 28 μg, an amount completely obscured by the large losses occurring during extraction procedure. We have performed metabolism studies with terminally ^{14}C-labeled retinol (unpublished) and have found that the excretion of $^{14}CO_2$ and urinary ^{14}C was depressed when retinoic acid was adminstered during the experiment, suggesting a sparing effect of retinoic acid on retinol. In any case, it cannot be decided on the basis of any results to date whether retinoic acid is the metabolically active form of the vitamin. This can be established when the vitamin A-dependent systems are isolated and shown to function only with retinoic acid to the exclusion of other vitamin A compounds.

ACKNOWLEDGMENTS

The authors thank Dr. R. J. Emerick for his valuable advice in preparation of the paper.

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Reproduction and Early Postnatal Growth of Progeny in Swine Fed a Protein-free Diet during Gestation

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ABSTRACT Studies were made to ascertain the effect of a "protein-free" diet containing 0.5% protein supplemented with vitamins and minerals on swine reproduction and subsequent growth and serum protein of their progeny. Litter size and individual pig birth weight were normal in gilts fed the all-corn diet. In experiment 1, gilts were introduced to dietary treatment before breeding; in experiment 2, 24 to 28 days after breeding. Two of three pregnant gilts fed the protein-free diet in experiment 1 died of perforated gastric ulcers on days 67 and 101 of gestation. The uterus of these 2 gilts contained, respectively, 10 and 11 grossly normal, viable fetuses at time of death. One gilt successfully completed gestation and farrowed 9 normal pigs averaging approximately 80% of the birth weight of pigs from control and all-corn fed gilts. When the protein-free diet was introduced on day 24 to 28 of pregnancy in experiment 2, body weight and total serum protein of pigs at birth were not significantly lower than those of pigs from control gilts. Milk production of gilts fed protein-free diets during pregnancy was adversely affected as indicated by the significantly lower 6-week body weight of their own and of pigs transferred to them at 2 days of age from control sows as compared with that of pigs nursing control sows of their own or from reciprocal transfer. Serum protein of pigs from gilts fed the protein-free diet was significantly lower than that of pigs from control gilts only at week 4. Pregnancy can apparently be maintained in the gilt deprived of dietary protein by dependence on maternal tissue stores of amino acids for growth of the fetus. Inception of the protein-free regimen at day 24 to 28 of pregnancy in the gilt appears to have far less adverse effect than inception before breeding. Part of this effect is probably related to the longer depletion period in the latter case, but the interruption of the estrous cycle in the absence of dietary protein seems to be a more immediate and specific endocrine effect.

Dietary requirements of protein and amino acids for normal growth of most mammalian species have been elucidated, but less is known of the importance of the maternal diet on the growth of the developing fetus. Leathem (1) recently reviewed the subject of nutrition-endocrine interrelationships in reproduction. Study of the laboratory rat (2) indicates that a level of dietary protein as low as 5% of the diet allows normal reproduction. Other work (3, 4) has suggested a slightly higher requirement. Number and birth weight of young rats from dams fed a protein-free diet during weeks 1, 2 or 3 of pregnancy have been shown to be reduced (5). A gestation diet containing 7% wheat protein produced greater mortality and smaller weanling weights than an 18% mixed protein diet (3). Chow and Lee (6) showed that restriction of the total amount of a normal diet fed female rats during gestation and lactation results in permanent growth-stunting of the offspring and more recently (7) demonstrated a difference in protein metabolism between rats from dams fed normal versus restricted diets. The relative importance of reduced protein intake as compared with the effects due to reduced intake of other nutrients in the above work is not known.

In swine, Evvard et al. (8) showed that 90 g of protein per animal daily produced lower birth weight and vigor than 200 g. Clawson et al. (9) found no difference in the reproductive performance of gilts fed 136 or 545 g of soybean protein daily in combination with either 1362 or 2724 g of total feed. Similarly, Rippel et al. (10) obtained normal reproduction in gilts and sows fed 5% protein diets in which the protein was provided by corn-soybean meal, sesame meal or gelatin, the last two of which are poor in essential amino acid balance. In nitrogen balance studies (11)

Received for publication September 22, 1967.

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TABLE 1

Composition of experimental diets (exps. 1 and 2)

Diet	Protein-free (PF)	Control (C)	Corn
Exp. no.	1 and 2	1 and 2	1
	%	%	%
Corn (ground, no. 2, yellow)	—	76.0	93.5
Glucose monohydrate [1]	90.5	—	—
Corn oil [2]	3.0	—	—
Soybean meal, 50% protein	—	18.0	—
Alfalfa meal, 17% protein	3.0	3.0	3.0
Limestone, ground	1.0	1.0	1.0
Dicalcium phosphate	1.5	1.0	1.5
Salt, trace-mineralized [3]	0.5	0.5	0.5
Vitamin supplement [4]	0.5	0.5	0.5

[1] Cerelose, Corn Products Company, New York.
[2] Mazola, Corn Products Company, New York.
[3] Supplied the following trace minerals: (ppm) Mn, 30; Fe, 10; Cu, 3; I, 0.8; Co, 0.75; Zn, 0.50; and SO_4, 12.
[4] Hopro R, Borden Company, New York. Supplied the following: (per kg of diet) riboflavin, 4.3 mg; niacin, 13.6 mg; pantothenic acid, 6 mg.; vitamin A palmitate, 2178 IU; vitamin D_2, 1307 IU; vitamin B_{12}, 10.9 μg; zinc, 85.8 ppm; butylated hydroxy toluene, 33 ppm.

with corn-soybean meal-cornstarch diets ranging from zero to 15% protein, nitrogen retention reached a plateau at 12.5% protein. A negative nitrogen balance of about 6.5 g per day was obtained in gilts fed the protein-free diet.

The purposes of the present studies were to determine: 1) the effects of a protein-free diet introduced before breeding on the estrous cycle of the gilt and on the growth and viability of the developing pig fetus, and 2) the effects of a protein-free diet introduced on day 24 to 28 of pregnancy on growth and viability of the fetus and on its postnatal growth and serum protein level.

METHODS AND MATERIALS

Experiment 1. Fifteen Yorkshire gilts approximately 7 months of age were divided at random into 3 groups of 5 each and assigned to the diets shown in table 1. The group designated protein-free (PF) was actually receiving approximately 9 g of protein daily from the 3% alfalfa meal which was included in the diet as a source of possible unidentified factors required for reproduction. This amount of protein can be considered negligible in terms of the relative amount needed for sow maintenance and development of the fetus. All gilts had previously shown at least one estrous period. They were kept in groups of 5 in 10-m × 30-m outdoor dirt lots equipped with a portable wooden shelter. All gilts were mated to a Yorkshire boar of known fertility during the first estrus following introduction to the experimental diets. Feed and water were supplied twice daily in cast-iron troughs large enough to accommodate all 5 gilts in each pen at once. The daily feed allowance per animal was 1.82 kg. By day 70, PF gilts were not consuming their entire daily allowance so that beginning on day 79 and continuing through pregnancy the amount given to this group was reduced to 1.36 kg per gilt daily. During the last 2 to 3 weeks of pregnancy even this amount was not consumed consistently, but no attempt was made to further adjust the allowance.

Gilts were removed individually to indoor farrowing crates on day 112 of pregnancy and continued on the same feeding schedule until parturition. After parturition, all gilts were fed the control diet (C) ad libitum. Those that died during pregnancy or failed to conceive were necropsied. The number of live and stillborn pigs, individual birth weights and 3-week weights of survivors were recorded for each litter. Pituitary glands from 2 normal and 2 nonpregnant PF gilts were removed at slaughter for histological examination and hormone assay.

Thyrotrophic hormone (TSH) was assayed using the uptake of ^{32}P in the thyroid of one-day-old cockerels as described by Lamberg (12). Gonadotrophic hormone

was assayed using uptake of ^{32}P by the testis in one-day-old cockerels as described by Breneman et al. (13).

Experiment 2. Eight Yorkshire gilts approximately 7 months of age were divided into 2 groups of 4 each after breeding to a Hampshire boar of known fertility. Assignment to group was made in pairs, based on the breeding date, so that reciprocal transfer of one-half of each litter could be accomplished at 2 to 3 days of age where possible, to test the effect of gestation diet on postnatal performance of the progeny. This reciprocal transfer of one-half of each litter within each pair of gilts was possible in 3 of the 4 gilt pairs. In the fourth pair, parturition was approximately 1 week apart for the 2 members, so that transfer was not feasible and the data from this pair are not reported here.

The PF and C diets were the same as used in experiment 1. The diets were introduced starting at day 24 to 28 of pregnancy and continued until parturition. Gilts were kept in groups of four in 5-m × 8-m indoor concrete-floor pens. Feed was given dry at a level of 1.82 kg daily in 2 equal feedings in a wooden self-feeder large enough to accommodate all gilts in each pen. Water was supplied from cast-iron troughs. At 112 days of pregnancy gilts were removed individually to farrowing crates as in experiment 1 and continued on the same feeding schedule until parturition when they were fed the control diet ad libitum. The number of live and stillborn pigs and individual birth weights were recorded for each litter. At day 2 or 3, one-half of the survivors in each litter were reciprocally transferred to a foster sow as described above. Blood was taken from the anterior vena cava of each pig at day 2 or 3 and at weekly intervals to 6 weeks of age. Individual body weights were recorded at each bleeding. Body weight of each gilt was recorded initially, at parturition and at 6 weeks postpartum when the pigs were weaned.

RESULTS AND DISCUSSION

Experiment 1. Body weights after the experimental diets had been fed for 78 days are shown in table 2 and reproductive performance is summarized in table 3. PF gilts failed to gain weight during this period, whereas the corn-fed and C gilts gained an average of 17 and 31 kg per gilt, respectively. Salmon-Legagneur and Rerat (14) have summarized data on weight changes and relative body composition of pregnant as compared with nonpregnant swine and have described "pregnancy anabolism" reflected in an increase in bone and muscle weight of the dam in addition to the increase in uterine weight and the weight of the developing fetuses. Bare maintenance of initial weight in PF gilts must have been associated with a loss rather than a gain in weight of the maternal tissues to a degree about equal to that of the placental membranes and developing fetuses at that stage.

TABLE 2

Effect of gestation diet on body weight of gilts (exps. 1 and 2)

Diet [1]	Protein-free (PF)	Control (C)	Corn
	Experiment 1		
No. of gilts	5	5	5
Body wt at start, kg	132	131	128
Body wt after 78 days, kg [2]	130	162	145
	Experiment 2		
No. of gilts	3	3	—
Body wt at start, kg	149	134	—
Body wt 1 day postpartum, kg	144	146	—
Body wt at 6 weeks postpartum, kg	151	144	—

[1] In experiment 1, each group of gilts was fed 1.82 kg of diet/gilt daily during the first 78 days. By day 70, gilts fed the protein-free (glucose) diet were not consuming their entire daily allowance. Therefore, beginning on day 79 and continuing until the end of pregnancy the amount supplied to this group was reduced to 1.36 kg/gilt daily. Toward the end of pregnancy even this amount was not consumed consistently. In experiment 2, each group of gilts was offered and consumed 1.82 kg of diet/gilt daily from day 24 to 28 of pregnancy to parturition.

[2] Includes both pregnant and nonpregnant gilts.

TABLE 3

Effect of quality and quantity of dietary protein on reproduction of gilts (exp. 1)

Diet	Protein-free (PF)	Control (C)	Corn
Protein, %	0	12	9
No. of gilts	5	5	5
No. mated	3 [1]	5	5
No. conceived	3	4	3
No. farrowed	1 [2]	4	2 [3]
Live pigs/litter	9	10.5	10.5
Stillborn pigs/litter	1	1	1
Individual pig birth wt, g	799	1074	1017
Survivors at 3 weeks	7	8.3	8.5
Body wt of survivors at 3 weeks, g	2858	4966	3750

[1] Two gilts were never observed in estrus after induction of experimental diet.
[2] One died of perforated gastric ulcer on day 67 of pregnancy; 10 grossly normal fetuses were present. One died of a diaphragmatic hernia and perforated gastric ulcer on day 101 of pregnancy; 11 grossly normal fetuses were present.
[3] One aborted on day 81 of pregnancy; 7 grossly normal fetuses were present.

Daily feed consumption of the C and corn-fed gilts was maintained at 1.82 kg per animal daily during the entire experimental period, but PF gilts failed to consume their entire daily allowance beginning at about day 70. Since all gilts that conceived did so at the first mating (during the first 3 weeks on experiment), this reduced appetite occurred despite pregnancy. Two of the 3 gilts fed the protein-free diet died suddenly, 1 on day 67 and the other on day 101 of pregnancy. Necropsy revealed, respectively, 10 and 11 grossly normal fetuses. The fetuses from the 67-day gilt were not weighed but those from the 101-day gilt averaged 551 g (range 325 to 655). This corresponds to normal value of 970 ± 29 g at 102 days (15). The smaller-than-normal size agrees with the observations of Evvard (8) with low protein diets. The remaining pregnant PF gilt steadily became more apathetic and by day 108 of pregnancy refused to eat or to rise except when forced to do so. Its daily feed intake had gradually declined to only 50 to 100 g after day 100. To avoid abortion or death it was changed to the high protein control diet on day 108. Its daily feed intake during the last 5 days of pregnancy with this diet was approximately 100 g. The gilt farrowed uneventfully on day 113 a litter of 9 live pigs and one stillborn. The average birth weight of the pigs in the litter was 799 g as compared with 1017 and 1074 for the pigs from corn-fed and C gilts, respectively.

Experiment 2. Body weights of gilts initially, at parturition and 6 weeks after parturition, are summarized in table 2. The greater weight gain during gestation of control gilts is in agreement with the results of experiment 1.

Number of pigs per litter, individual pig birth weight and average 6-week weight and weekly serum protein level of pigs to 6 weeks of age are summarized in table 4. Growth curves during this period are shown in figure 1. There were no statistically significant differences between the two dietary groups in number of live pigs per litter, individual pig birth weight or total serum protein of the pigs at 2 to 3 days of age, although the mean for each criterion favored the control group. A larger experiment might have revealed differences, but the important point is that normal reproductive performance and viability of the progeny were possible in the absence of dietary protein during the final two-thirds of pregnancy when the bulk of fetal growth occurs. The lower total serum protein level of protein-free gilts as compared with controls midway through the third trimester of gestation (6.0 vs. 7.6 g per 100 ml) indicates depletion of labile protein reserves of the former group to satisfy fetal requirements.

The reciprocal transfer of one-half of each PF litter to a foster C gilt and vice versa at 2 to 3 days of age was made to test the effect of gestation diet on lactation performance. The mean body weight of progeny was significantly different among

TABLE 4

Effect of a protein-free diet for gilts during gestation on litter size, birth weight, growth rate and serum protein level to 6 weeks of age (exp. 2)

Diet	Protein-free			Control		
Protein, %	0			12		
No. of gilts	3			3		
Live pigs/litter	8.6			9.6		
Individual pig birth wt, kg	1.18(26) [2]			1.23(29)		
	Postnatal treatment of progeny [1]					
	Protein-free	Control	Combined	Protein-free	Control	Combined
Body wt at 6 weeks, kg [3]	8.14(12) [2]	10.05(12)	9.09(24)	8.09(15)	9.67(13)	8.93(28)
Serum protein, g/100 ml						
Week 1	5.50	5.67	5.58(24)	5.92	5.82	5.87(29)
2	5.45	5.98	5.69	5.37	5.83	5.61
3	5.16	5.34	5.25	4.99	5.39	5.21
4 [2]	5.16	5.72	5.44	5.33	5.70	5.53
5	4.94	5.54	5.23	5.29	5.41	5.36
6	4.97	5.26	5.13(24)	5.26	5.33	5.30(28)

[1] At 2 to 3 days of age one-half of each litter from gilts fed the protein-free diet during gestation was transferred to a foster dam fed the control diet during gestation and vice versa.
[2] Numbers in parentheses indicate number of observations making up the mean. Two pigs from gilts fed the protein-free diet died during the first week and one from a gilt fed the control diet died during the fourth week, accounting for the discrepancy in number of observations between week 1 and week 6.
[3] Significant difference among groups ($P < 0.01$).

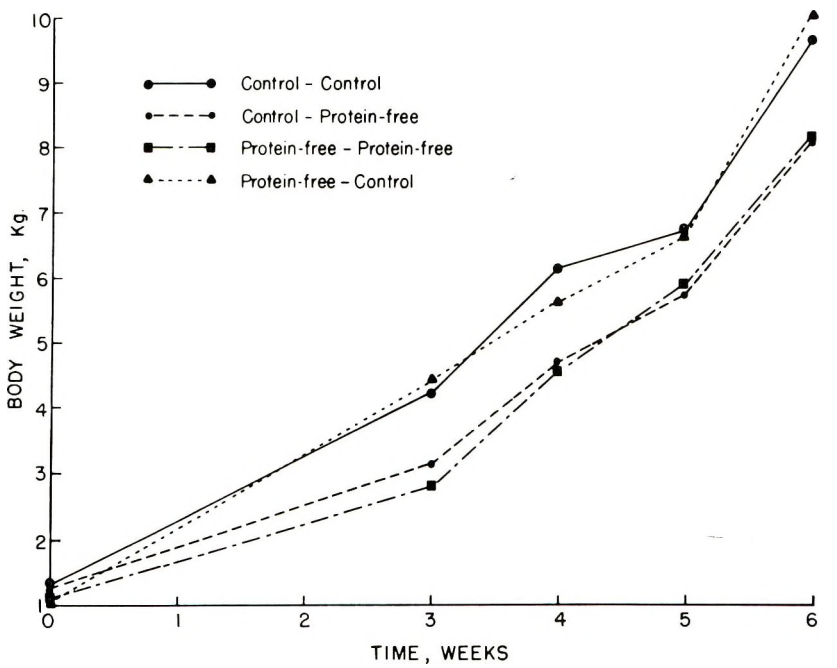

Fig. 1 Effect of gestation diet of dam and postnatal assignment of suckling pigs on body weight gain of the pigs. The term (control or protein-free) preceding the hyphen in the legend refers to the gestation diet of the dam; the term succeeding the hyphen refers to the gestation diet of the sow to which the pig was assigned during the 6-week suckling period. All sows were fed the control diet throughout lactation.

treatments ($P < 0.01$) at week 6. However, the difference was not due to prenatal nutrition (9.09 kg vs. 8.93 kg for PF and C pigs, respectively) but to whether nursing a PF or C gilt. The mean weight of PF pigs nursing PF gilts was 8.14 kg; that of PF pigs nursing C gilts, 10.05 kg; that of C pigs nursing PF gilts, 8.09 kg; and that of C pigs nursing C gilts, 9.67 kg. Analysis of variance of weekly body weights revealed these same differences at weeks 2 and 4, indicating that the effect originated early in the suckling period. This is interpreted as a difference in milk yield of PF and C gilts and is probably related to appetite during lactation, although feed consumption was not recorded to document it, or to an endocrine-related difference in development of mammary tissue between PF and C gilts. The lower serum protein and body weight of PF gilts than of C gilts could be considered predictive of such a difference in lactation performance.

The nutrition of the fetus is determined by the placental exchange area, the composition of the maternal blood and kinetic aspects of transfer against a concentration gradient. Rippel et al. (10) found a reduction in total serum protein and serum albumin of pregnant gilts fed low protein diets, but suggested that changes in serum protein profile are not a sensitive index of amino acid needs except in detecting pronounced deficiencies.

The similarity in reproductive performance of C gilts and corn-fed gilts in experiment 1 indicates that lysine, tryptophan and total protein of the diet are not critical dietary constituents for reproduction in pigs. The similar performance of PF and C gilts in experiment 2 is further evidence that maternal protein intake is not critical

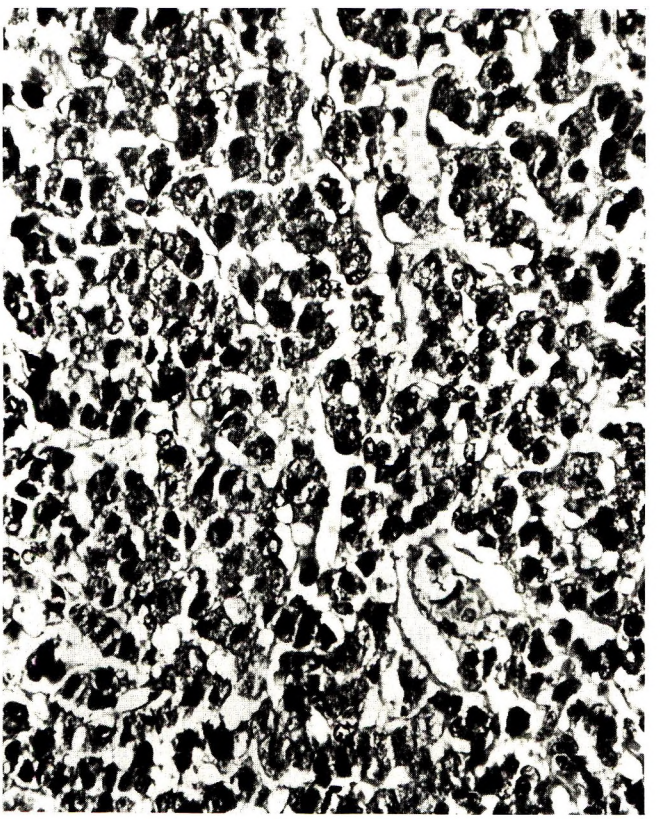

Fig. 2 Pituitary from protein-depleted gilt. Note the lack of granulation of the basophilic cells and increase in small pyknotic nuclei. H & E. ×750.

during the last two-thirds of gestation. The amino acid requirements for viability of the developing pig fetus apparently can be met almost entirely from amino acids and nitrogen originating from the tissues of the dam. Intrauterine protein and amino acid demands apparently override extrauterine demands.

Death of the protein-deficient gilts in experiment 1 was due to perforated gastric ulcers in the 67-day gilt and to perforated gastric ulcers and diaphragmatic hernia in the 101-day gilt. Whether the gastric ulcers were related to the consumption of the protein-free diet is not known.

The 2 gilts in the protein-free group that failed to conceive were never observed in estrus after being placed on the experiment. Nelson and Evans (2) observed in rats that most animals had only one estrus after introduction of a protein-free diet and frequently would not mate during this estrus. This cessation of the normal reproductive cycle is probably endocrine-related. Leathem (1) has discussed these relationships in laboratory rats.

TABLE 5

Levels of luteinizing hormone (LH) and thyrotrophic hormone (TSH) in pituitary gland of gilts fed normal or protein-free diets (exp. 1)

Gilt no.	Diet	LH [1]	TSH [2]
62–8	Nonprotein	1.102	0.4864
90–3	Nonprotein	1.476	0.6110
121–10	Control	1.443	—
121–1	Control	—	1.007

[1] Given as μg MHLH B_{31}/mg fresh pituitary.
[2] Given as milli units NIH TSH-B_3/mg fresh pituitary.

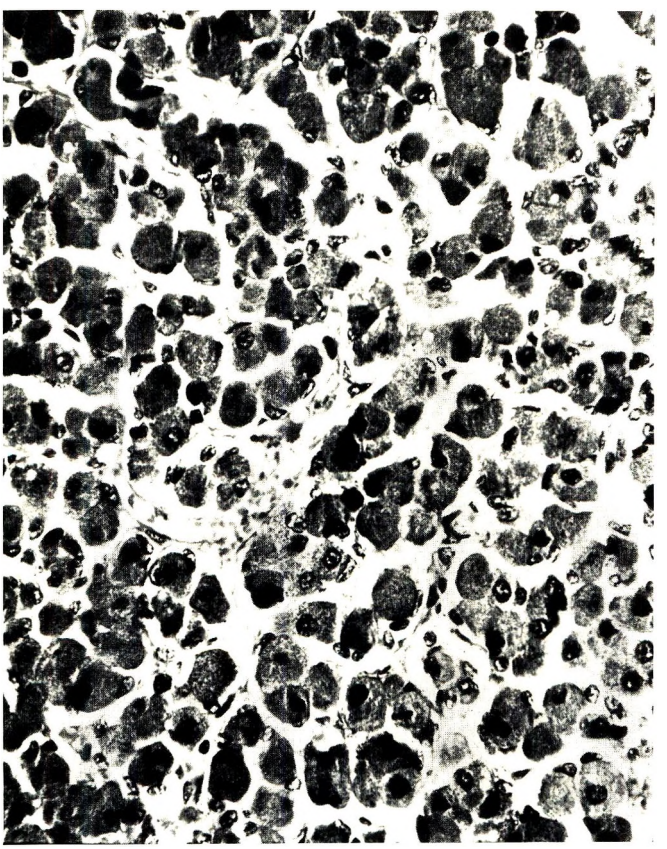

Fig. 3 Pituitary from control gilt. Note the large, well-granulated thyrotrophic basophils. H & E. ×750.

The results of the bioassay of thyrotrophic hormone (TSH) and luteinizing hormone (LH) in the 4 pituitary glands are given in table 5. Although indexes of precision for the 2 assays were fairly good ($\lambda = 0.23$ for LH assay, $\lambda = 0.26$ for TSH assay) the 95% confidence limits were such that differences were not demonstrated in hormone content between the nonprotein and control-fed pigs. Thus, there appears to be a discrepancy between the histologic picture (figs. 2 and 3) seen in nonprotein and control fed pigs and the actual hormone content. Srebnik (16) has indicated that there may be accumulation of pituitary hormones even in protein-deficient animals. This could occur even with rather negligible secretion rates if no release were taking place. Further support for this has come from Kalivas and Nelson (17) who demonstrated maintenance of pregnancy in protein-deficient rats by giving injections of reserpine and concluded that the effect was due to release of stored prolactin from the pituitary gland and from Berg (18) who accomplished this by transitory feeding of protein during 2 stages of gestation and suggested an endocrine relationship.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance of Phyllis Chapman, Jack Logomarsino, James O'Connor, Robert Banis, Dale Forsyth and Raymond Carpenter in carrying out these studies.

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Intake and Digestion of Nutrients by the Bovine under Climatic Stress[1]

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ABSTRACT The intake and digestion of nutrients by heifers of a good breed imported into a hot and humid region and of the indigenous, low-producing, stunted animals of that area were compared to assess the possible relationship of difference in metabolic activity to heat tolerance, and also with respect to alimentary function and digestive efficiency. Metabolism trials were conducted in the spring, summer, autumn and winter, and the effects of regional feeds and seasons on feed and water intakes, digestibility of protein, fats and carbohydrates, and balances of nitrogen, calcium and phosphorus were studied. The local animals, adapted to the climate, had higher feed and water intakes per unit of body weight, but their digestion coefficients for dry matter, crude protein, fats, and total carbohydrates were significantly lower than those of the imported animals. Nitrogen, calcium, and phosphorus balances were also lower in local animals. Between the 2 diets, animals preferred the diet of their home area.

In climatic regions where animals have to contend with high environmental temperature and relative humidity, various physiological reactions maintain them in a condition of thermostability (1–6). The indigenous cattle in such tropical areas like Malaya, South Africa, and some parts of India are stunted in growth and are low producers of milk, meat, and work. Because of various factors prevailing there, particularly high temperature and humidity (1, 7, 8), even imported high-producing animals suffer degeneration.

A number of investigators have made comparative studies of a few physiological reactions on different breeds of cattle, mostly of temperate origin, but no attempt has been made to make a comparative study of the metabolic behavior of various breeds of growing cattle in a hot and humid climate, particularly in an effort to compare the improved types or to compare an imported and a local breed or cross-breed. Since nutritional process, genetic constitution, and heat regulation are in many respects interdependent (6, 9, 10, 11), Phillips (7) stressed the importance of finding the relationship of difference in metabolic activity to heat tolerance and also alimentary function and digestive efficiency.

Among Indian breeds, native cattle of Bengal, a hot and humid region, are short in stature and are low milk-producers (fig. 1). Hariana cattle (fig. 2) of East Punjab area are tall in stature and their production of milk and work is comparatively heavy. In the present paper a comparative study is reported of the food and water intake, digestibility of protein, fats and total carbohydrates, and nitrogen and mineral balances of imported Hariana heifers and low-producing local cattle, fed local and imported diets, in each of the 4 seasons, that is, spring, summer, autumn, and winter. Calcutta, which is situated at the sea coast in the tropics and has a hot and humid climate for most of the year, was selected as the site for conducting the experiment.

MATERIALS AND METHODS

Sixteen female calves similar in age, body weight, and general condition were selected from a large herd of high-producing Hariana cattle maintained at their home tract, Hissar (East Punjab, India; lat. 29° 10″ N; long. 15° 46″ E) and were brought to Calcutta (West Bengal, India; lat. 22° 34″ N; long. 80° 24″ E), a place

Received for publication September 22, 1967.

[1] This study was supported in part by the funds of the research project "Investigation of the Causes of Degeneration of Cattle in Humid Climate," sponsored by the Indian Council of Agricultural Research, New Delhi.

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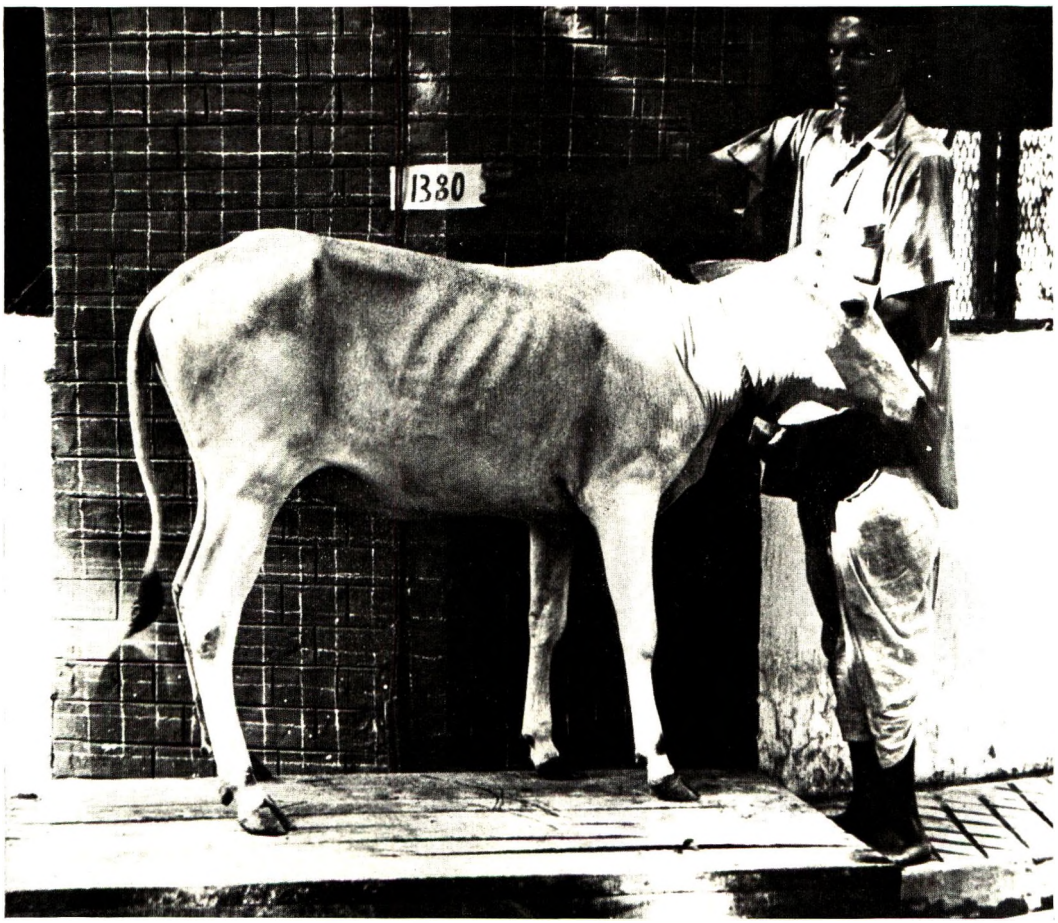

Fig. 1 One of the 16 Bengal breed (indigenous) animals of Calcutta after one year of preliminary feeding of diet P.

known for low productivity of cattle. Another 16 female calves of the same age, of indigenous Bengal breed, were obtained locally from a large herd at Calcutta. The imported Hariana calves were matched and divided at random into 2 groups of 8 each; animals of the Bengal breed were divided in a similar manner (table 1). One group of each breed was fed a typical Punjab diet (P). The feeds for this diet were obtained from Hissar. The remaining 2 groups, one of each breed, were fed a typical Bengal diet (B), the feeds for this diet being obtained locally at Calcutta. The composition and chemical analysis of the 2 diets are given in table 2. All animals were fed in accordance with the Morrison standard (13), and the ration schedule was adjusted every week on the basis of the body weights of the animals. A trace mineral mixture and vitamin A supplement were given to all animals.

The 4 groups were maintained on their dietary regimens for a preliminary period of about 410 days and then a series of metabolism trials was conducted. To obtain a general picture of the metabolic behavior of the 2 breeds, four metabolism trials were spread over a period of one year and were synchronized with the 4 seasons, spring, summer, autumn, and winter. Each metabolism trial lasted 10 days, during which records of feed and water intake, and feces and urine voided by individual

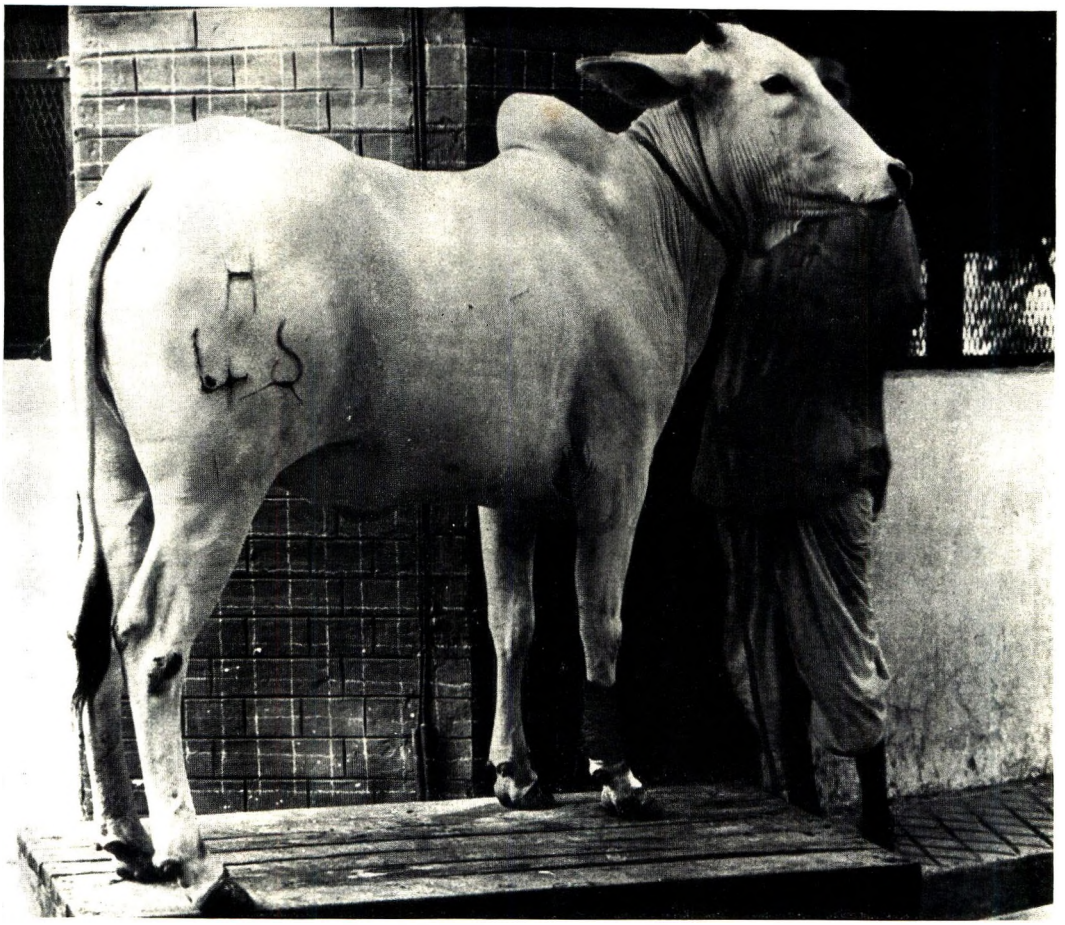

Fig. 2 One of the 16 Hariana breed (imported) animals of the Hissar area after one year of feeding diet P at Calcutta.

animals were kept, along with environmental data. The feces, urine, and feed residues were sampled every 24 hours, and a suitable aliquot was preserved. The cumulative samples for each animal were analyzed at the end of each metabolism trial. Methods of the AOAC (14) were used for analyzing feed, water, feces, and urine. Feed and water intakes, apparent digestion coefficients, and mineral and nitrogen balances were calculated.

RESULTS

The data on environmental temperature and relative humidity during the metabolism trials in the 4 seasons are summarized in table 3 and the body weight of the animals when grouped and before each of the metabolism trials are given in table 1.

Table 4 shows a summary of the results of the four metabolism trials. During the one-year preliminary feeding period, one Hariana and one Bengal animal fed diet B died, leaving seven in each of the 2 groups fed the B diet as compared with 8 animals of each breed fed the P diet. During the last metabolism trial (winter) another Hariana animal fed diet B was sick and it was not included in the winter season experiment. Missing values for these animals were calculated (15). The data were analyzed statistically by analysis of variance (16) and the results are given in table 5.

TABLE 1

History of experimental animals

Animal no.	Body wt when grouped	Age	Metabolism trials Body wt			
			Spring	Summer	Autumn	Winter
	kg	*days*	*kg*	*kg*	*kg*	*kg*
		Hariana heifers fed Punjab diet [1,2]				
12	69	690	244	260	280	319
147	61	690	206	234	243	264
48	89	720	251	278	297	318
51	95	742	232	261	271	307
74	74	730	224	240	251	275
49	78	720	253	274	290	324
50	66	724	169	184	193	234
63	73	774	213	225	234	251
		Hariana heifers fed Bengal diet [1,2]				
131	64	695	died	—	—	—
144	60	687	157	170	174	199
43	90	739	256	274	272	294
61	89	720	220	229	220	sick
54	72	743	216	237	239	265
57	87	752	248	267	272	307
64	58	748	225	239	245	262
53	81	766	232	241	256	297
		Bengal heifers fed Punjab diet [3,4]				
3	35	696	101	114	131	156
1367	30	712	94	101	116	135
1566	34	715	107	120	136	157
1373	44	726	111	117	135	157
1393	46	733	126	133	146	164
1377	50	744	143	151	172	193
1402	41	762	114	124	141	162
1390	39	774	120	129	155	187
		Bengal heifers fed Bengal diet [3,4]				
5	36	696	62	70	84	114
6	28	698	died	—	—	—
1380	40	714	79	87	96	118
1351	44	733	118	132	135	155
1343	45	746	120	133	141	154
1366	50	747	117	130	145	162
1387	45	761	112	124	133	165
1375	40	772	118	136	153	173

[1] The animals were selected from a large herd at the Livestock Farm Hissar, grouped and placed on their dietary regimen 414 days before the spring experiment.
[2] The age is given at the time of the spring trial, that is, 414 days after grouping.
[3] The animals were selected from Kanchra Para dairy farm, Calcutta, grouped and placed on their dietary regimen 406 days before the spring experiment.
[4] The age is given at the time of the spring trial, that is, 406 days after grouping.

In all the 4 seasons, dry matter intake and water intake per unit of weight were significantly higher for the short-statured Bengal animals as compared with the Hariana animals. Water intake per unit of dry matter ingested did not differ significantly. The imported Hariana animals showed significantly higher apparent digestion coefficients for dry matter, crude protein, ether extracts, and total carbohydrates. Daily balances of nitrogen, calcium and phosphorus were also higher for Hariana animals in comparison with Bengal animals.

Between the 2 diets, imported Punjab diet (P) and local Bengal diet (B), Hariana animals ingested more of the former, and Bengal animals, more of the latter. Apparent digestibility of crude protein and nitrogen balances were higher for animals fed diet P within the same breed. Irrespective of dietary treatment the dry matter

intake of both breeds was higher in spring and winter, and lower in summer and autumn. Water intake was highest in summer and lowest in winter.

DISCUSSION

A higher dry matter intake by Hariana animals was expected because they were larger and heavier in comparison with Bengal animals of the same age. But on a unit body weight basis, the stunted Bengal animals, which were poor in general condition, had a significantly higher dry matter intake. This appears anomalous. With the food intake of Bengal animals better than that of Hariana animals, they should not be less productive and unhealthy since both groups of animals were kept free of parasites by regular administration of phenothiazine and fecal examination. However, a study of the apparent digestion coefficients of dry matter, crude protein, fats and total carbohydrates shows that these are significantly higher for Hariana animals (tables 4 and 5). The water consumption of these shorter animals on a kilogram body weight basis was also significantly higher. This supports the hypothesis that increased water consumption may exert a flushing effect on the alimentary tract (17).

Lowered efficiency of animal production in hot and humid areas has been attributed to the complex formed by interactions of climate, nutrition, and management. With adequate supply of nutrition and proper management significant improvement is possible (8, 18–20). However, the adaptation to hot and humid environments necessary for the survival of animals imported from cool areas involves physiological changes which also place a limit on production (19). Lee (3) reported that despite a high plane of nutrition and proper management to keep animals free from parasites, the level of production in tropical areas remains much lower as compared with those of temperate climate.

In the present study low-producing Bengal animals did not appear to benefit from a typical diet of a high-producing area, with the feeds also being imported from that area. The short stature of indigenous animals and hence comparatively greater surface area for heat dissipation probably

TABLE 2

Composition of diets [1]

	Punjab diet [2]		Bengal diet [3]	
	Conc mix	Jowar	Conc mix	Paddy straw
Crude protein, %	23.97	3.82	24.01	3.24
Ether extract, %	6.86	1.52	8.21	1.04
Crude fiber, %	10.08	33.70	10.90	33.65
Nitrogen-free extract, %	53.07	53.62	43.38	47.70
CaO, %	0.40	0.61	0.39	0.70
P_2O_5, %	1.77	0.26	2.28	0.12

[1] In addition each animal was given per day 3000 IU of vitamin A as shark liver oil, and 28 g of the following mineral mixture: (in %) manganese (as manganese carbonate), 0.165; iron (as iron oxide), 0.170; copper (as copper carbonate), 0.033; iodine (as potassium iodide), 0.007; cobalt (as cobalt carbonate), 0.010; and sodium chloride (as common salt), 99.615.

[2] Punjab diet, consisting of a concentrate mixture containing 34% mustard cake, 52% crushed gram, and 14% wheat bran; roughage was dried jowar (Andropogon sorghum) fed ad libitum; feeds obtained from Hissar.

[3] Bengal diet, consisting of a concentrate mixture containing 40% mustard cake, 50% crushed gram, and 14% rice bran; roughage was paddy straw fed ad libitum; feeds obtained from Calcutta area.

TABLE 3

Environmental temperature and relative humidity during metabolism trials

Temperature			Relative humidity			
Mean maximum	Mean minimum	Extreme range	Mean daily	8 AM	5 PM	Mean
°	°	°	°	%	%	%
			Spring experiment—March 7 to 17			
35	22	37–19	30.6 ± 0.56 [1]	78.1	35.2	56.4 ± 3.02
			Summer experiment—May 18 to 28			
36	27	38–25	318 ± 0.50	72.9	65.8	69.4 ± 3.58
			Autumn experiment—August 18 to 28			
32	26	33–25	29.0 ± 0.25	87.9	83.5	85.7 ± 1.35
			Winter experiment—January 16 to 26			
27	14	29–10	20.6 ± 0.56	70.03	50.4	59.9 ± 3.03

[1] Values are mean ± SD.

TABLE 4

Summary of results of four seasonal experiments

Diet	Breed	Intake			Digestibility				Balance		
		Dry matter	Water	Water/ unit dry matter ingested	Dry matter	Crude protein	Ether extract	Total CHO	N	CaO	P_2O_5
		g/kg body wt	*g/kg body wt*		%	%	%	%	*g*	*g*	*g*
					Spring						
P [1]	Hariana	20.1 ± 0.35 [2]	82.64 ± 3.13	4.11 ± 0.40	50.17 ± 0.95	52.88 ± 0.74	72.76 ± 0.81	53.61 ± 0.95	9.30 ± 0.91	3.42 ± 0.56	5.95 ± 0.91
P	Bengal	23.5 ± 0.69	95.11 ± 3.31	4.05 ± 0.13	43.40 ± 0.89	47.05 ± 0.43	67.01 ± 0.83	45.31 ± 1.04	6.06 ± 0.26	1.73 ± 0.46	4.08 ± 0.51
B [2]	Hariana	18.3 ± 0.42	81.14 ± 3.22	4.42 ± 0.13	52.87 ± 0.36	50.58 ± 0.40	72.55 ± 1.10	57.88 ± 0.62	4.05 ± 0.52	2.10 ± 0.73	4.93 ± 1.53
B	Bengal	23.9 ± 0.86	103.12 ± 2.02	4.31 ± 0.16	47.19 ± 0.78	45.38 ± 0.74	68.13 ± 0.92	51.59 ± 0.92	2.79 ± 0.51	2.55 ± 0.48	4.26 ± 0.38
					Summer						
P	Hariana	19.4 ± 0.59	91.20 ± 1.58	4.70 ± 0.10	46.61 ± 0.53	48.89 ± 0.62	74.16 ± 0.68	49.37 ± 0.62	8.06 ± 0.69	0.81 ± 0.36	1.91 ± 0.66
P	Bengal	20.9 ± 0.48	98.26 ± 2.67	4.69 ± 0.08	40.57 ± 0.71	41.82 ± 0.54	71.95 ± 0.39	45.02 ± 0.68	2.76 ± 0.33	0.41 ± 0.21	0.67 ± 0.34
B	Hariana	17.73 ± 0.45	91.36 ± 1.32	5.15 ± 1.13	46.10 ± 0.77	47.36 ± 0.55	74.62 ± 0.89	52.82 ± 0.74	2.79 ± 0.82	1.14 ± 0.18	3.10 ± 0.81
B	Bengal	22.4 ± 0.81	113.24 ± 3.08	5.06 ± 0.14	42.24 ± 0.48	41.12 ± 0.46	70.52 ± 0.49	49.15 ± 0.56	1.85 ± 0.48	0.70 ± 0.21	0.03 ± 0.21
					Autumn						
P	Hariana	19.3 ± 0.63	69.98 ± 2.19	3.63 ± 0.86	48.62 ± 0.43	50.17 ± 0.47	70.90 ± 0.84	52.88 ± 0.53	6.16 ± 1.19	1.51 ± 0.89	1.91 ± 0.32
P	Bengal	21.4 ± 0.46	76.68 ± 2.77	3.58 ± 0.07	42.69 ± 0.58	43.32 ± 0.69	65.34 ± 0.60	47.12 ± 0.38	−0.20 ± 0.40	−0.09 ± 0.03	0.47 ± 0.44
B	Hariana	16.2 ± 0.59	66.51 ± 1.75	4.11 ± 0.15	45.92 ± 0.56	47.95 ± 0.19	74.68 ± 0.58	52.14 ± 0.84	2.66 ± 0.78	−0.42 ± 0.09	1.16 ± 0.60
B	Bengal	22.8 ± 1.33	88.99 ± 4.52	3.90 ± 0.08	41.12 ± 0.49	41.72 ± 0.37	69.10 ± 0.54	48.60 ± 0.35	0.03 ± 0.38	−0.69 ± 0.45	1.04 ± 0.70
					Winter						
P	Hariana	20.2 ± 0.39	65.67 ± 1.53	3.25 ± 0.07	52.48 ± 0.52	53.86 ± 0.77	71.36 ± 0.43	53.59 ± 0.56	16.06 ± 1.77	2.33 ± 1.04	3.58 ± 0.68
P	Bengal	22.5 ± 0.56	67.55 ± 3.45	3.00 ± 0.09	45.32 ± 0.66	45.50 ± 1.22	62.97 ± 0.84	47.54 ± 0.66	7.65 ± 0.72	2.17 ± 0.34	1.35 ± 0.36
B	Hariana	18.7 ± 0.54	77.85 ± 3.30	4.16 ± 0.15	51.94 ± 0.63	50.25 ± 0.96	73.64 ± 0.32	54.49 ± 1.84	9.27 ± 3.23	3.26 ± 0.77	1.73 ± 0.40
B	Bengal	24.1 ± 1.04	72.63 ± 3.37	3.01 ± 0.13	47.54 ± 0.94	45.57 ± 1.35	63.34 ± 0.60	50.90 ± 0.91	3.13 ± 0.77	1.60 ± 0.39	0.83 ± 0.31

[1] Composition of Punjab diet (P) and Bengal diet (B) and their proximate analysis are reported in table 2.
[2] Mean ± SD.

TABLE 5

Analysis of variance; mean squares

Source of variation	df	Intake			Digestibility				Balance		
		Dry matter	Water	Water/ unit of dry matter ingested	Dry matter	Crude protein	Ether extract	Total CHO	N	CaO	P_2O_5
		g/kg body wt	*g/kg body wt*		%	%	%	%	*g*	*g*	*g*
					Spring						
Breed	1	161.47**	2787.40**	0.00	312.50**	36.16**	194.63**	435.93**	43.58*	3.90	15.46
Diet	1	5.40	102.33	0.88	87.99**	193.75**	3.56	222.97**	144.00**	0.08	1.22
Breed × diet	1	8.38	307.00	0.02	2.18	109.52	5.36	6.70	6.54	7.81	1.84
Error	17	2.18	101.76	0.17	3.27	3.63	7.00	5.54	5.61	2.02	6.30
					Summer						
Breed	1	80.16**	1954.10**	0.00	213.22**	383.58**	80.65**	148.39**	120.08**	10.58**	33.46**
Diet	1	0.17	565.28**	1.67	1.20	11.25	3.23	100.01**	48.63**	52.99**	0.46
Breed × diet	1	21.29**	579.90**	0.00	6.20	0.16	7.43	0.01	16.41**	4.58*	5.10
Error	16	2.06	49.03	0.10	2.53	5.27	4.06	2.54	1.42	0.65	3.25
					Autumn						
Breed	1	150.16**	2051.56**	0.02	226.52**	316.76**	243.60**	175.60**	137.94**	13.01**	4.14
Diet	1	8.76	214.41	1.71	38.45**	26.90**	95.32**	0.86	27.56	7.49	0.08
Breed × diet	1	38.64**	711.47**	0.00	2.89	2.48	0.02	9.41	32.93*	3.37	4.19
Error	16	3.51	72.49	0.146	2.11	2.33	3.69	2.78	7.22	1.95	2.26
					Winter						
Breed	1	128.76**	882.03**	0.19	274.13**	323.21**	647.64**	191.64**	408.83**	16.92	17.06*
Diet	1	0.01	146.19	0.59	6.28	17.97	16.79	37.05*	250.09**	1.86	14.50*
Breed × diet	1	23.67**	97.46	0.20	13.73*	32.04	2.98	9.75	12.81	1.68	3.30
Error	15	2.19	49.06	0.065	2.44	7.32	2.88	7.13	24.97	5.44	2.06

* $P < 0.05$.
** $P < 0.01$.

keeps them in better physiological harmony with the hot and humid environments as compared with the larger, higher-producing imported animals. In areas of great heat stress, the climate appears to favor small animals (20).

Worstell and Brody (6) reported that the higher the production level and the larger the individual, the greater the cold tolerance and lower heat tolerance. Heat tolerance of guinea pigs also varies inversely with body size (21).

A lowered metabolic rate due to decreased thyroid gland activity in a hot climate has been reported in animals (4, 22). Hancock and Payne (19) reported that the apparent efficiency of feed conversion was lowest when the environmental conditions were most severe in a hot and humid area. In this study nitrogen and calcium balances for the short-statured animals of the hot and humid area were significantly lower than those of the high-producing animals. This, along with lower digestion coefficients of protein, fats and carbohydrates, may partly account for the low productivity of the indigenous cattle of hot and humid areas in the tropics. Animals of a good breed, maintained at their home tract, have been found to have a higher food intake, digestion of nutrients, and balance of nitrogen, as compared with those of the same breed imported and maintained in a hot and humid area (17).

Seasons appeared to exert a significant influence on the food intake and apparent digestion coefficient of dry matter, irrespective of the diet and breed. This is in confirmation of earlier studies (23).

ACKNOWLEDGMENTS

The author is grateful to Dr. N. D. Kehar, Director of the Indian Veterinary Research Institute, for financial support and interest in this work and, for help in the statistical analysis, the author is grateful to Dr. V. G. Panse, Statistical Division, I.A.R.I., New Delhi and Sheldon Kugler of Syntex Research.

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Plasma Lysine Titers in the Chick in Relation to Source of Lysine and Mode of Administration

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ABSTRACT Plasma lysine increased in a linear manner when fasted chicks were fed, ad libitum for 30 minutes, a crystalline amino acid diet containing graded concentrations of lysine. The same trend was noted when a single meal from either a crystalline amino acid mixture or dried egg white was force-fed in the form of a slurry. When a constant dose of lysine (25 mg/meal) was administered in a series of multiple meals, plasma lysine increased in a linear fashion up to the final (16th) meal when the crystalline amino acid mixture was used, whereas plasma lysine plateaued at the eighth meal of dried egg white. However, when the crystalline amino acid mixture was a component of the complete diet, the concentration of plasma lysine plateaued at the eighth meal also.

In previous work reported from this laboratory use has been made of a reference diet containing crystalline amino acids to examine the effect of varying the dietary concentration of one or more amino acid on the plasma amino acid titers of the very young chick (1). In subsequent studies the plasma amino acid pattern of chicks fed the reference diet was related to that of chicks fed diets containing intact proteins in an effort to detect the limiting amino acids therein (2–4). In the feeding phase of the assay, chicks having undergone a 4-hour fast were fed 12 meals (0.8 g of diet/meal), spaced 30 minutes apart over a period of 6 hours. Blood for analysis was taken 30 minutes after the ingestion of the terminal meal. This feeding procedure afforded an opportunity for the plasma pattern to reach a "steady state" in response to a given diet.

However, when the same diet, containing graded levels of lysine, both above and below the chick's requirement, was fed ad libitum over an extended period of time (6–7 days), plasma lysine remained constant and low at all suboptimal dietary concentrations but started to accumulate in the plasma in a linear manner once the chick's requirement for growth had been satisfied (5). Valine and arginine were shown to behave in a like manner. This assay technique made it possible to estimate, from the shape of the plasma lysine curve, the requirement of the chick for this amino acid. Since plasma concentration did not differentiate between diets slightly deficient in lysine and those severely deficient in this amino acid, this technique will not measure the concentration of lysine in the diet. In contrast, ad libitum feeding of the same diets to fasted chicks for a greatly reduced period (30 minutes) resulted in a linear accumulation of plasma lysine over the complete range (0.6–1.1%) of dietary concentrations fed (6). The same relationship was shown to hold for valine as well.

The present investigation was undertaken to explore in greater detail the influence that various diet types and feeding systems exert on plasma lysine titers in the young chick.

PROCEDURE

Stock. Male chicks originating from a mating of New Hampshire males × Columbian females were used in all assays. The number of chicks assigned to each treatment is indicated in the table footnotes. In all but one instance (nonfasted groups of exp. 2) the chicks had undergone an overnight fast (15–16 hour) just before being offered the experimental diets. Chicks, 16 and 13 days of age were used in experiments 1 and 2, respectively, whereas in all remaining experiments they were 20 to 21 days of age at time of the assay.

Received for publication October 9, 1967.

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Plasma. Unless stated otherwise in the tables, blood (approximately 3 ml/chick) was obtained by heart puncture 30 minutes after feeding the last meal. Following centrifugation, free amino acids were separated from the plasma by dialysis over 16 hours at a temperature of approximately 4°. Plasma lysine concentration (μg/ml ± SE) was determined on duplicate samples of the dialysate using *Leuconostoc mesenteroides* as the test organism.

Diets. In the first experiment, the chicks were fed the reference diet (lysine variable) on an ad libitum basis for 30 minutes, whereas in all other instances, lysine, irrespective of source, was force-fed to individual chicks in the form of a slurry. The slurried meals were introduced directly into the ingluvies via pipette.

RESULTS

Experiment 1. Diet A (table 1), containing a complete mixture of crystalline amino acids but with varying concentrations of dietary lysine (0–3.2%), was fed in the dry form on an ad libitum basis to groups of 9 chicks per treatment for 30 minutes. The absolute intake of lysine was calculated from the feed consumption data.

The results (table 2) demonstrate that with this procedure of feeding, lysine intake and plasma lysine were highly correlated (r = 0.95). The accumulation of lysine in the plasma was found to be linear and the regression equation Y = 23.37 + 2.27X adequately represents the relationship between plasma concentration (Y) expressed as micrograms per milliliter and milligrams of lysine intake (X). Omitted from the calculation of the regression equation are the data pertaining to the first group of chicks. This treatment was incorporated in the experiment for the sole purpose of measuring the build up of plasma lysine that occurs when chicks are subjected to extended periods of fast (7). The re-establishment of protein synthesis is marked by an immediate reduction in plasma lysine provided the supplemental level of dietary lysine does not exceed the chick's requirement.

Experiment 2. Both fasted (16 hours) and nonfasted chicks received in a single meal either 1) a complete mixture of crystalline amino acids (table 1) or 2) dried egg white in amounts that would provide lysine intakes of 25, 50 and 75 mg. The former dose (25 mg) would approximate the amount of lysine that fasted chicks

TABLE 1

Composition of diet

	% of diet
Cornstarch	55.95
Amino acid mixture [1]	19.48
Corn oil	15.00
Salt mixture [2]	5.37
Cellulose [3]	3.00
$NaHCO_3$	1.00
Choline chloride	0.20
Vitamins [4]	+
Antioxidant [5]	+
	100.00

[1] Composition of crystalline amino acid mixture as a percentage of total diet: L-arginine·HCl, 1.21; L-histidine·HCl·H_2O, 0.41; L-lysine·HCl, 1.19; L-tyrosine, 0.45; L-tryptophan, 0.15; L-phenylalanine, 0.50; DL-methionine, 0.35; L-cystine, 0.35; L-threonine, 0.65; L-leucine, 1.20; L-isoleucine, 0.80; L-valine, 0.82; L-proline, 0.20; glycine, 1.20; L-glutamic acid, 10.00; total, 19.48.

[2] Klain et al. (9); $ZnCo_3$ substituted for $ZnCl_2$.

[3] Solka Floc, Brown Company, Chicago, Illinois 60603.

[4] Klain et al. (9).

[5] Santoquin, Monsanto Company, St. Louis.

TABLE 2

Correlation between lysine intake and plasma lysine (exp. 1)

Dietary lysine	Feed consumption/chick	Lysine consumption/chick	Plasma lysine
%	g	mg	μg/ml
— [1]	2.54	none	53 ± 10 [2]
0.1	2.54	2.5	27 ± 3
0.2	2.47	4.9	31 ± 4
0.4	2.47	9.9	48 ± 7
0.8	2.73	21.8	82 ± 10
1.6	2.54	40.6	109 ± 8
3.2	2.54	81.3	209 ± 14

[1] Diet A (table 1) devoid of L-lysine·HCl.

[2] Average for 9 chicks/treatment ± SE.

TABLE 3

Single meal feeding of either crystalline lysine or dried egg white to fasted and nonfasted chicks (exp. 2)

Lysine dose	Plasma lysine			
	Crystalline amino acid mix		Egg white	
	Fasted	Nonfasted	Fasted	Nonfasted
mg	*μg/ml*	*μg/ml*	*μg/ml*	*μg/ml*
25	204 ± 20 [1]	101 ± 20	61 ± 13	108 ± 8
50	216 ± 29	139 ± 13	113 ± 12	115 ± 16
75	332 ± 36	235 ± 19	143 ± 12	117 ± 6

[1] Average of 3 chicks/treatment ± SE.

would voluntarily consume when fed diet A containing 0.95% lysine during 30 minutes (table 2). Blood samples were taken for analysis 30 minutes and 120 minutes after feeding the crystalline lysine and egg white meal, respectively.

In the case of fasted chicks, each increment of lysine intake resulted in a stepwise increase in plasma lysine irrespective of whether the lysine came from the crystalline amino acid mixture or from dried egg white (table 3). The same trend was observed for the nonfasted chicks fed the crystalline amino acid mixture but not for those fed dried egg white. At each dosage level of lysine in the series involving the fasted chicks, the concentration of plasma lysine was much greater for chicks fed the crystalline amino acid mixture than it was for those fed dried egg white.

Experiment 3. In this experiment, 75 mg of lysine/chick were administered in a single meal either in the form of the crystalline amino acid mixture or as dried egg white. Blood samples were taken at various times following the meal as indicated in table 4. Plasma lysine increased rapidly and reached a peak 20 to 30 minutes after the crystalline amino acid meal had been fed, and decreased sharply thereafter. With the dried egg white meal only minor differences in plasma lysine concentration were noted over the range of zero to 4 hours. As in the previous assay a much greater concentration of plasma lysine was associated with the crystalline lysine meal than with the dried egg white meal.

Experiment 4. In this trial a constant dose of lysine was administered in a series of force-fed meals spaced 30 minutes apart. In one series the source of lysine (25 mg/meal) was either that present in the complete crystalline amino acid mixture or that present in dried egg white. In the second series, the source of lysine (10 mg/meal) was the same as above except that in both instances the lysine, whether from the crystalline amino acid mixture or dried egg white, was a component of the complete diet (table 1).

When the meal consisted of the reference standard mixture of crystalline amino acids containing lysine, plasma lysine continued to increase in a stepwise manner with the number of meals fed, without exhibiting any trend to plateau. Regression analysis indicated that each dose (25 mg/meal) increased plasma lysine about 11 μg/ml. In contrast, when crystalline lysine was incorporated in the complete diet, plasma lysine plateaued after having fed 8 meals containing 10 mg of crystalline lysine each.

TABLE 4

Change in plasma lysine concentration following the administration of a single dose of lysine (75 mg) as either crystalline lysine or dried egg white (exp. 3)

Time [1]	Plasma lysine	
	Crystalline amino acid mix	Egg white
min	*μg/ml*	*μg/ml*
0	70 ± 8 [2]	91 ± 28
10	168 ± 8	—
20	209 ± 21	—
30	201 ± 14	75 ± 7
40	187 ± 16	—
50	105 ± 5	—
60	—	99 ± 19
90	—	103 ± 26
120	—	100 ± 16
150	—	98 ± 25
180	—	78 ± 8
240	—	91 ± 6

[1] Time of blood sampling after feeding lysine meal.
[2] Average of 5 chicks at each time period ± SE.

TABLE 5

Plasma lysine titers following the feeding of successive meals of different types (exp. 4)

No. meals fed	Plasma lysine			
	AA mixture [1] (25 mg Lys/meal)	Egg white [2] (25 mg Lys/meal)	Complete diet with AA mixture [3] (10 mg Lys/meal)	Complete diet with egg white replacing AA mix [4] (10 mg Lys/meal)
	μg/ml	*μg/ml*	*μg/ml*	*μg/ml*
0	76 ± 7 [5]	78 ± 8	65 ± 3	65 ± 3
2	154 ± 8	136 ± 13	102 ± 6	60 ± 13
4	201 ± 14	134 ± 16	132 ± 13	88 ± 12
6	209 ± 22	156 ± 11	116 ± 16	83 ± 2
8	232 ± 4	188 ± 12	147 ± 15	118 ± 1
10	268 ± 15	188 ± 4	162 ± 6	118 ± 15
12	291 ± 25	164 ± 13	124 ± 15	124 ± 7
16	312 ± 21	192 ± 21	147 ± 12	117 ± 9

[1] The complete crystalline amino acid mixture (table 1) was force-fed to each of 4 chicks in an amount to provide 25 mg lysine/meal.
[2] Dried egg white was force-fed to each of 4 chicks in an amount to provide 25 mg lysine/meal.
[3] The complete diet (table 1) containing the complete mixture of crystalline amino acids was force-fed to each of 3 chicks in an amount to provide 10 mg lysine/meal.
[4] The complete diet with dried egg white (18.07%) replacing the crystalline amino acid mixture was force-fed to each of 3 chicks in an amount to provide 10 mg lysine/meal.
[5] Mean ± SE.

When dried egg white was the source of lysine, the concentration of plasma lysine was maximized after the eighth meal irrespective of whether the protein was fed alone or incorporated in a nutritionally adequate diet. The plasma lysine titers remained quite constant thereafter.

DISCUSSION

When fasted chicks were fed ad libitum for a short period of time (30 minutes) a complete diet containing graded amounts of crystalline lysine, plasma lysine was shown to be correlated with the absolute intake of lysine in a positive manner. These results parallel those noted previously (6). Likewise the same trend was noted when lysine intake was regulated by force-feeding, in a single meal, either a slurry of the crystalline amino acid mixture or a slurried meal of dried egg white to provide comparable amounts of lysine. Feeding the complete crystalline amino acid mixture in a series of successive meals, spaced 30 minutes apart, while holding the dose of lysine per meal constant (25 mg), also resulted in a linear accumulation of lysine in the plasma. However, when egg white was fed in a series of multiple meals in a comparable manner, the concentration of plasma lysine plateaued after the eighth meal and remained relatively constant up to the final meal (16th). In the final assay, complete diets containing either the crystalline amino acid mixture or dried egg white were fed to fasted chicks in a series of multiple meals with the lysine per meal (10 mg) held constant. The data (table 5) show that the "steady state" of plasma lysine concentration was reached at the eighth meal in both instances. Presumably with this technique a single blood sample taken at any time during the "steady state" would yield a valid estimate of the utilizable lysine in an intact protein when related to the concentration of plasma lysine achieved with the crystalline amino acid reference standard. Preparing and force-feeding the slurried crystalline amino acid diet presents fewer complications and is less time-consuming than tableting diets of this type (8).

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Hepatorenal Lesions in Rats Fed a Low Lipotrope Diet and Exposed to Aflatoxin [1,2,3]

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ABSTRACT A diet low in methionine and choline was tested in rats to determine 1) the effect of a low lipotrope diet on DNA synthesis in the renal epithelium; 2) which of the lipotropes is most closely associated with the diet-associated renal lesion; and 3) the effect of superimposing aflatoxin, also associated with a renal lesion, on the low lipotrope diet. The low lipotrope diet caused a marked increase in DNA synthesis in the renal epithelium after 6 weeks but not after 3 weeks. Choline had the least beneficial effect on the renal lesion, whereas methionine was most effective in preventing it. When choline, methionine, and vitamin B_{12} were all included in the diet at normal levels, the renal lesion was prevented. Aflatoxin had no appreciable effect on the diet-associated renal lesion but had a damaging effect on the livers of rats fed the low lipotrope diet. It was concluded that 1) DNA synthesis in the injured renal epithelium was increased rather than impaired; 2) methionine was more closely associated with the renal lesion than was either choline or vitamin B_{12}; and 3) aflatoxin had no appreciable effect on the renal lesion but resulted in a more severe liver response in rats fed the low lipotrope diet.

During the course of a series of investigations with diets low in lipotropic factors (1) it was observed that rats fed a diet containing only 0.1% choline, 0.3% methionine, and neither cystine nor vitamin B_{12} could go through the period of most rapid growth with only a slight depression in weight gain. Despite the marginal level of lipotropic factors, their livers were morphologically normal and did not accumulate excessive amounts of fat. Although all other organs and tissues appeared normal on gross examination, the urinary bladders of many of the rats fed the low lipotrope diet contained uncalcified mucoid structures and the proximal tubules of the kidney had severe degenerative lesions. A majority of the epithelial cells of the straight portion of the tubule were grossly enlarged and contained bizarre nuclei with intranuclear inclusion bodies; such a lesion suggests a preneoplastic state. Since a similar lesion had been observed in the kidneys of ducklings (2) and rats (3) fed aflatoxin-contaminated peanut meal, the condition seemed worthy of further investigation.

Aflatoxin is the generic term applied to a complex of metabolites elaborated by some strains of the ubiquitous mold *Aspergillus flavus*. Four major components of the complex have been identified (4) and at least one, the B_1 fraction, is hepatotoxic and carcinogenic to a number of species of animals (2, 5, 6). The mold metabolites have been found as contaminants in a number of natural food products and may constitute a public health hazard (7).

Since aflatoxin is a potent hepatotoxin and carcinogen and has, in addition, been associated with renal lesions, we have examined the effects of superimposing aflatoxin on a diet which induces renal lesions but has not previously been found to appreciably affect the liver in an adverse fashion.

MATERIALS AND METHODS

This series of investigations consisted of 3 trials. 1) A total of 60 rats, half of which were killed after 3 weeks and half after 6 weeks, was maintained with a low lipotrope diet to determine the effect of the diet on DNA synthesis in the renal epithelium, as measured by the incorporation of tritiated thymidine into nuclear deoxyribonucleic acid (DNA). 2) A total of 100 rats was fed the low lipotrope diet for 6 months to determine which of the lipo-

Received for publication August 28, 1967.

[1] This study was supported by contract no. PH 43-62-468 and Public Health Service Research Grant no. AM-11158 from the National Institute of Arthritis and Metabolic Diseases.

[2] Presented in part at the annual meeting of the Federation of American Societies for Experimental Biology, Chicago, 1967.

[3] This manuscript is Contribution no. 1112 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology.

tropic factors were most closely associated with the renal lesion. 3) A total of 70 rats was fed the respective diets for 12 months and intubated with aflatoxin during the early weeks of the experiment to observe the effect of superimposing this potent hepatotoxin and carcinogen on both low lipotrope (107) and lipotrope-supplemented (108) diets. In addition, some of those animals fed diet 107, with and without aflatoxin, were fed diet 108 for the last 3 months of the experiment to try to determine whether the renal lesion was reversible.

Charles River Cesarean-derived (CD) weanling male rats were used in all experiments. They were housed singly in screen-bottom cages in air conditioned quarters; feed and water were supplied ad libitum. Composition of the diets is shown in table 1. Individual supplements were added to the basal diet (107) as 0.6% methionine, 0.6% choline chloride, or 50 μg vitamin B_{12}/kg of diet. Animals for autoradiographic studies were injected with tritiated thymidine, 1μCi/g of body weight. They were killed 2 hours later and emulsion autoradiographs were prepared and processed according to methods routinely used in our laboratory (8). All tubule cells in the affected area of the kidney were counted and the number labeled was expressed as a percentage of the total. The aflatoxin B_1 used in these studies was more than 99.5% pure according to chromatography on thin-layer silica-gel plates and molar extinction coefficient (2.2×10^4 at 363 mμ); it was dissolved in dimethylsulfoxide (DMSO), 100 μg/ml, and intubated daily, 5 days/week. Two hundred and forty micrograms, as twenty-four 10-μg doses, were given to each animal.

TABLE 1

Composition of the diets

	Diet 107 [1]	Diet 108 [2]
	%	%
Isolated soybean protein	20.0	20.0
Sucrose	67.8	66.8
Salts mix [3]	5.0	5.0
Vitamin mix [3]	2.0	2.0
Cottonseed oil	5.0	5.0
DL-Methionine	0.1	0.6
Choline chloride	0.1	0.6
		μg/100 g diet
Vitamin B_{12}	—	5.0
Total lipotrope content, g/kg	4.0	15.0

[1] Basal diet.
[2] Supplemented diet.
[3] Newberne, P. M., and V. R. Young 1966 Effect of diets marginal in methionine and choline with and without vitamin B_{12} on rat liver and kidney. J. Nutr., *89*: 69.

Tissues for microscopic study were processed in a routine manner and stained with hematoxylin and eosin. For trial 2 the animals were killed at the end of the 6-month period, and the tissues were processed for microscopic study and assessed on an arbitrary basis with grading from zero to 4 according to the severity of the renal lesion.

RESULTS

Table 2 lists results of counting the renal tubule cells which incorporated tritiated thymidine after 3 and 6 weeks of feeding diets 107 and 108. The lesion was not established at the earlier period, and hence there was no significant difference in the number of tubule cells which incorporated tritiated thymidine. After 6 weeks, however, there was a significantly larger number of cells incorporating thymidine in the inner cortex of rats fed the low lipotrope

TABLE 2

Effect of diet at 3 and 6 weeks on incorporation of ^{3}H-thymidine into renal tubule cell nuclei [1]

Avg ^{3}H-labeled tubule cell nuclei	3 weeks		6 weeks	
	Diet 107	Diet 108	Diet 107	Diet 108
	%	%	%	%
Inner cortex	4.2	3.3	* 1.5 ± 0.2	* 0.2 ± 0.1
Outer cortex	2.6	2.2	0.8 ± 0.2	0.2 ± 0.1

[1] Where applicable, values are given ± SE.
* Significant difference: $P < 0.01$.

diet. Furthermore, as indicated by thymidine incorporation, even the very large, bizarre nuclei continued to synthesize DNA. The difference between the number of cells incorporating thymidine at 3 and 6 weeks is a normal consequence of a slowed rate of kidney growth during the 6-week period.

Effects of supplementation of either choline, methionine, vitamin B_{12}, or of all three are shown in figure 1 and table 3. Figure 1 shows that no individual supplement was sufficient to support body weight at the same level as all three but that vitamin B_{12} or methionine most nearly approached the level achieved by all three. Clearly, the condition is not a simple choline deficiency since supplementation with this lipotrope made little difference in the 6-month body weight. It is of interest that the basal diet supported a linear growth pattern over the 6-month period and that at 6 months there was little difference in body weights despite the different dietary treatments.

The data in table 3 parallel to a degree those illustrated in figure 1. The important observation is that the severity of the renal lesion was inversely related to dietary methionine and vitamin B_{12}, with some evidence that methionine had a more beneficial or protective effect.

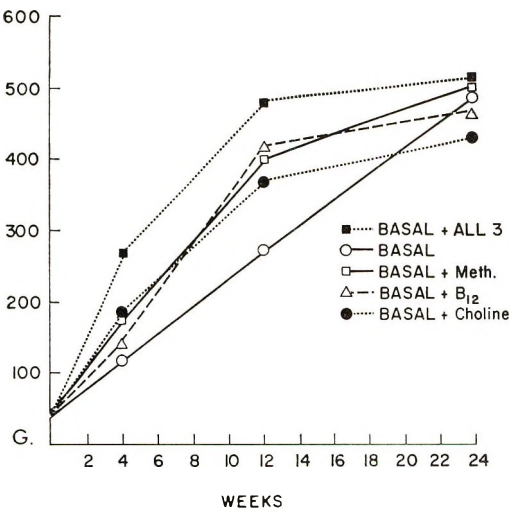

Fig. 1 Graph illustrating the effect on body weight of supplementation of the low lipotrope diet with individual lipotropes. Each of the 3 lipotropes caused an early increase in body weight but there was little difference at the end of 6 months.

TABLE 3

Effect of supplementation of the basal diet on the severity of the renal lesions

Dietary treatment	Avg severity of renal lesions
Diet 107 [1]	3.8 ± 0.2
Diet 107 + choline	3.8 ± 0.2
Diet 107 + methionine	2.0 ± 0.3
Diet 107 + vitamin B_{12}	3.0 ± 0.3
Diet 108 [2]	0.0

[1] Basal diet.
[2] Basal diet supplemented with choline, methionine, and vitamin B_{12}.

Table 4 lists the results of the 12-month experiment in which both groups of animals, lipotrope-deficient and lipotrope-supplemented, were exposed to the carcinogenic activity of aflatoxin B_1. It appears clear from these data that the low lipotrope diet made the liver more vulnerable to the effects of aflatoxin. Some animals died during the course of the 12-month study but ample numbers survived to indicate the potentiating effect of the low lipotrope diet. Aflatoxin did not appear to influence the renal lesion in either direction.

Histopathology. There was no evidence of fat accumulation in the periportal zone of the liver, typical of methionine deficiency, or in the centrolobular zone, characteristic of choline deficiency. Therefore, lesions described in the literature (9) as being associated with lipotrope deficiency were not observed in this series of animals fed the low lipotrope diet.

A section of a control kidney is shown in figure 2; the renal lesion associated with the marginal lipotrope diet is illustrated in figure 3. Degenerative changes are evident in all epithelial cells, and many of the nuclei are enlarged and contain prominent nucleoli or intranuclear inclusion bodies (arrows). There is also an accumulation of debris in the lumen of the tubules. Although the nuclei were enlarged and obviously abnormal, they retained the facility for synthesizing DNA, as shown by incorporation of tritiated thymidine (fig. 4). Cells not actively synthesizing DNA are shown dimly in the background.

Those animals fed the respective diets for the entire 12-month period developed

TABLE 4

Effects of lipotrope deficiency on the response of the liver and kidney to aflatoxin

Dietary treatment	Liver			Kidney
	Early change	Transitional change	Liver cell carcinoma	Epithelial changes
Diet 107 [1]	0/19	0/19	0/19	19/19
Diet 107 + aflatoxin	8/17	4/17	5/17	17/17
Diet 108 [2]	0/13	0/13	0/13	0/13
Diet 108 + aflatoxin	5/14	2/14	0/14	0/14

[1] Low lipotrope diet.
[2] Lipotrope-supplemented diet.

lesions varying from early degenerative or hyperplastic changes to frank liver cell carcinoma. Figure 5 illustrates the gross and microscopic (inset) appearance of a typical aflatoxin-induced hepatocellular carcinoma. A large nodule of carcinoma is indicated by the dark arrow, and the white arrow points out a small nodule which, after microscopic study, was classified as a transitional change (table 4). The early liver changes, which we now believe to be preneoplastic, include focal fatty change seen in some areas (fig. 6) and scattered localized areas of parenchymal cell hyperplasia (fig. 7). The latter stained deeply with hematoxylin, there was considerable mitotic activity, and the basophilic cytoplasm indicated increased protein synthesis.

Additional, more advanced changes leading to carcinoma of the liver were observed in some animals fed either the low lipotrope or the lipotrope-supplemented diet and intubated with aflatoxin. Four of seventeen fed the low lipotrope diet and 2 of 14 fed the supplemented diet developed the transitional lesion. Figures 8 and 9 illustrate the characteristic transitional and nodular regeneration stages considered to be the intermediate forms that probably progress to liver cell carcinoma if given sufficient time and proper circumstances.

Figure 10 is a photomicrograph of the renal tubules of an animal intubated with aflatoxin B_1 and fed the low lipotrope diet (107) for 12 months. Although the lesion is severe it is no more advanced and has characteristics no different from lesions found in the tubules of animals fed only diet 107 (fig. 3). Thus, aflatoxin did not appear to affect the renal lesion induced by the low lipotrope diet. The same lesion was observed in the small group of rats fed the low lipotrope diet (107) for 9 months and the supplemented diet (108) for 3 months and then killed. The same was true whether or not the rats had been dosed with aflatoxin. The only difference was the more frequent presence of occasional large, abnormal nuclei with marked degenerative changes in the renal epithelium of rats exposed to aflatoxin and fed the low lipotrope diet (fig. 11). Thus, it appears that the lesion in the kidney is slow to reverse once it is induced and that aflatoxin may result in some additional derangement in nuclear metabolism.

DISCUSSION

Diets consumed by population groups, particularly in technologically developing countries, are often low in protein and limiting in sulfur-containing amino acids. Although fragmentary, evidence is accumulating that in many areas of the world, populations subject to dietary deficiencies are also exposed to dietary contaminants including aflatoxin (3, 7, 10). There is little factual information to support the notion that human populations are predisposed to hepatotoxic agents by dietary alterations, but the results of studies in animals are convincing.

It has been reasonably well-established that toxic manifestations related to the administration of drugs, chemicals, food additives, and other substances may depend to a significant degree on the composition of the diet (11–15). When compared with those fed a diet composed of natural food products, animals fed purified diets were found to have more severe toxic manifesta-

tions. Furthermore, a shorter induction time has been reported for tumors when purified diets were used in experiments with 2-acetylaminofluorene (16–18); there was no suggestion as to the nature of the protective factors in the natural foods. In our own laboratory (19) we have observed that a cirrhogenic diet can influence the incidence of aflatoxin-induced liver tumors in rats. Madhavan and Gopalan (20) claim to have increased the incidence of liver lesions in rats caused by aflatoxin by lowering the dietary protein level; Engel (21) has reported a similar effect with other agents and conditions. There is little doubt that animals fed diets low or deficient in lipotropic factors have less hepatic reserve (22) than those fed normal diets and that this may result in decreased ability to metabolize or detoxify noxious agents.

Administration of aflatoxin in a single dose sharply decreases protein synthesis in rat liver (23). However, protein synthesis in the liver of choline-deficient rats is affected little, if at all, by the dietary treatment (24). Aflatoxin interferes in some manner with DNA metabolism (8, 25); perhaps through mechanisms related to nucleic acid or protein synthesis it exerts a more pronounced carcinogenic effect on animals fed the low lipotrope diet. Ethionine has a profound effect on protein synthesis in rat liver and is a potent liver carcinogen (26).

The low lipotrope diet used in this series of investigations is marginal, but not deficient, in methionine and choline. Observations of body weight and renal lesions (fig. 1 and table 3) of rats fed the basal diet supplemented with individual lipotropes suggest that methionine is more closely associated with the syndrome than is choline. Liver and kidney changes observed in this study do not approximate those described by György and Goldblatt (27) and by Hartroft (9) as characteristic for choline deficiency. Although choline may be a factor in the observed response of rats in our study, it does not appear to be the primary one.

The earlier reports by Salmon et al. (28), in which carcinoma of the liver in rats was attributed to choline deficiency, are now open to question. Their diets contained peanut meal and, according to more recent information (2, 3, 5, 6, 10), much of the feed-grade peanut meal sold on the open market in recent years was contaminated by aflatoxin. Thus, one cannot say with certainty that this potent carcinogen was not a factor in the choline-deficiency cirrhosis and liver carcinoma reported by Salmon et al. This is particularly cogent in view of our recent disclosure of the minute quantities of aflatoxin required to induce a 100% incidence of liver carcinoma in rats (29). Using Fischer-strain rats and purified aflatoxin B_1, we found that as little as 15 ppb fed continuously for 12 to 18 months is sufficient to induce liver carcinoma in all rats of both sexes. Male rats developed carcinoma sooner than females but in the final analysis the female was merely able in some way to delay the time of onset of malignancy rather than to prevent it. Perhaps the neoplastic change in the liver of rats fed the supplemented diet in the present study was delayed rather than prevented and, if given sufficient time, a higher incidence in this group might have been observed.

The renal lesion associated with the low lipotrope diet was not appreciably affected by aflatoxin. Although the low lipotrope diet resulted in nuclear changes in the renal epithelium suggestive of preneoplasia, none of the animals developed renal adenomas even when aflatoxin was superimposed. A significant incidence of renal adenomas had been observed in rats fed aflatoxin-contaminated peanut meal (2) but the relationship was only one of association and may not have been directly attributable to aflatoxin.

It seems unlikely that the renal lesion associated with the low lipotrope diet was the result of a cysteine analogue, such as the dichlorovinylcysteine found in soybean protein as a result of extracting it with trichlorethylene. This analogue has been reported as the cause of lesions similar, if not identical, to those observed in the present study (30). The soybean protein used in this study was processed in a manner that would preclude formation of dichlorovinylcysteine but the presence of some other toxic factor cannot be ruled out. However, in acute experiments preliminary

extraction studies have revealed nothing that is toxic to rats. We are continuing these investigations.

There is no obvious explanation for the difference in response to aflatoxin of rats fed low lipotrope and lipotrope-supplemented diets. However, the results lend support to the growing body of evidence that an inadequate diet can influence development of neoplastic disease. They also further emphasize the need to expand studies on the interrelationship of diet and those carcinogens found in natural food products.

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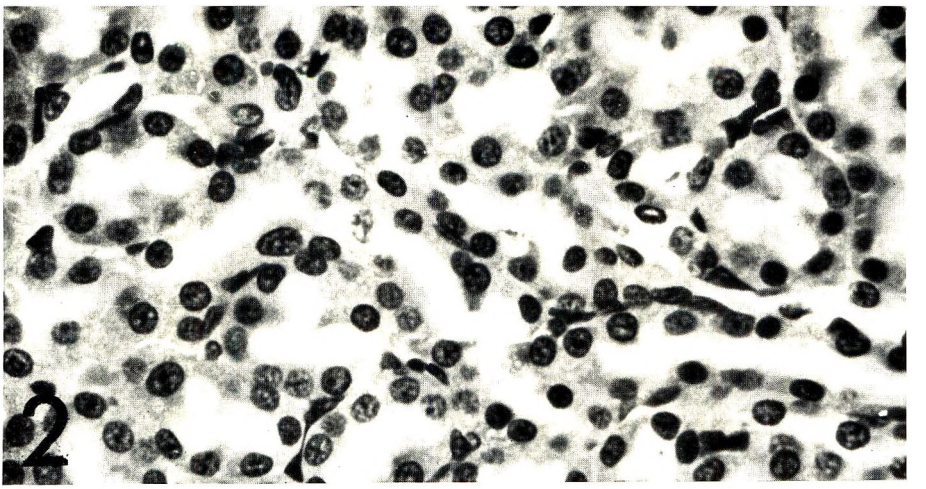
PLATE 1

EXPLANATION OF FIGURES

2 Section from the inner cortex of the kidney of a rat fed the supplemented diet (108). Tubular epithelium is normal and the lumen of the tubules patent. H & E. × 400.

3 Section from the inner cortex of the kidney of a rat fed the low lipotrope diet (107). Note the enlarged tubular epithelial cells, 2 of which contain large nucleoli and an intranuclear inclusion body (arrows). The lumen is filled with casts and debris from the degenerating epithelium. Compare with figure 2; both are the same magnification. H & E. × 400.

4 Kidney section taken from the same location as in figure 3 and prepared for emulsion autoradiography. Note the greatly enlarged nucleus which, as shown by the dark silver grains over it, had taken up a large amount of tritiated thymidine. This indicates that, despite its bizarre appearance, the cell is still capable of synthesizing DNA. Prepared with Kodak NTB2 emulsion, processed, and stained with hematoxylin and eosin following a 3-week exposure period. H & E. × 870.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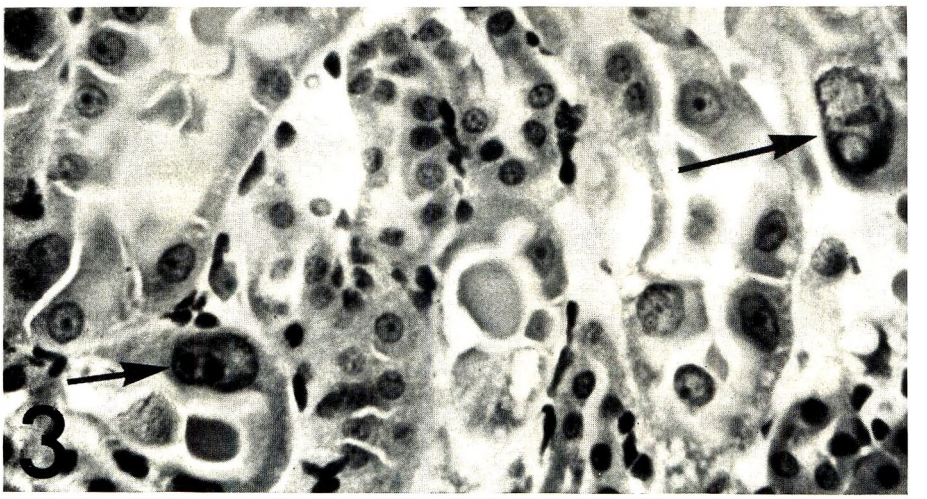
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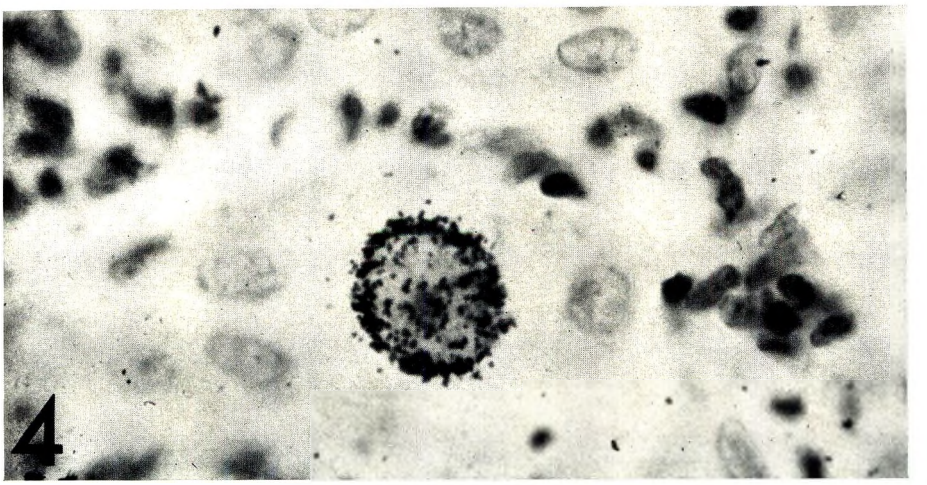
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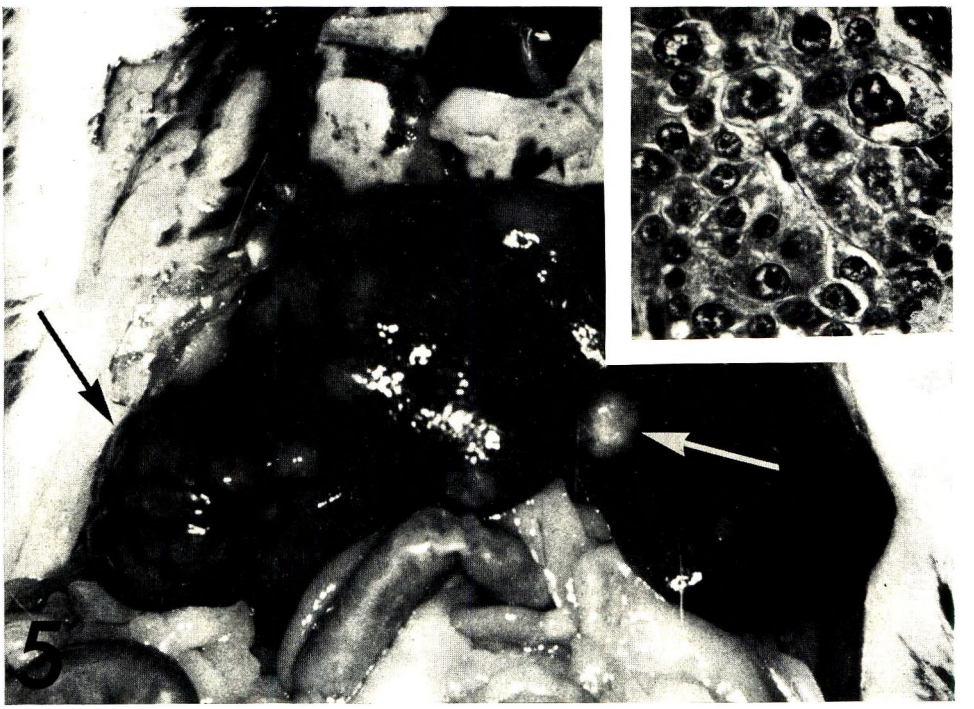
PLATE 2

EXPLANATION OF FIGURES

5 Characteristic appearance of the liver tumor induced in rats fed the low lipotrope diet and intubated with 240 μg of aflatoxin B_1. Inset (H & E. × 870) shows the hepatocellular nature of the neoplasm.

6 Focal fatty change in the liver of a rat fed the low lipotrope diet and intubated with 240 μg of aflatoxin B_1. These lesions were the first detectable alteration in liver morphology attributable to aflatoxin. They were more often seen about the periportal zone and were sometimes adjacent to an area of hyperplasia similar to that illustrated in figure 7. H & E. × 210.

7 Focal area of parenchymal cell hyperplasia in the liver of a rat exposed to aflatoxin. Liver cells stained deeply with hematoxylin and mitotic figures were frequent about the periphery of the focus. Figures 6 and 7 illustrate the 2 lesions we refer to as early, preneoplastic changes. H & E. × 290.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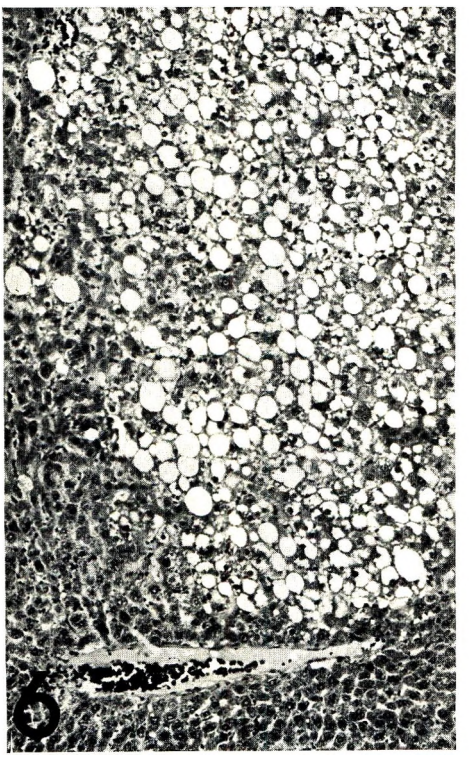
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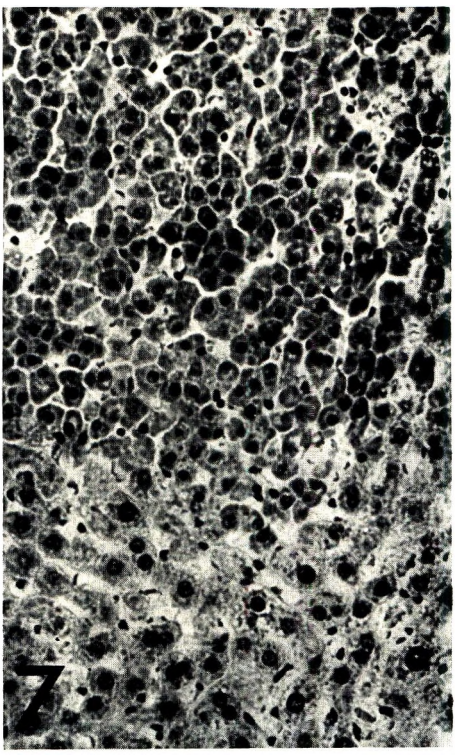
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PLATE 3

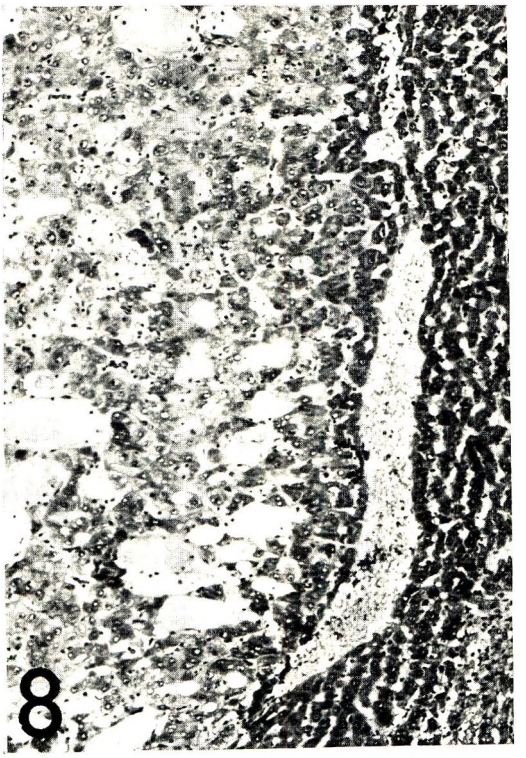
EXPLANATION OF FIGURES

8 A large focus of transitional cells in the liver of a rat exposed to aflatoxin. The cells are large and are arranged in trabeculae about distended vascular spaces. More nearly normal liver is on the right of the photograph. H & E. × 200.

9 A nodular lesion from the liver of a rat exposed to aflatoxin. This type of change is considered transitional but is not as advanced as the lesion illustrated in figure 8. H & E. × 120.

10 Section from the kidney of a rat fed the low lipotrope diet (107) for 12 months and intubated with aflatoxin B_1. The lesion is no more severe than that seen in an animal fed only diet 107 (fig. 3). H & E. × 400.

11 Section from the kidney of a rat dosed with aflatoxin, fed the low lipotrope diet (107) for 9 months followed by the supplemented diet (108) for 3 months, and then killed. Abnormal, degenerate nuclei (arrow) were more frequent in those animals fed the low lipotrope diet and dosed with aflatoxin than in animals fed only the low lipotrope diet. In neither case was the renal lesion reversed after 3 months on the supplemented diet. H & E. × 400.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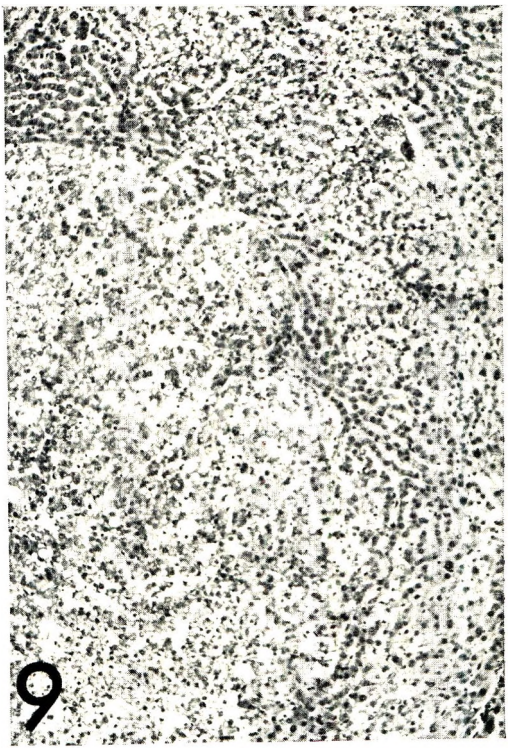
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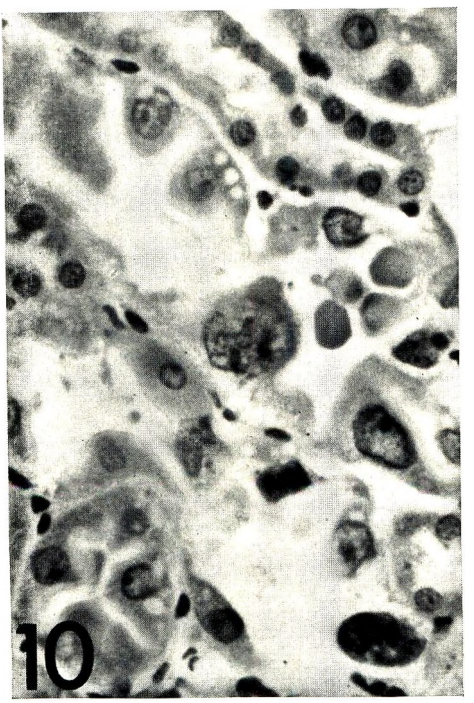
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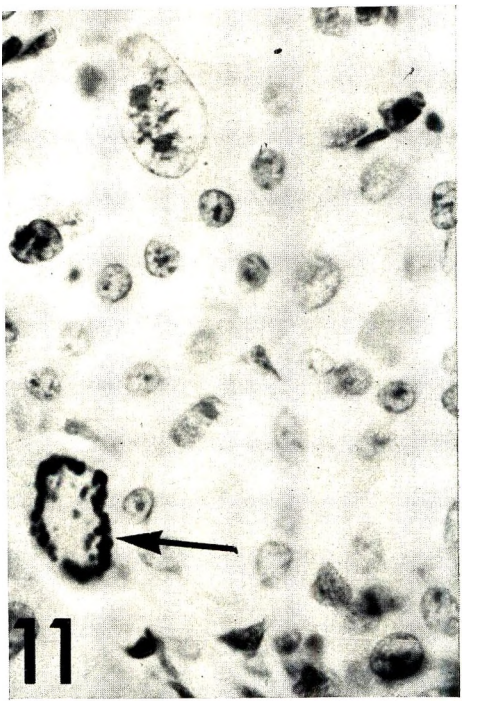
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Zinc Deficiency in the Rat: Effect on serum and intestinal alkaline phosphatase activities[1,2]

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ABSTRACT In three separate experiments zinc deficiency was produced in weanling rats fed a low zinc diet (0.9 ppm Zn) containing spray-dried egg white solids as the protein source. Preliminary studies with this diet showed that high levels of supplementary biotin were required to prevent symptoms of biotin deficiency from appearing. Symptoms of zinc deficiency included severe growth retardation and a rough, thin hair coat, but no other gross lesions. Parakeratotic lesions were confined to the tongue and esophagus, and there was no evidence of spermatogenesis in the seminiferous tubules of the testes. A reduction in intestinal alkaline phosphatase activity was noted which was not affected by the reduced food intake of the zinc-deficient animals. In addition, the in vitro incubation of duodenal homogenates from zinc-deficient animals with various concentrations of zinc between 1×10^{-5} and 1×10^{-3} M failed to increase alkaline phosphatase activity to that of the positive controls. The decrease in intestinal alkaline phosphatase activity in the zinc-deficient animals was found to occur when two different substrates were used. It is concluded that the concentration of this enzyme is reduced in the duodenum of the zinc-deficient rat. However, the reduction in serum alkaline phosphatase activity was apparently the result of inanition since restricted-fed control animals also showed a reduced enzyme activity.

Since the known biochemical roles played by zinc in mammalian metabolism are diverse, some difficulty has been experienced by workers attempting to find some of the important biochemical lesions produced by an inadequate intake of this element. The recent work of Theuer and Hoekstra (1) suggests that the primary effect of zinc deficiency lies in the area of protein metabolism but not in carbohydrate or lipid metabolism.

A logical approach to a study of the biochemical effects of zinc deficiency should include some quantitative measure of the activities of the various zinc-containing enzymes. Since there are about fifteen to twenty enzymes from different sources which require this element for activity (2), the problem becomes complex. Moreover, the nature of the zinc-binding to the enzyme would be of importance since a firmly bound zinc metallo-enzyme such as alcohol dehydrogenase or glutamic dehydrogenase might not be expected to result in altered activity in the zinc-deficient animal unless an impaired synthesis of the enzyme itself were involved. In this connection Hsu et al. (3) and Macapinlac et al. (4) have shown that the activity of liver alcohol dehydrogenase was unaffected in the zinc-deficient rat. However, Hsu and his associates observed a decrease in pancreatic carboxypeptidase A activity in zinc-deficiency (3). The zinc atoms of carboxypeptidase are not as firmly bound to the enzyme protein as those of liver alcohol dehydrogenase. Thus, in carboxypeptidase the loss of zinc appears to be directly proportional to the loss of enzymatic activity (5).

There appears to be little doubt that the activity of alkaline phosphatase is decreased in the zinc-deficient animal. Decreases in the activity of this enzyme have been reported in rat intestine and kidney (6), pig serum (7), calf serum (8) and turkey bone (9). However, the reduction in the activity of plasma alkaline phosphatase in the zinc-deficient rat has been found to result almost entirely from the depressed food intake produced by the deficiency (4).

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) is present in many animal and human tissues and fluids. In addition, alkaline phosphatase enzymes obtained from different tissues may have different substrate

Received for publication November 13, 1967.

[1] Published with the approval of the director of the Michigan Agricultural Experiment Station as Journal article no. 3973.

[2] We thank Merck Sharp and Dohme Research Laboratories for their courtesy in supplying the vitamins used in the various diets.

affinities as well as different electrophoretic mobilities (10). Moog et al. (11) have observed multiple forms of this enzyme in the mouse duodenum. In view of this it seemed worthwhile to study the effects of zinc-deficiency on the activity of intestinal alkaline phosphatase, using different substrates and, in one case, different buffers. Values for serum alkaline phosphatase are also reported in some instances. Moreover, since there appears to be some question as to whether the lowering of alkaline phosphatase activity is due to effects of zinc deficiency per se or the resulting inanition, a separate experiment is reported on the effects of dietary restriction on serum and intestinal alkaline phosphatase.

EXPERIMENTAL

Animals and diets. Weanling male rats of the Sprague-Dawley strain were obtained from a local breeding farm at 21 days of age and immediately placed on experiment.[3] They were kept in individual stainless steel cages with free access to deionized distilled water. The zinc-deficient diet used in shown in table 1. This diet contained by analysis 0.8 to 0.9 ppm zinc. For all positive control groups zinc carbonate was added to the basal diet to provide an additional 50 ppm of zinc. The vitamin-glucose mixture of the basal diet was modified by increasing the level of biotin so that the complete diet contained 4 mg/kg of this vitamin. This increase was necessary since preliminary studies with the unmodified diet containing 0.2 mg of biotin/kg resulted not only in growth failure but the appearance of a scaly seborrheic type of dermatosis, often during the second week of the experiment. Other symptoms included progressive alopecia, particularly in the areas around the mouth and eyes, and in some instances a spastic gait and kangaroo-like posture. In general, all the overt symptoms suggested a clinical manifestation of biotin deficiency. Biotin deficiency symptoms were never noted in the zinc-supplemented groups even though some of the animals remained on experiment as long as 8 weeks. The use of the modified vitamin-glucose mixture containing high levels of biotin did not alter the poor growth shown by the zinc-deficient group, but resulted in marked improvement in growth of the zinc-supplemented group. No abnormal skin changes were observed as a result of feeding the modified zinc-low diet for as long as 6 weeks except that mild erythemia of the paws was noted in a few cases. Histopathological studies revealed no changes in the skin except that it was somewhat thickened. Parakeratotic lesions were confined to the esophagus and tongue, and there was no evidence of spermatogenesis in the seminiferous tubules of the testes.

TABLE 1

Composition of diet

	g/kg
Glucose monohydrate [1]	577.9
Egg white solids (spray-dried)[2]	200
Corn oil	100
Cellulose [3]	30
Salt mix [4]	37
Vitamin-glucose mix [5]	50
Vitamin A and D conc [6]	5
α-Tocopherol	0.1

[1] Cerelose, Corn Products Company, Argo, Illinois.
[2] General Biochemicals, Inc., Chagrin Falls, Ohio.
[3] Solka Floc, Brown Company, Berlin, New Hampshire.
[4] Phillips, P. H., and E. B. Hart, J. Biol. Chem., 109: 657, 1935; reagent grade salts were used and $ZnCl_2$ was omitted.
[5] Composition similar to that used by Forbes, R. M., and M. Yohe. J. Nutr., 70: 53, 1960, except that the level of biotin was increased from 0.004 to 0.080 g/kg of mixture to prevent biotin deficiency.
[6] Vitamin A and D concentrate: 2000 IU vitamin A and 250 IU vitamin D/g.

In the first 2 experiments all the animals were fed to appetite, whereas in the third, a restricted control group was included in an effort to determine whether food intake was a factor in the lowering of alkaline phosphatase due to zinc deficiency.

Assay procedures. After the 3-week experimental feeding period the rats were placed under light anesthesia with ether, and killed by removing as much blood as possible by heart puncture. In some cases the blood was centrifuged and the serum used for alkaline phosphatase determinations; the duodenum was used for alkaline phosphatase determinations. In the preparation of the intestinal homogenate a section of the duodenum most proximal to the pylorus representing 15% of the total length of the small intestine was taken.

[3] Spartan Research Animals, Inc., Haslett, Michigan.

This section was cut into small pieces with scissors and a weighed sample taken sufficient to make a final concentration of 10% in deionized water. The whole was placed into the 50-ml chamber of an Omni-Mixer [4] and homogenized at 16,000 rpm for 5 minutes in an ice bath. The resulting supernatant was used for the assay, although in many instances further dilution with cold deionized water was required.

The intestinal alkaline phosphatase assays were determined using 2 substrates, sodium β-glycerophosphate (12) as modified by Moog (13), and disodium *p*-nitrophenylphosphate (14).[5] In the latter method 2 buffers were used, namely, Veronal and 2-amino-2-methyl-1-propanol. Each assay method was carried out under optimal conditions for each substrate.

Several different methods for the determination of alkaline phosphatase were used because the enzyme has been shown to exist in a number of forms when subjected to starch-gel electrophoresis (15) and it was desirable to ascertain whether zinc deficiency would cause a depression of the same order of magnitude with each method used.

At the time the animals were killed the small intestine was immediately removed and washed by forcing cold deionized water through the lumen with a plastic wash bottle. The tissue was placed in small polyethylene bags, quick-frozen on dry ice and stored at −20° until the analysis was performed. Preliminary studies showed that the highest intestinal alkaline phosphatase activity was in the first 1- to 2-g segments of the duodenum proximal to the pylorus; accordingly, tissue from this segment was used for the assay. Thus, although the term intestinal alkaline phosphatase will be used, the tissue used for assay more correctly corresponded with the pyloric end of the duodenum.

In the instances where serum alkaline phosphatase determinations were carried out the method of Bessey et al. (14) was used.

The results presented in this paper represent three separate experiments each of 3 weeks' duration, and data relative to the growth performance of the zinc-deficient and positive control groups are presented for all experiments even though their performance was similar in each instance. In addition, data were examined by analysis of variance, with statistical significance of treatment differences being determined by the multiple range test of Duncan (16).

RESULTS

Experiment 1. The effect of the zinc-deficient diet on growth and intestinal alkaline phosphatase is shown in table 2. The marked effect of zinc deficiency on growth and diet consumption is evident. The marked and statistically significant reduction of intestinal alkaline phosphatase is of the order of 50% if the average value obtained for the positive control group is considered normal. However, the possibility exists that feeding of supplementary zinc at the level of 50 ppm caused increased activity of the enzyme above normal values. Hove et al. (6) have reported a 40 to 100% increase of crude intestinal alkaline phosphatase activity by the in vitro addition of zinc ions. In an investigation of the effects of divalent metals on the activity of alkaline phosphatase obtained from calf duodenum, Hofstee (17) re-

[4] Ivan Sorvall, Inc., Norwalk, Connecticut.
[5] Obtained from Sigma Chemical Company, St. Louis.

TABLE 2

Effect of zinc deficiency on growth and intestinal alkaline phosphatase

Group [1]	Zn diet	Avg initial wt	Avg final wt	Total diet consumed	IAP [2]
		g	*g*	*g*	
1	Deficient	48 ± 0.8[3]	82 ± 2.7	115 ± 8.3	24.9 ± 1.7
2	Supplemented	48 ± 0.9	198 ± 8.0[a]	292 ± 11.2[a]	51.1 ± 1.9[a]

[1] Sixteen male rats/group.
[2] Intestinal alkaline phosphatase values expressed in μg phosphorus liberated from sodium β-glycerophosphate/min/mg protein at 38° and pH 9.4 in Veronal buffer.
[3] Mean ± SE.
[a] Significantly greater than least values ($P < 0.01$).

ported that preincubation of the enzyme mixture with zinc ions would activate the system. In view of this it was decided to determine whether the addition of zinc in appropriate concentrations to the duodenal homogenates in vitro would increase the activity of intestinal alkaline phosphatase in both the zinc-deficient and positive control animals. Accordingly, six duodenal homogenates were selected at random from each of the 2 groups in experiment 1, and the zinc content was determined by atomic absorption spectrophotometry. The results indicated a lower zinc concentration in the duodenum of the zinc-deficient rats. The values for the 6 zinc-deficient and 6 positive control animals were (mean ± SE): 124 ± 4 and 191 ± 20 mg Zn/g protein, respectively. When this difference in zinc content was calculated on the basis of the amount present in the tissue homogenate used it was of the order of 2.8×10^{-6} M zinc. Accordingly, several zinc concentrations were selected and added, as zinc acetate, to the same six duodenal homogenates from each group used for the zinc assay. The intestinal alkaline phosphatase assays were performed using the method described previously with sodium β-glycerophosphate as the substrate. The alkaline phosphatase activities were determined both immediately after the zinc additions and after incubation of the mixture for 24 hours at 4°. The results are presented in table 3. Although the addition of 1×10^{-5} M zinc appeared to increase the intestinal alkaline phosphatase activity within each group at both the zero and 24-hour incubation time, these increases are not statistically significant. However, the more important result of these assays is that the addition of zinc ions to duodenal homogenates of the deficient rats did not increase the enzyme activity to that of the positive controls. This shows that the lowered activity in zinc deficiency is not caused by a lack of sufficient ionic zinc but instead, to a reduced quantity of the enzyme itself. The addition of 1×10^{-3} M zinc proved to be inhibitory to the enzyme activity within each group.

Experiment 2. As indicated previously, alkaline phosphatase has been shown to exist in a number of different forms. Moreover, Moog et al. (11) and Moog (13) have found that the alkaline phosphatase of mouse duodenum will hydrolyze various substrates at different rates. Whether zinc deficiency would result in a more or less uniform lowering of enzyme activity as determined by different procedures was studied in the second experiment. In addition, serum alkaline phosphatase determinations were made by the method cited previously (14).

Thirty 21-day-old male rats were divided into 2 groups, with half receiving the low zinc diet (table 1) and the other half receiving the same diet supplemented with zinc at $ZnCO_3$ (53 ppm zinc by analysis). The source of the rats as well as their care and feeding were the same as in the first experiment.

Severe growth depression was evident by the end of the third week, at which time the animals were killed, blood samples

TABLE 3

Effect of the in vitro addition of zinc on intestinal alkaline phosphatase activity [1]

Zn added	Zinc-deficient		Zinc-supplemented	
	Incubation period, hours		Incubation period, hours	
	0	24	0	24
moles				
—	27.9 ± 2.4 [2]	28.4 ± 2.3	54.0 ± 1.9	54.2 ± 2.8
1×10^{-6}	29.1 ± 3.2	32.1 ± 3.1	55.3 ± 2.3	56.8 ± 3.2
1×10^{-5}	32.0 ± 3.0	33.4 ± 3.0	57.1 ± 2.4	58.2 ± 3.1
1×10^{-4}	31.4 ± 2.4	31.4 ± 2.5	56.2 ± 2.6	54.5 ± 2.8
1×10^{-3}	21.6 ± 3.0[a]	20.6 ± 2.7[a]	46.7 ± 2.8[a]	43.6 ± 2.4[a]

[1] Intestinal alkaline phosphatase values expressed in μg phosphorus liberated from sodium β-glycerophosphate/min/mg protein at 38° and pH 9.4 in Veronal buffer.

[2] Mean ± SE.

[a] Significantly less than the other values within the same group ($P < 0.01$).

TABLE 4

Effect of zinc deficiency on growth and intestinal alkaline phosphatase activity as determined by three different procedures

Group [1]	Zn diet	Avg initial wt	Avg final wt	Total diet consumed	Buffer system		
					Glycine [2]	2-amino-2-methyl-1-propanol [2]	Veronal [3]
		g	*g*	*g*			
1	Deficient	52 ± 0.5 [4]	79 ± 2.7	120 ± 5.8	7.5 ± 0.7	48.0 ± 5.2	26.7 ± 2.1
2	Supplemented	52 ± 0.6	196 ± 6.6[a]	298 ± 9.4[a]	13.6 ± 1.2[a]	115.9 ± 6.9[a]	51.4 ± 1.9[a]

[1] Fifteen male rats/group.
[2] Values used are expressed as the number of enzyme units that will liberate one μmole of p-nitrophenol/hr/mg protein at 38° and pH 9.98.
[3] Values expressed as μg phosphorus liberated from sodium β-glycerophosphate/min/mg protein at 38° and pH 9.4.
[4] Mean ± SE.
[a] Significantly greater than least values ($P < 0.01$).

obtained and the small intestine was removed and assayed for intestinal alkaline phosphatase. The important results of this experiment are shown in table 4. Again, as in the first experiment, the growth and appetite depressions due to zinc deficiency were marked.

The significant reduction of intestinal alkaline phosphatase in the zinc-deficient animal appears to be of the same order of magnitude with each of the 3 buffers used. Individual intestinal alkaline phosphate values also show that the lowest activity obtained with one buffer system and substrate was also the lowest when determined by the other 2 methods. This same characteristic was also true for the highest activity values. It thus appears that substrates such as sodium β-glycerophosphate or disodium-*p*-nitrophenylphosphate both indicate a marked reduction in enzyme activity. Of the 2 buffers used with disodium-*p*-nitrophenylphosphate substrate, 2-amino-2-methyl-1-propanol appears to show the largest percentage decrease in activity due to zinc deficiency. Lowry et al. (18) reported this buffer to be superior to Veronal or glycine for alkaline phosphatase determinations.

The determination of serum alkaline phosphatase on all the animals in experiment 2 showed the following mean and standard error values (expressed in Bessey-Lowry units): 5.1 ± 0.5 for the zinc-deficient and 12.1 ± 1.0 for the positive control animals. These differences were statistically significant ($P < 0.01$).

Experiment 3. This experiment was designed to determine whether the lower activity of serum and intestinal alkaline phosphatase observed in the zinc-deficient animals was due to the marked reduction in food consumption or was, in fact, a specific effect of zinc deficiency.

Twenty-one 21-day-old male rats were divided into three equal groups. Group 1 received the low zinc diet (table 1); group 2 the same diet supplemented with 50 ppm zinc, but with the dietary intake restricted to the amount consumed daily by group 1; and group 3 received the zinc-supplemented diet in unrestricted amounts. At the end of the third week the animals were killed, and serum and intestinal alkaline phosphatase were determined by previously described methods. The results of this experiment are shown in table 5. The reduction in dietary intake of the controls (group 2) caused a significant reduction in the activity of serum alkaline phosphatase, but not of intestinal alkaline phosphatase. The results obtained with serum alkaline phosphatase are similar to observations previously reported for plasma alkaline phosphatase by Macpinlac et al. (4) for the zinc-deficient rat. However, the lowering of intestinal alkaline phosphatase indicates a specific response to zinc deficiency. Although the results of Fishman et al. (19) show that a major portion of serum alkaline phosphatase in the normal rat is of intestinal origin, this may not be the case in the zinc-deficient animal.

DISCUSSION

The significant reduction in intestinal alkaline phosphatase activity is not due to

TABLE 5

Serum and intestinal alkaline phosphatase activity as influenced by zinc deficiency and zinc supplementation with and without dietary restriction

Group	Diet	Avg initial wt	Avg final wt	Total diet consumed	Alkaline phosphatase		
					Serum [1]	Intestinal	
						Glycine buffer [2]	2-amino-2-methyl-1-propanol buffer [2]
		g	*g*	*g*			
1	Zn-deficient	57 ± 0.5 [3]	78 ± 1.6	127 ± 2.9	5.5 ± 1.0	8.0 ± 0.8	46.4 ± 4.0
2	Restricted-fed control	57 ± 0.7	95 ± 1.3[a]	126 ± 0.5	7.4 ± 0.4	16.0 ± 1.4[a]	99.4 ± 10.3[a]
3	Ad libitum-fed control	58 ± 0.8	211 ± 6.7[a]	307 ± 6.7[a]	14.9 ± 1.8[a]	15.2 ± 1.2[a]	86.6 ± 7.9[a]

[1] Values expressed in Bessey-Lowry units.
[2] Values are expressed as the number of enzyme units that will liberate one μmole of *p*-nitrophenol/hr/mg protein at 38° and pH 9.98.
[3] Mean ± SE.
[a] Significantly greater than corresponding value of zinc-deficient group ($P < 0.01$).

lack of zinc ions per se or to inanition, but appears to result from lowered concentration of the enzyme itself. This is evidenced by the failure to obtain an increase in enzyme activity following incubation of duodenal homogenates obtained from zinc-deficient rats with appropriate concentrations of zinc ions. A similar situation with respect to plasma alkaline phosphatase was reported by Heaton (20) who observed decreased concentration of the enzyme in the magnesium-deficient rat.

No attempt was made in the present investigation to study the effect of the in vitro addition of zinc ions on serum alkaline phosphatase since the reduction in activity of this enzyme in the serum was apparently due almost entirely to inanition resulting from the zinc deficiency.

Intestinal alkaline phosphatase appears to be heterogeneous with respect to activity toward different substrates (11), and also to various physical methods of separation such as starch-gel electrophoresis (15). However, Moss (21) in a study of human intestinal alkaline phosphatase, has indicated that while structural differences may exist, the enzymes are functionally similar. In the present investigation, zinc deficiency produced about the same degree of depression in the activity of intestinal alkaline phosphatase with the two substrates used.

ACKNOWLEDGMENT

We thank Dr. D. L. Whitenack, Department of Animal Pathology of this institution, for his assistance with the histopathology of the deficient animals.

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Pancreatic Adaptation to Change in Dietary Protein Source in Rats Fed at Different Frequencies [1]

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ABSTRACT The rates at which the pancreatic concentrations of chymotrypsinogen, trypsinogen, amylase, and nucleic acid adapt to the substitution of whole-egg protein for casein at the 15% level of the diet were determined over a 2-week period using rats fed 1) ad libitum, and 2) for 1-hour intervals spaced 12 hours apart (space-fed). Chymotrypsinogen and trypsinogen increased almost immediately, with maximal adaptation occurring within 2 to 4 days. Four to seven days were required for amylase to adjust, by increasing, to the change in protein source. There was some evidence to indicate that the mechanisms mediating amylase and protease induction were not identical although the direction of adaptation was the same for all 3 enzymes. Enzyme and RNA levels in rats fed the same diet did not differ significantly regardless of feeding frequency on any specific day of the experiment although, on the average, more chymotrypsinogen and trypsinogen, but not amylase, were found throughout the experimental period in rats fed the egg protein diet at intervals. The introduction of whole-egg protein into the diet caused pancreatic RNA but not DNA to increase as much as 66% within 2 to 3 days.

Elaboration of enzymes by the exocrine pancreas of the rat has been shown to adapt to the composition of the diet given. Kinetics of the adjustment to a change in dietary carbohydrate-to-protein ratio in rats fed ad libitum were described by Howard and Yudkin (1) who reported that protease activity decreased within 24 hours whereas amylase adaptation required 1 to 7 days when the casein-to-sucrose ratio in the diet was changed from 60:20 to 20:60. Ben Abdeljlil and Desnuelle (2) observed that pancreatic adjustment to a change in starch-to-casein ratio from 75:15 to 20:70 started without delay and required 5 to 7 days. The specific activity of chymotrypsinogen increased about threefold, and that of amylase decreased by more than half in the experiments of the latter group.

The protease concentration of the pancreas of rats fed "meals" increased almost as much in 3 days when whole-egg protein was substituted for casein at the 15% level of the diet (3, 4) as when casein was increased three- to fourfold. Amylase, however, was not affected significantly by egg protein substitution within 3 days. It was postulated that the response to whole-egg protein was related to the superior amino acid composition of this protein (3, 4). The possibility that a protein component of whole egg, such as egg white trypsin inhibitor, might stimulate the exocrine pancreas was not excluded although addition of egg white trypsin inhibitor to hydrolyzed protein diets did not affect the pancreatic response to those diets (4).

Before the factors effecting the pancreatic response to egg can be further elucidated, it is important to follow this response over an adaptive period longer than 3 days and shorter than one to determine the time required to produce a maximal as well as a minimal response. Because the various experiments mentioned above were performed using rats fed at different frequencies, it appears necessary to determine whether pancreatic adaptation to diet differs when rats are fed at intervals as opposed to when they are fed ad libitum. (Meal feeding would cause digestive tract loading, whereas ad libitum feeding would lead to more frequent pancreatic stimulation.) These were the objectives of the experiments reported herein. In addition, the effect of whole-egg protein on the nucleic acid composition of the pancreas was determined over a 2-week period to assess whether changes in the protein synthetic equipment of the pancreatic cell might be involved in the adaptive process.

Received for publication August 29, 1967.
[1] This work was supported in part by Public Health Service Research Grant no. HD-02207 from the National Institute of Child Health and Human Development.

EXPERIMENTAL

Albino male rats of the Charles River CD strain, initially weighing between 150 and 175 g, were used. The rats were fed a 15% casein diet (table 1) for a one-week pre-experimental period. Control rats were killed by chloroform inhalation and their pancreases obtained for analysis at the end of the pre-experimental period, or they were maintained with the casein diet for assigned periods of time during the experimental period and then were killed and autopsied. Experimental rats were given the whole-egg protein diet (table 1) for varying periods of time during the experimental period. The design of the 2 experiments conducted is detailed below.

Experiment 1. Two methods of feeding were used in this experiment — groups, comprised of 4 rats each, were fed 1) ad libitum throughout the study, or 2) were allowed to eat all they would consume in one-hour periods spaced 12 hours apart (space-fed) during the pre-experimental and experimental periods. The whole-egg protein diet was given to the experimental groups for 0.5 up to 14 days (equivalent to 1 to 28 feedings for the space-fed animals). Death was induced 11 hours after the termination of the last feeding period in the case of the space-fed animals or 11 hours after food was removed from the cages of the animals fed ad libitum. The pancreases were then removed and prepared for analysis.

TABLE 1

Composition of diets fed

	Casein diet	Whole-egg protein diet
	%	%
Sucrose	70.0	66.4
Casein [1]	15.0	—
Whole-egg powder [1]	—	18.6
Cellulose	5.0	5.0
Cottonseed oil	5.0	5.0
Salt mix [2]	4.0	4.0
Vitamin mix in sucrose [3]	1.0	1.0

[1] The casein and whole-egg powder provided approximately 13.9 and 13.5 g protein per 100 g diet according to data obtained in a series of analyses of these proteins. The whole-egg powder, hexane-extracted, was composed of about 6% white trypsin inhibitor.

[2] Phillips, P. H., and E. B. Hart. J. Biol. Chem., *109:* 657, 1935; obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Water-soluble vitamins were supplied in the diet at the following levels: (mg/kg) thiamine, 20; riboflavin, 20; pyridoxine, 14; Ca pantothenate, 140; inositol, 700; niacin, 140; folic acid, 1.8; biotin, 0.7; vitamin B_{12}, 0.12; choline chloride, 1500; and ascorbic acid, 700. Fat-soluble vitamins were supplemented weekly at the following levels: *dl*-α-tocopherol, 4.2 mg; menadione, 0.007 mg; vitamin A (crystalline, gelatin-coated), 1400 IU and vitamin D (calciferol), 175 IU.

Experiment 2. Rats were allocated to groups of 5 rats each. Every 12 hours during the pre-experimental period, the rats were each given 6.5 g of the casein diet, which they usually ate within an hour. Food cups were removed from the cages after an hour and weigh-back, if any, was recorded. The whole-egg protein diet was fed in the same manner during the experimental period. At the end of the one-week pre-experimental period, groups of control rats, which had been fed the casein diet only, were killed at the following hours after the initiation of the 1-hour feeding period: 1, 2.5, 5, and 12 (the time at which food ordinarily would also be given and thus equivalent to zero time). Groups of experimental rats were killed at the same time-intervals after 1 feeding and after 7 feedings of the whole-egg protein ration.

Preparation of pancreas samples. After removal, each pancreas was freed, as much as possible, from surrounding fat and connective tissue and then was homogenized in ice-cold 0.25 M sucrose solution. Aliquots of the exact size needed for the determinations to be run were pipetted into test tubes and frozen. It had been determined previously that the outcome of the analyses to be run was not affected appreciably by the length of the freezing period used.

Chemical analyses. For the enzyme analyses, frozen aliquots, containing about 100 mg of wet pancreas, were thawed and made up to a final volume of 10 ml with 0.2 M tris buffer, pH 8.1, containing 0.05 M $CaCl_2$. Fifteen milligrams of purified enterokinase were added to activate chymotrypsinogen and trypsinogen. The activation step was carried out at 4° for 18 to 24 hours. Particulate matter settled during this time-period; enzyme assays were run, using aliquots of the supernatant.

Chymotryptic and tryptic activities were estimated using N-acetyltyrosine ethyl ester (ATEE) and *p*-toluene sulfonyl-L-arginine methyl ester (TAME), respectively. The 2 assays, described in detail in a previous

paper (3), were accomplished by measuring the rate at which hydrogen ion was released during hydrolysis of the esters by means of the change in the color of the indicator *m*-nitrophenol.

Amylase activity was measured using the starch-iodine method developed by Smith and Roe (5).

The RNA and DNA content of a 200-mg sample of wet pancreas was determined by a modification of the Schneider (6) technique using orcinol and diphenylamine reagents to analyze for ribose and desoxyribose, respectively.

RESULTS

Method of reporting enzyme activity. Pancreatic enzyme activity is expressed in the literature in several ways: for example, as total enzyme activity per pancreas (4, 7), activity per milligram of pancreatic protein (2), activity per milligram of dry pancreas (1), activity per 100 g body weight (8), and activity per microgram of DNA phosphorous (9). Because the method of expressing activity may well affect interpretation of the data, trypsinogen, as measured in rats space-fed whole-egg protein for 0.5 to 14 days (1 to 28 feedings) is expressed for comparison in 3 ways in table 2. The data reported indicate that trypsinogen steadily increased in the pancreas after whole-egg protein was substituted for casein in the diet, reaching a maximum between the second and fourth days except when activity was reported as total per pancreas. The variability and interpretation of the data were essentially the same regardless of whether trypsinogen was reported as total per pancreas, per 100 g body weight, or per milligram of DNA. However, factors which might influence interpretation of results are the age-related changes in pancreas composition. The enzymic as well as the nucleic acid composition of the rat pancreas increases with age or weight until the rat has attained an age of more than 7 weeks but less than 13 weeks or weight of more than 200 g but less than 400 g (results of unpublished experiments). Since experiment 1 lasted 2 weeks (length of experimental period) and since maximal pancreatic growth presumably was not attained by the experimental animals during this time-period, growth-related changes in the pancreas might obscure changes related to dietary alteration. To correct this, values obtained in experiment 1 are reported as total enzyme or nucleic acid per pancreas per 100 g body weight. Enzyme activity in experiment 2 is reported, as in previous papers, as total per pancreas since there was little weight change during the experimental period and since identical curves were obtained when data were plotted graphically regardless of the manner in which enzyme activity was reported.

Adaptation to dietary protein change over a 2-week period. The growth of rats fed the 2 diets at different frequencies is shown in figure 1. Food consumption of rats fed ad libitum and at intervals averaged 17.7 and 11.2 g/day, respectively,

TABLE 2

Adaptation of trypsinogen to substitution of whole-egg protein for casein in the diet

No. days egg protein fed	*p*-Toluene sulfonyl-L-arginine methyl ester (TAME) hydrolyzed		
	μmoles/min/pancreas	*μmoles/min/pancreas/100 g body wt*	*μmoles/min/mg pancreatic DNA*
—	451 ± 81 [1,2]	273 ± 38	103 ± 20
0.5	483 ± 131	270 ± 77	106 ± 28
1	529 ± 63	340 ± 47	137 ± 19
1.5	813 ± 63	476 ± 127	244 ± 32
2	683 ± 139	454 ± 105	223 ± 56
4	1359 ± 245	673 ± 104	337 ± 78
7	918 ± 154 (619 ± 52) [3]	498 ± 24 (337 ± 19)	335 ± 34 (127 ± 7)
14	1416 ± 260 (718 ± 154)	592 ± 112 (304 ± 61)	303 ± 68 (196 ± 44)

[1] SE of mean.
[2] All rats were space-fed the 15% casein diet for 1 week ending at day zero.
[3] For comparison, trypsinogen values for rats space-fed the casein diet until day 7 or day 14 of the experimental period are shown in parentheses.

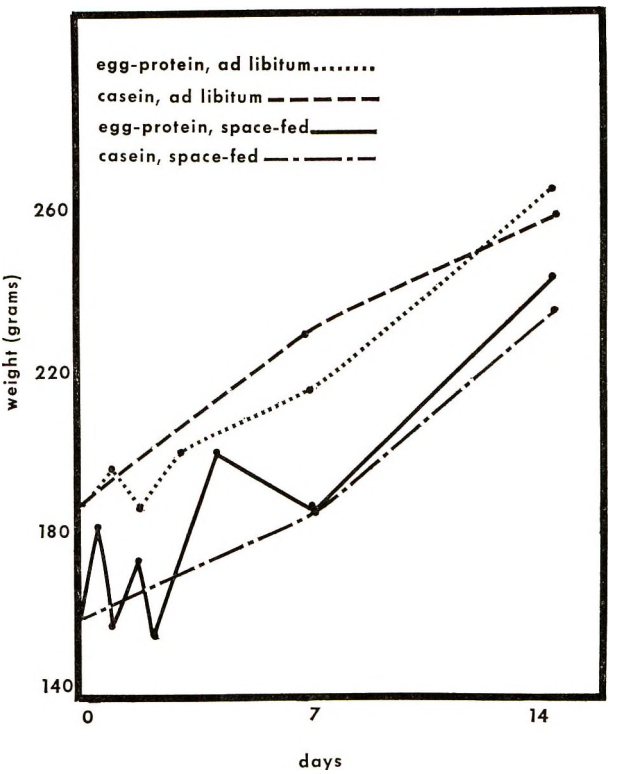

Fig. 1 Mean body weights of rats at various times during the experimental period. All rats were fed the casein diet during a one-week pre-experimental period ending at day zero shown above. Two methods of feeding were used.

during the pre-experimental and experimental periods. Food consumption of the space-fed animals was especially low during the pre-experimental period; the weights of the space-fed animals at day zero reflect this. Indicative of the erratic food consumption observed when rats were first offered the egg protein diet are the fluctuations in body weight of the experimental groups during the first days of the experimental period. Subsequently, it has been found that when rats are trained to eat a meal of about 6.5 g before the experimental period, this unnecessary source of variation can often be eliminated.

The adaptation of pancreatic constituents to the substitution of whole-egg protein for casein in the diet is illustrated in figures 2–5. During the 2-week experimental period, the chymotrypsinogen content of the pancreas (fig. 2), as calculated on a constant body weight basis, remained relatively constant in control rats maintained with the casein diet, whereas this zymogen increased 164% and 94%, respectively, between days zero and 14 of the experimental period when rats were fed egg protein at intervals and ad libitum. As with trypsinogen, highest levels of chymotrypsinogen in space-fed rats were reached the fourth day. When rats were fed ad libitum, the concentration of chymotrypsinogen in the pancreas plateaued by the third day but then appeared to increase slightly during the second week. Consistently more chymotrypsinogen was observed in the pancreas of rats fed whole-egg protein at intervals. However, differences between the 2 feeding procedures were not statistically significant on any specific day of the experimental period.

That the amylase concentration of the pancreas was also affected by the substitution of whole-egg protein for casein in the

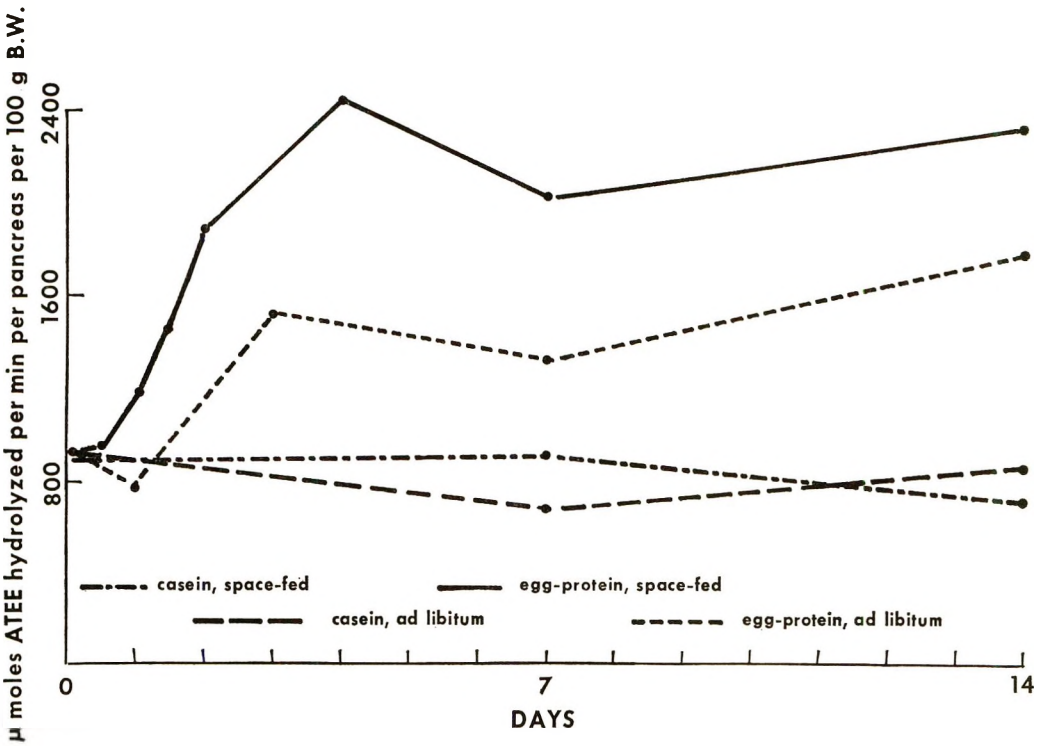

Fig. 2 Mean pancreatic concentration of chymotrypsinogen per 100 g body weight during adaptation to the whole-egg protein diet or during maintenance with the pre-experimental casein diet. Pre-experimental period ended on day zero shown above. Two feeding methods were used.

diet is shown in figure 3. Amylase, as calculated on a constant body weight basis, increased 140% and 53%, respectively, between day zero and day 14 in rats fed at intervals and ad libitum. In contrast with chymotrypsinogen, feeding frequency did not affect the amylolytic response to whole-egg protein substitution in a consistent manner.

Adaptation of amylase required about 7 days for completion in space-fed rats; maximal adaptation apparently was not achieved by the end of the 2-week experimental period in rats fed ad libitum. Results of previous studies (4) indicated that substitution of whole-egg protein for casein in the diet does not affect amylase synthesis in space-fed rats. Results of this study appear to be conflicting. However, the earlier studies were of short-term duration (3 days) at the end of which time, amylase adaptation, unlike protease adaptation, was not complete according to the data presented here.

Changes in the RNA and DNA content of the pancreas during the experimental period are shown in figures 4 and 5. Pancreatic RNA per 100 g body weight (fig. 4) increased 66% and 38%, respectively, during the first 2 days in rats fed the whole-egg protein diet at intervals and ad libitum. Adaptation of RNA was essentially complete by the second or third day and appeared to precede, to some extent, enzymic adaptation.

There was a tendency for the ratio between pancreatic RNA and body weight to decrease during the latter part of the experimental period in all groups except the one fed the casein diet ad libitum. This may have been a growth-related change.

Pancreatic DNA was somewhat difficult to extract from the pancreas for analysis and hence results must be interpreted with care. However, DNA whether reported as total per pancreas or per 100 g body weight (fig. 5), apparently was not affected by the substitution of whole-egg protein for

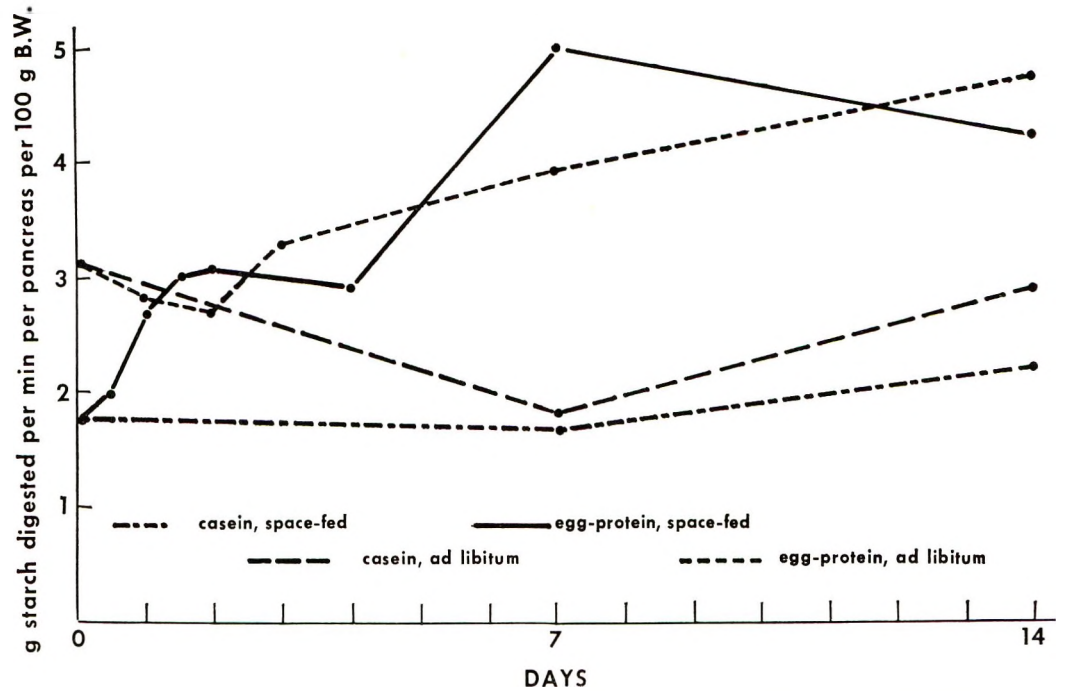

Fig. 3 Mean pancreatic concentration of amylase per 100 g body weight during adaptation to the whole-egg protein diet or during maintenance with the pre-experimental casein diet. Pre-experimental period ended on day zero shown above. Two feeding methods were used.

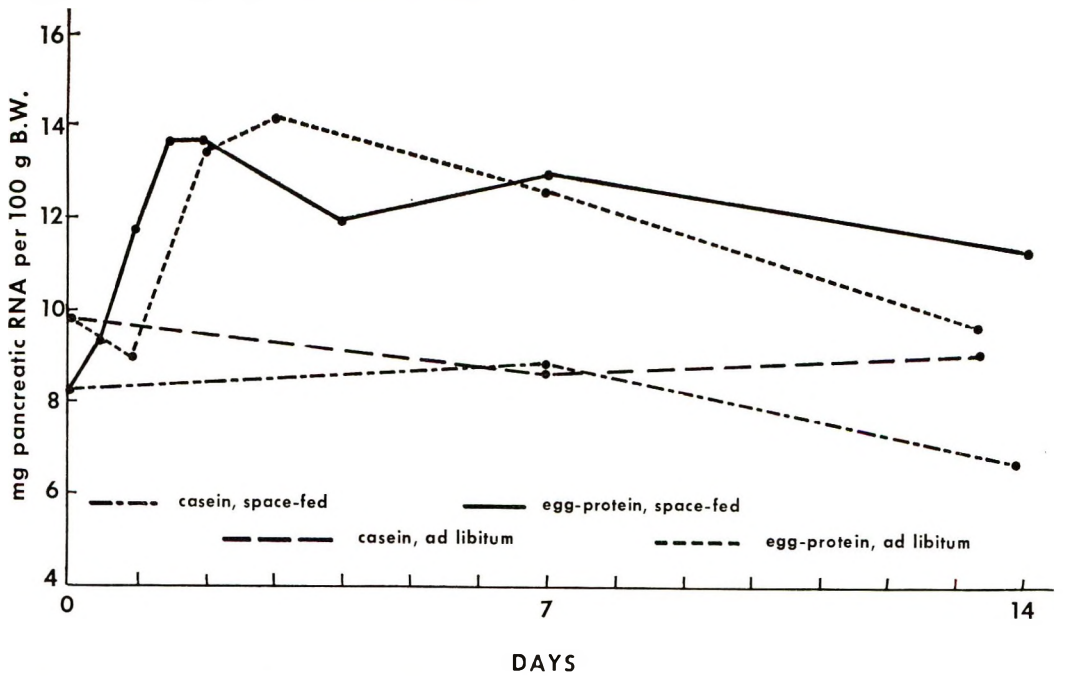

Fig. 4 Mean pancreatic concentration of RNA per 100 g body weight during adaptation to the whole-egg protein diet or during maintenance with the pre-experimental diet of 15% casein. Pre-experimental period ended on day zero shown above. Two feeding methods were used.

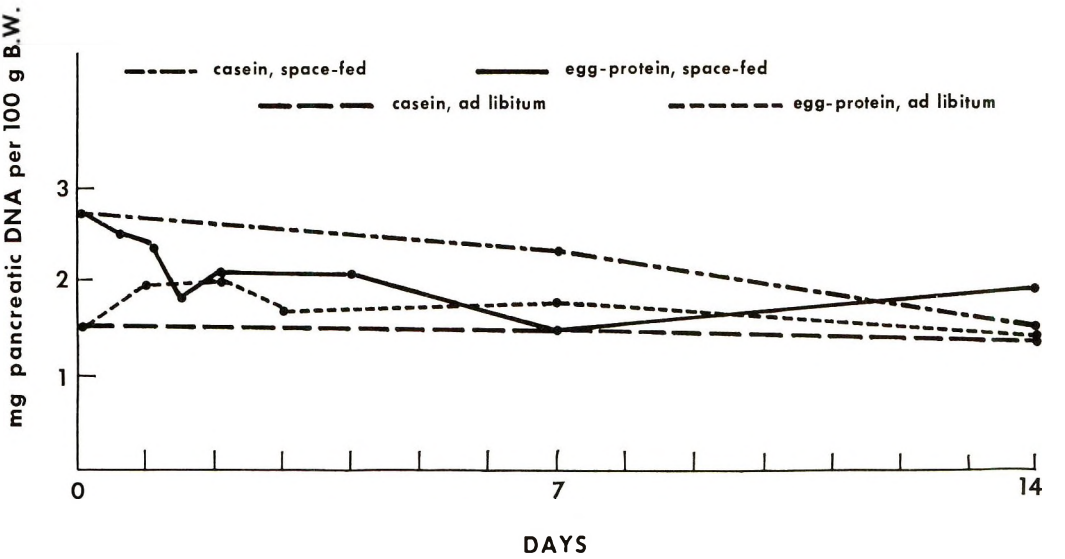

Fig. 5 Mean pancreatic concentration of DNA per 100 g body weight during adaptation to the whole-egg protein diet or during maintenance with the pre-experimental casein diet. Pre-experimental period ended on day zero shown above. Two feeding methods were used.

casein in the diet. This observation indicated that whole-egg protein stimulated pancreatic hypertrophy but not hyperplasia although rats, at least the age of the ones used in this experiment, apparently can undergo pancreatic cell division. At days zero and 7 of the experimental period, the difference in DNA concentration between rats fed casein at intervals and ad libitum was statistically significant ($P < 0.05$).

Pancreatic enzymes during absorption of a meal. This study, in which the pancreas was assayed at various times after rats were fed a 6.5-g meal, was performed for 2 reasons: to determine the minimal length of time required to produce a response to a meal and to study enzyme concentration in the pancreas during absorption as well as during fasting. Since results obtained in experiment 1 indicated that enzyme levels in rats space-fed the casein diet for 7 and 10 days (equivalent to days zero and 3, respectively, of the experimental period), would not differ appreciably, only one casein control series was run in experiment 2.

The extent of change in enzyme concentration during the 12-hour period following the initiation of feeding did not exceed 40% for amylase or 33% for chymotrypsinogen. Enzyme activity was lowest 5 hours after feeding.

Although the pancreatic concentration of chymotrypsinogen was reduced slightly 1 to 2 hours after the first feeding of the egg protein diet, between 5 and 12 hours an average of 31.5 ± 2.3% more chymotrypsinogen was noted in rats fed egg protein than in rats fed casein. Hence, the response to dietary change was rapid.

The behavior of amylase was in direct contrast with that of chymotrypsinogen. An average of 16.2 ± 1.5% less amylase was noted in the pancreas between 5 and 12 hours after the first feeding of egg protein. The difference in the behavior, between 5 and 12 hours, of the 2 enzymes was statistically significant ($P < 0.01$).

After a 3-day period of adaptation to whole-egg protein (during which time maximal adaptation of amylase would not be achieved) the higher level established for both enzymes in the pancreas was maintained in the absorptive as well as the postabsorptive state.

DISCUSSION

Three points should be considered in discussing the results of these experiments: 1) the similarity between the kinetics of

pancreatic adaptation to a change in dietary protein source and to a change in protein-to-carbohydrate ratio, 2) the effect of feeding frequency on pancreatic enzymes, and 3) the role of RNA in the adaptation process.

Adaptation kinetics. Data reported herein indicate that the pancreatic concentration of amylase, chymotrypsinogen, and trypsinogen adapted to the dietary substitution of whole-egg protein for casein and to a change in dietary carbohydrate-to-protein ratio (as reported by Howard and Yudkin (1)) at approximately the same rates. Adaptation to whole-egg protein began almost immediately and required for completion, at least in space-fed animals, approximately 3 to 4 days for chymotrypsinogen and trypsinogen and as many as 7 days for amylase. It was of interest that the amylase content of the pancreas was augmented as much or more when egg protein was substituted for casein in the diet as when the carbohydrate content of the diet was increased three- to fourfold (1, 2) and that this augmentation had approximately the same time requirement for completion. The observations that amylase adapted more slowly to a change in dietary protein, and that, under the more controlled conditions achieved in experiment 2, amylase was reduced in the pancreas 5 to 12 hours after whole-egg protein was given in place of casein in the diet, whereas chymotrypsinogen was elevated (a behavioral difference that was statistically significant) are evidence that the mechanisms regulating induction of amylase and protease are not identical.

Feeding frequency. Whether feeding frequency affected adaptation is debatable. The space-feeding method could be standardized more exactly than the ad libitum method because it was difficult to measure the exact time lapse between eating and removal of the pancreas in rats fed ad libitum. Despite the observation that enzyme adaptation appeared to continue into the second week of the experimental period in rats fed ad libitum, the enzyme content of the pancreas of rats fed ad libitum and at intervals did not differ significantly at 7 or at 14 days. In fact, as previously mentioned, levels of pancreatic enzymes did not differ significantly between groups of rats fed at different frequencies at any specific time during the experimental period. Additional information on the relative effect of interval versus ad libitum feeding is given in table 3. The data shown there was calculated for rats fed each diet by dividing the average amount of pancreatic constituent, as measured at a specific time during the experimental period in rats fed ad libitum, by the average amount as measured at a corresponding time-period in space-fed rats. The ratios thereby obtained were averaged, and hence the data in table 3 represent the average ratio of ad libitum-fed to space-fed over the entire experimental period. The 99% confidence limits show the variability of these ratios. If feeding frequency had no effect on the results, the ratio should be one. In 2 instances the ratio is sufficiently different from one that it appears that feeding frequency had an effect on the results. These are the ratios calculated for chymotrypsinogen and trypsinogen in rats fed whole-egg protein. Once again, the difference in the behavior of protease and amylase was significant ($P < 0.05$). The results also indicate that it was the interaction between feeding frequency and dietary protein that affected the various pancreatic constituents and not feeding frequency per se.

TABLE 3

Relative effect of interval vs. ad libitum feeding on pancreatic constituents

Diet	Amylase	Chymotrypsinogen	Trypsinogen	RNA	DNA
Casein	1.38 ± 0.49 [1,2,3]	1.00 ± 0.36	1.03 ± 0.36	1.18 ± 0.28	0.70 ± 0.28
Whole-egg protein	0.99 ± 0.18 [4]	0.66 ± 0.07	0.77 ± 0.10	0.95 ± 0.18	0.91 ± 0.21

[1] 99% confidence limits.
[2] Amylase per pancreas per 100 g body weight of rats fed ad libitum divided by amylase per pancreas per 100 g body weight of space-fed rats.
[3] Ratio for rats fed casein was determined at days zero, 7 and 14 of the experimental period and averaged
[4] Ratio for rats fed whole-egg protein was determined at days 1, 2, 3-4, 7 and 14 and averaged.

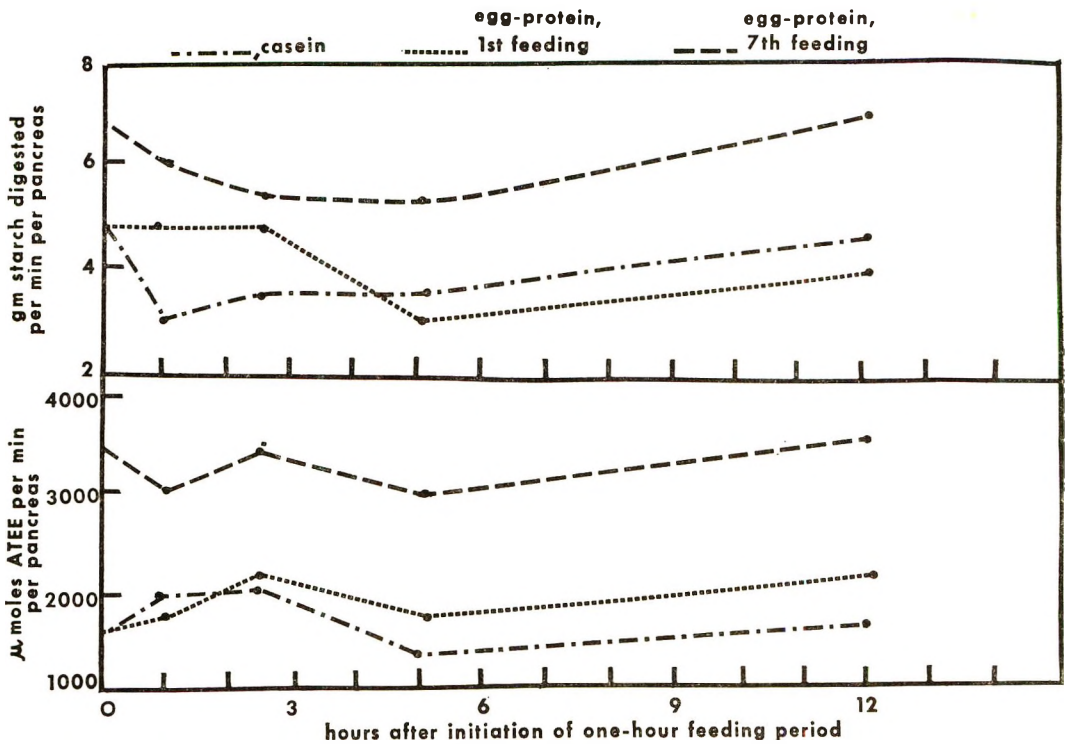

Fig. 6 Mean pancreatic concentration of amylase (top) or chymotrypsinogen (bottom) at various hours after feeding. All rats were fed the casein control diet during a one-week pre-experimental period. Enzymes were assayed in control rats after the last meal of the pre-experimental period. The experimental period consisted of 1 or 7 feedings of the whole-egg protein diet.

Role of RNA in adaptation process. It has been reported that withdrawal of protein from the diet of rats does not affect the amount of RNA per pancreatic cell (10), whereas enzyme synthesis but not RNA concentration is increased by the addition of amino acids to pigeon pancreas slices incubated in vitro (11). Therefore, the observation that a higher level of RNA was established in the pancreas when the source rather than the quantity of protein in the diet was altered is of interest. In line with the observation that whole-egg protein caused RNA to increase in the pancreas is the finding of Allison et al. (12) that RNA:DNA ratios in the liver were greater when egg albumin as opposed to casein was fed to rats at levels below 18% of the diet. These authors noted that the RNA concentration in the liver corresponded to the nutritive value of the protein fed. However, the explanation for the effect of whole-egg protein on pancreatic RNA may not be this simple. Results of unreported research show that pancreatic RNA levels are affected more by the presence of egg white trypsin inhibitor in the diet than by amino acid mixtures simulating egg protein.

The concomitance of the relationship between the increase in pancreatic RNA and exocrine enzymes is not clear, although the data obtained in experiment 1 suggest that the establishment of a higher RNA level in the pancreas may accompany or perhaps precede an induction of digestive enzymes.

ACKNOWLEDGMENT

The author thanks Edith Lerner for assistance with the RNA and DNA determinations.

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Effect of Infection on Skeletal Muscle Ribosomes in Rats Fed Adequate or Low Protein [1,2]

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ABSTRACT The effect of *Salmonella typhimurium* infection on protein metabolism in the skeletal muscle of the hind leg was studied in rats in 2 experiments. In experiment 1 rats were fed an adequate-protein diet and killed at zero, 1, 2, 3, and 5 days after infection. In vivo uptake of ^{14}C-leucine by muscle ribosomes into nascent peptides was measured; and ribosomes were studied by sucrose gradient analysis. Infection reduced the uptake of radioactivity by ribosomes and resulted in lowered levels of heavy polyribosomes and increased proportions of the light ribosome species. In experiment 2 weanling rats were prefed a low protein or adequate-protein diet for 9 days and then infected. In vivo and in vitro synthetic capacity of muscle ribosomes was studied at zero, 1, 2, 3, or 7 days after infection. The in vivo and in vitro protein-synthesizing capacity of the ribosomes was reduced by the infection in both dietary groups. Clear-cut differences in the variables studied were not observed between the dietary groups during the first 3 days after infection. The changes are discussed in relation to the metabolic nitrogen loss occurring during infection.

Recently, increasing interest has been focused on the interaction between nutrition and infection (1, 2). It has been well-established that a generalized infection leads to an increased rate of urinary nitrogen excretion and a depletion of body protein in experimental animals (3, 4) and man (5, 6). Although the source of this increased nitrogen loss has not received detailed study, the available information suggests that muscle supplies a major portion of it (5).

Even though a number of studies have been concerned with the effects of a generalized bacterial infection on hepatic protein synthesis (7–9), there is little information on the specific biochemical changes in muscle following infection. Therefore, we have begun a series of investigations concerning the response of muscle protein metabolism following *Salmonella typhimurium* infection in the rat. The present report emphasizes the ribosome in view of its central role in the protein synthesis of mammalian tissues. The capacity for in vivo and in vitro incorporation of labeled amino acid into nascent peptides by ribosomes and the sedimentation of muscle ribosomes in sucrose gradients were investigated to gain further information on the host-metabolic response to infection and the effects of previous protein nutrition on the biochemical response of muscle.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain [4] were housed individually in suspended galvanized cages with wire-mesh bottoms. The composition of the diets is shown in table 1. Diet was placed in the animal rooms each day, and the unused food was discarded. Water was available ad libitum.

The bacterial strain producing the infection was isolated from a natural infection by the M.I.T. Nutritional Pathology Laboratory. Rats were infected by intraperitoneal injection with about 5.5×10^7 viable organisms. The organism was isolated from the livers of these animals on days 2 and 5 post-infection and subsequently typed by the Communicable Disease Center of the United States Public Health Service, Chamblee, Georgia.

The design of the 2 experiments is shown in table 2. The first consisted of 3 groups of rats fed the 18% casein diet. The mean initial weight of the rats in each

Received for publication October 16, 1967.

[1] This work was supported by Contract no. DA-49-193-MD-2560 with the U.S. Army Medical Research and Development Command. Taken from a thesis submitted by S. C. Chen in partial fulfillment for the Ph.D. degree in Nutritional Biochemistry and Metabolism, Massachusetts Institute of Technology.

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TABLE 1

Composition of low protein and adequate-protein diets fed to rats infected with S. typhimurium

	Low protein	Adequate protein
	% *diet*	
Casein	5.0	18.0
Methionine	0.06	0.3
Dextrin	52.96	44.2
Sucrose	26.48	22.0
Cottonseed oil [1]	10.0	10.0
Salt mix [2]	5.0	5.0
Vitamin mix [3]	0.5	0.5

[1] Wesson Oil, Wesson Oil Sales Company, Fullerton, California.
[2] Composition as described by Rogers and Harper (25); purchased from General Biochemicals, Incorporated, Chagrin Falls, Ohio.
[3] Composition as described by Rogers and Harper (25). In addition, choline was added as an aqueous solution to the diet at a level of 0.3 g/100 g diet.

group was 80 g. In addition, as an initial control 4 rats were killed on day of infection. Two groups of rats were moved to an isolation room and inoculated with *S. typhimurium*. One group was used to assess the cumulative death rate. Noninfected controls were pair-fed at 12-hour intervals to the intake of their infected mates. Four rats were killed from each group (18-I-1 and 18-C-1) at selected times following infection.

The second experiment consisted of 2 groups of 90 rats each—one fed the low protein (5% casein) diet and the other, the adequate-protein (18% casein) diet. Rats in the latter group were pair-fed to those fed the low protein diet. The mean initial weight of the rats was 58 ± 2 g. On the morning of the ninth day of the experiment, 80 animals from each group were infected with the bacterium described above. The remaining 10 rats in each group were used as initial controls. Following infection, rats in each dietary group were killed at the intervals described in the Results section. Pair-feeding was maintained during the first 2 complete days after infection, but because of the greater mortality rate of rats in the low protein group, all rats thereafter were fed ad libitum.

Since it would be very difficult to examine the whole muscle mass of the animal, the muscles of both hind legs were pooled and studied. The assumption was made that they reflect changes which occur in the whole muscle mass. The preparation of muscle ribosomes, in good yield, for the in vivo and in vitro studies, as well as the method for the sedimentation analysis of muscle ribosomes, have been described in detail previously (10). The incubation system used for the in vitro studies was as follows: (in μmoles) 5 tris (hydroxymethyl) amino methane (pH 7.6), 10 $MgCl_2$, 70 KCl, 2 adenosine triphosphate, 1 guanosine triphosphate, 15 creatine phosphate; 20 μg creatine phosphokinase [5]; 1.0 mg protein of pH 5 enzyme fraction; 10 mμmoles of a complete amino acid mixture containing 1 μCi of a uniformly labeled ^{14}C-amino acid mixture [6]; and 0.1 mg RNA from the ribosome preparation. The complete incubation system was carried out in a total

[5] Grade A, from Calbiochem, Los Angeles.
[6] NEC-445 from New England Nuclear Corporation, Boston. One microcurie of the ^{14}C-amino acid mixture supplied the following: (in mμmoles) 0.67 L-alanine; 0.34 L-arginine; 0.5 L-aspartic acid; 0.5 glycine; 0.06 L-histidine; 0.21 L-isoleucine; 0.59 L-leucine; 0.62 L-glutamic acid; 0.26 L-lysine; 0.22 L-phenylalanine; 0.25 L-proline; 0.33 L-serine; 0.05 L-tyrosine; 0.41 L-valine; and 0.32 L-threonine. In addition 1 mμmole each of nonradioactive L-asparagine, L-glutamine, L-methionine, L-cysteine, and L-tryptophan were included in 1.0 ml of incubation mixture.

TABLE 2

Design of experiments 1 and 2 for the study of the effects of S. typhimurium *infection on muscle protein metabolism in rats*

Exp. no.	Group	No. rats/group	Dietary casein	Experimental infection	Food intake
			%		
1	18-C-1	20 [1]	18	absent	Pair-fed to 18-I-1
1	18-I-1	20	18	present	ad lib.
1	18-I-1b	20	18	present	ad lib.
2	5-I-2	80 [2]	5	present	ad lib.
2	18-I-2	80 [2]	18	present	Pair-fed to 5-I-2

[1] In addition a group of 4 rats was used as an initial control.
[2] In addition 10 rats were used as initial controls.

volume of 1 ml. The pH 5 enzyme fraction was prepared as described previously (10) from livers of normal rats purchased specifically for this purpose. Assay results were linear with concentration of ribosomes up to 0.25 mg ribosomal RNA. Further details and measurement of incorporation of radioactivity have been described (10).

For the in vivo measurement of isotope uptake into nascent peptides by muscle ribosomes, rats were given intraperitoneally 10 μCi/100 g body weight DL-leucine-1-^{14}C (specific activity 2.75 mCi/mole, in 0.1 ml physiological saline). A preliminary experiment established that peak specific activity of the ribosome fraction after leucine-^{14}C injection (cpm/mg ribosomal RNA) occurred at 3 hours and that a marked decline in the specific activity was observed within a further 3 hours. Therefore, 3 hours after isotope administration, rats were killed, and the muscles from both hind legs removed. Ribosomes were extracted by the method described previously (10).

Fresh muscle was prepared for analysis of free leucine, isoleucine and valine concentrations by the procedure of Tallan et al. (11) and subsequent ion-exchange chromatography using a Technicon amino acid analyzer, with norleucine as the internal standard. DNA and RNA were extracted by the method of Munro and Fleck (12). RNA was determined using yeast RNA (Sigma Type XI, purified) as a standard. For skeletal muscle, hydrolysis of DNA at 75° for 45 minutes was found to give maximal yields and reproducible values for DNA. The method of Giles and Meyers (13) was used for quantitation of DNA, and protein was determined by the method of Lowry et al. (14).

RESULTS

Food intake in the rats was reduced to a variable degree after infection. The greatest reduction was observed in experiment 2 during the second day after infection with mean intake being 28% of the pre-infection value. Subsequently mean food

TABLE 3

Cumulative numbers of rats dying of S. typhimurium *infection during the experimental period* [1]

Exp. no.	Group [2]	No. days after infection				
		1	2	3	5	7
1	18-C-1	0	0	0	0	0
1	18-I-1	0	0	0	1	3
1	18-I-1b	0	0	1	1	5
2	5-I-2	9	19	32	34	37
2	18-I-2	1	2	14	18	20

[1] In addition to those killed.
[2] Group descriptions are given in table 2.

TABLE 4

Effects of S. typhimurium *infection in rats fed 18% casein on the concentration of RNA, DNA and protein of hind leg skeletal muscle* [1] *(exp. 1)*

No. days after infection	Group 18-C-1 [2]					Group 18-I-1 [2]				
	DNA	RNA	RNA/DNA	Protein	Protein/DNA	DNA	RNA	RNA/DNA	Protein	Protein/DNA
	mg/g wet muscle					*mg/g wet muscle*				
0	0.352	1.68	4.77	121.3	344.6	0.356	1.71	4.80	122.4	343.8
1	0.342	1.58	4.62	118.5	346.4	0.342	1.30	3.80	110.1	329.1
2	0.321	1.56	4.86	117.5	366.0	0.399	1.28	3.21	101.2	253.6
3	0.341	1.54	4.52	117.0	343.1	0.369 [3]	1.41 [3]	3.82	110.3 [3]	298.9
5	0.345	1.56	4.52	119.0	344.9	0.375 [3]	1.54 [3]	4.10	112.1 [3]	298.9

[1] Mean values of duplicate analyses on a pooled sample from 4 rats unless specified.
[2] Group designations are given in table 2.
[3] Pooled sample from 3 rats.

TABLE 5

Effects of S. typhimurium *infection in rats fed 5% or 18% casein on the concentration of DNA and protein of hind leg skeletal muscle* [1] *(exp. 2)*

No. days after infection	Group 18-I-2 [2]					Group 5-I-2 [2]				
	DNA	RNA	RNA/DNA	Protein	Protein/DNA	DNA	RNA	RNA/DNA	Protein	Protein/DNA
	mg/g wet muscle					*mg/g wet muscle*				
0	0.310	1.72	5.54	116.6	376.1	0.328	1.64	5.00	116.7	355.8
1	0.386	1.60	4.13	119.8	310.4	0.294	1.25	4.25	115.8	393.8
2	0.389	1.61	4.13	118.9	305.6	0.303	1.31	4.32	111.8	368.9
3	0.326	1.87	5.73	115.4	353.9	0.323	1.35	4.18	101.3	313.6
7	0.385	1.81	4.70	105.3	273.5	0.324 [3]	1.22 [3]	3.76	104.5 [3]	322.5

[1] Mean values of duplicate analyses on a pooled sample from 5 rats unless specified.
[2] Group designations are given in table 2.
[3] Pooled sample from 3 rats.

intake increased to 55 to 80% of pre-infection values.

The deaths due to infection which occurred during these 2 experiments are summarized in table 3. Changes in the gross chemical composition of muscle following infection during the 2 experiments are summarized in tables 4 and 5. Table 4 shows that muscle RNA concentration (mg/g wet muscle) and the RNA/DNA ratio decreased after infection. On the second post-infection day (exp. 1), the RNA/DNA ratio was only 65% of that in noninfected rats. However, the RNA/DNA ratio tended to increase on the third day and approached normal values on the fifth day of infection. Similar results were obtained during the second experiment for changes of the RNA/DNA ratio in rats given the 18% casein diet (table 5) except that a normal value was found for the group killed after 3 days of infection. The small increase in DNA per gram of wet tissue observed for infected rats fed the adequate-protein diet is presumably related to a reduction in the concentration of various cellular constituents. For rats given the 5% casein diet (table 5), the RNA/DNA ratio decreased from a pre-infection value of 5.00 to 3.76 by the seventh day.

The protein/DNA ratio decreased in rats fed the 18% casein diet following *S. typhimurium* infection in both experiments, reflecting a depletion of cellular protein in this tissue. In rats fed the 5% casein diet, the protein/DNA ratio appeared to decrease after the first 2 days of infection (table 5), suggesting that cellular protein depletion may have occurred later in this dietary group.

Of the branched-chain amino acids, leucine and isoleucine changed in a parallel fashion in the rats fed 18% casein (table 6). The authors observed significantly lowered values during the first 2 days post-infection in rats given the 5% casein diet and about normal values on the third day. For rats given the 18% casein diet, the levels of leucine and isoleucine appeared to increase on the first day and decline thereafter.

Infection reduced the in vivo incorporation of radioactivity from ^{14}C-leucine into nascent peptides on muscle ribosomes as shown in figure 1. During days 1, 2, and 3 after infection the incorporation shown by infected rats was 55, 20 and 41%, respectively, of the noninfected control lev-

TABLE 6

Concentration of the free branched-chain amino acids in pooled muscle following S. typhimurium *infection in rats (exp. 2)*

No. days after infection	Amino acid		
	Leucine	Isoleucine	Valine
	μmoles/kg fresh muscle		
	5% casein diet		
0	100 [1]	46	100
1	35	21	36
2	29	20	35
3	125	71	120
	18% casein diet		
0	100	67	155
1	146	100	116
2	99	68	109
3	80	51	92

[1] Pooled muscle was obtained from 5 rats in each group.

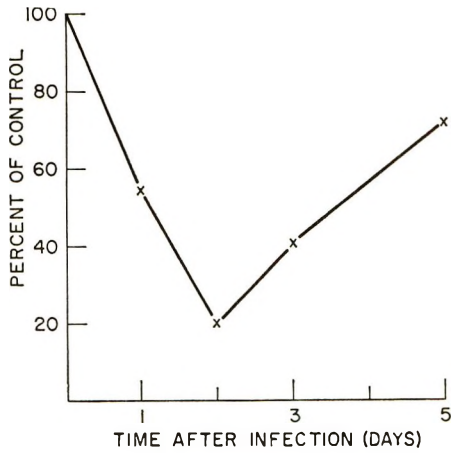

Fig. 1 Effect of *S. typhimurium* infection on muscle protein synthesis in vivo of rats fed 18% casein, as measured by incorporation of ^{14}C-leucine into nascent peptides of muscle ribosomes. Control values (dpm incorporated/mg ribosomal RNA) obtained with pair-fed non-infected rats were assigned a rating of 100%. Each point shown was obtained with a pooled muscle sample from 4 rats (exp. 1).

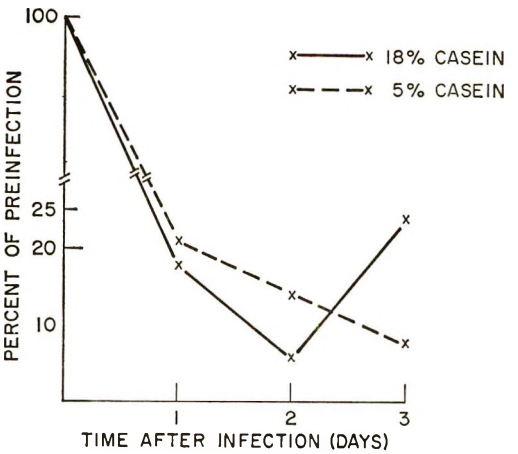

Fig. 2 Effect of *S. typhimurium* infection on muscle protein synthesis in vivo of rats fed 5% and 18% casein diets, as measured by incorporation of ^{14}C-leucine into nascent peptides of muscle ribosomes. Pre-infection values (dpm incorporated/mg ribosomal RNA) were assigned a rating of 100%. Each point represents the results obtained from a pooled muscle sample from 5 rats (exp. 2).

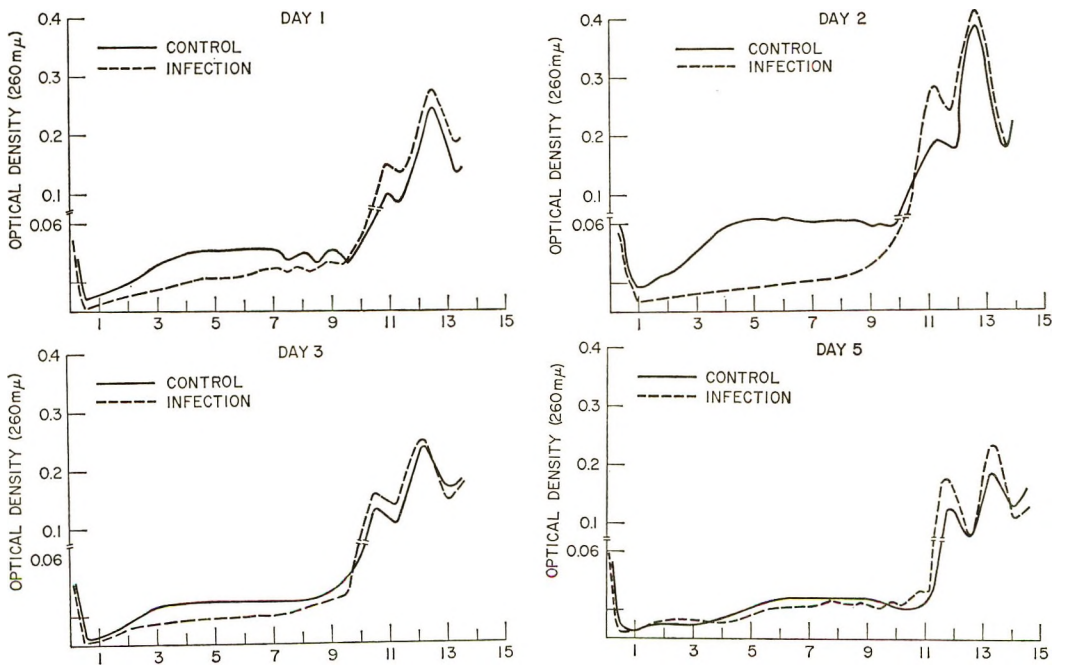

Fig. 3 Effect of *S. typhimurium* infection on the sedimentation of rat skeletal muscle ribosomes at 1, 2, 3, and 5 days after infection. A detergent-treated post-mitochondrial supernatant (usually 0.7 ml) was layered over a linear (15–40%) sucrose gradient prepared in 0.25 M KCl, 0.01 M $MgCl_2$ and 0.01 M tris-HCl (pH 7.6)). The gradients were centrifuged at 25,000 rpm in a Spinco SW 25.1 rotor for 2 hours. Each curve represents the profile obtained from 0.4 g initial muscle equivalent prepared from a pooled muscle sample from 4 rats. The control profiles were obtained with pair-fed non-infected rats (exp. 1). The gradient profile was monitored at 260 mμ and recorded automatically using a flow-cell with a 2-mm light path and a Gilford absorbance recorder. Further details are given in the text.

els. On the fifth day after infection, incorporation was about 70% of the control value, suggesting a return toward normal protein synthetic activity in this tissue. A similar response pattern was obtained in the second experiment for infected rats fed the 18% casein diet (fig. 2). For rats given the 5% casein diet, incorporation was also markedly reduced during the first 3 days of study after infection. However, for this group there was no apparent trend of a return toward pre-infection values during the initial 3 days after infection.

Sucrose gradient analysis of muscle ribosomes was undertaken during experiment 1 to elucidate further the biochemical lesion which might be associated with the decrease in muscle protein synthesis following infection. These results are shown in figure 3. The absorbance profile for ribosomes isolated from muscle one day after infection is characterized by a lower proportion of the heavy ribosome aggregates and a higher proportion of the light ribosome species compared with pair-fed noninfected controls. A similar but more dramatic change in the profile is apparent for the infected rats on the second day of the study. By the third day, the difference in the absorbance profiles of muscle ribosomes between infected and noninfected rats was less marked and on the fifth day, the profiles from both groups were similar.

These findings correlate well with the in vivo uptake of ^{14}C-leucine shown in figure 1 and suggest that the decreased uptake of labeled leucine observed in vivo may be the consequence of a decreased level of polyribosomes in the muscle cell. However, the decreased uptake during the first 3 days after infection appeared to be greater than the decrease in the level of polyribosomes as shown by sucrose gradient analysis.

For this reason the in vitro amino acid-incorporating ability of muscle ribosomes was studied in the second experiment. These results are shown in figure 4. In the infected rats given the 5% casein diet, a rapid decrease in the in vitro activity of the ribosomes occurred during the first 3 days and this decrease appeared to be maintained throughout the entire 7 days of observation. In the adequately fed rats,

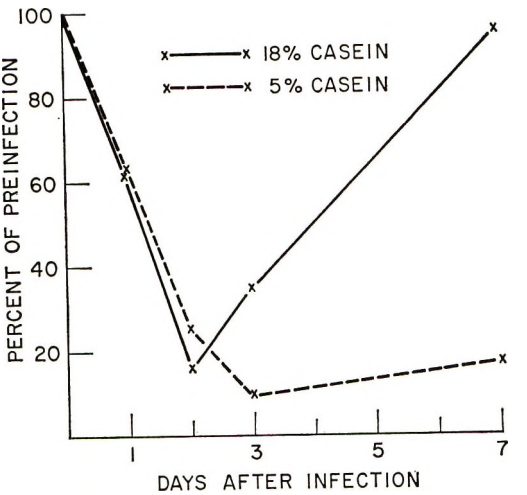

Fig. 4 Effect of *S. typhimurium* infection on the in vitro protein-synthesizing capacity of muscle ribosomes from rats fed either 5% or 18% casein diets. Pre-infection values (dpm incorporated/mg ribosomal RNA) were assigned a rating of 100%. Ribosomes were prepared, as described in the text, from a pooled muscle sample from 5 rats at each time-period except for the 7-day point in the 5% casein group which was from 3 rats. The incubation system contained 0.1 mg ribosomal RNA and the values represent the mean of duplicate determinations.

however, infection reduced the in vitro synthetic capacity of muscle ribosomes during the first 2 days and then the activity appeared to return to pre-infection values before the seventh day. A comparison of the results shown in figures 2 and 4 suggests a close correlation between the results obtained for the in vitro and in vivo uptake of labeled amino acid into nascent peptides by muscle ribosomes under the present experimental conditions.

DISCUSSION

The loss of body protein during the reaction to infection is accompanied by an increased excretion of potassium and creatinine (5). Physical injury and other causes of stress have been shown to cause a similar metabolic loss (15, 16). Fleck and Munro (17) have suggested that in physical injury the increased urinary nitrogen comes from the carcass and probably muscle. The results of Beisel (5) suggest that the increased urinary nitrogen loss following *Pasteurella tularensis* infec-

tion in man may also derive from the muscle of the body.

Few reports have appeared of specific alterations in tissue protein synthesis of the host during infection and the influence of previous protein nutrition on these changes. The information available suggests that protein synthesis is increased in the liver during infection (7–10). The results of Lust (7) suggest, however, that muscle protein synthesis is depressed. The present findings demonstrate that protein synthesis by ribosomes is reduced in muscle following infection of rats with *S. typhimurium*. They support previous findings which show a decreased in vivo uptake of ^{14}C-leucine into a muscle microsomal fraction prepared from mice infected with *Diplococcus pneumoniae* (7).

The agreement between the present in vitro and in vivo studies strengthens the view that the synthetic activity of ribosomes in muscle is reduced following infection, which presumably leads to a reduced rate of muscle protein synthesis. The decreased concentration of muscle protein following infection may, therefore, be a consequence of changes in the protein synthetic capacity of the ribosome population in this tissue. These changes, as well as the decrease in muscle protein content, suggest that a diversion of amino acids from muscle into the circulation probably occurs, enabling the liver and perhaps other visceral organs to maintain an enhanced level of protein synthesis during the course of infection.

The mechanism(s) responsible for the decrease in the level of muscle polyribosome following infection is not known. Glucocorticoids have been implicated in the stress response to infection, and we have observed a decreased level of muscle polyribosomes following hydrocortisone administration in rats (18). However, Beisel and co-workers (5, 19) have suggested that increased activity of the adrenal cortex is too brief and small to account for the catabolic nitrogen response which follows infection.

The nature of the change in the synthetic capacity of the ribosomes following infection is also not known. Recently, Decken (20) has shown that the in vitro synthetic capacity of hepatic polyribosomes is influenced by previous nutritional conditions. He suggests that this variation is not due to differences in the messenger RNA content of the system. Also Sox and Hoagland (21) have suggested that the intrinsic activity of hepatic polyribosomes is changed during starvation and refeeding and have considered the possibility that polysome activity in protein synthesis may be influenced by regulatory mechanisms residing in the polysome or neighboring structures. However, the present experiments do not provide an explanation for the observed differences in ribosome activity following infection.

Stirewalt et al. (22) have pointed out that the function of ribosome proteins is not well-understood, and it is possible that these play regulatory roles and are influenced by hormones. Therefore, the cellular changes produced by infection may also exert an effect on the function of ribosome proteins. The present studies were designed to evaluate the host-metabolic response to infection and hence do not establish whether infection exerted a direct or indirect effect on muscle ribosomes. This effect would be difficult to establish in the intact animal.

In the present experiments infected rats given the low protein diet died earlier than infected rats given an adequate, 18% casein diet. However, no clear-cut differences in the biochemical variables studied for assessing muscle protein metabolism were detectable in these dietary groups during the first 3 days after infection. This may be due to the fact that the present studies were limited to a moderate-to-severe infection; further extensive investigations will be required in order to determine whether the changes in muscle protein synthesis following infection are dependent upon both prior level of protein nutrition and the intensity of infection.

The present findings indicate that muscle protein synthesis is reduced following infection and therefore provides a partial explanation of the catabolic nitrogen loss as a constant reaction to infection (4–6). We have concentrated on only one aspect of muscle protein synthesis; other steps need to be examined during the stress re-

sponse in infection. Furthermore, Levenson and Watkin (24) have suggested that increased catabolism occurs in the carcass of the injured rat, and possibly the catabolic aspect of muscle protein metabolism plays an important role in the metabolic nitrogen loss during an infection. Therefore, this aspect of muscle metabolism should receive detailed study in an attempt to provide a clear understanding of the changes in the gross chemical composition of muscle and impoverishment of the cells in nitrogen which accompany an infection.

ACKNOWLEDGMENT

The authors acknowledge the assistance of Mrs. Jane Macdonald.

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Effects of Cupric Ion and Digitonin in vitro on Erythrocytes and Liver Mitochondria from Essential Fatty Acid-deficient Rats

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ABSTRACT Liver mitochondria and erythrocytes were isolated from essential fatty acid-deficient rats and the effect of cupric ion and digitonin on swelling and lysis was investigated. Both membranes were more resistant to the action of cupric ion than those isolated from rats fed a corn oil diet. In the presence of digitonin, deficient mitochondria swelled more rapidly and to a greater extent than the normal mitochondria. There appeared to be no difference in the susceptibility of normal and deficient erythrocytes to digitonin-induced lysis.

Earlier work in this laboratory (1) and elsewhere (2, 3) suggested that an essential fatty acid deficiency in rats is accompanied by an alteration of mitochondrial membranes. This has been presumed due, in part at least, to changes in fatty acid moieties of the phospholipid (4). In the present investigation the nature of the changes associated with the deficiency has been examined further by comparing normal and essential fatty acid-deficient rats with respect to cupric ion and digitonin effects, in vitro, on mitochondria and erythrocytes.

Erythrocytes were included in the present study since it has been recognized for some time that significant species differences exist in the behavior of erythrocyte membranes toward various lipid-soluble substances. A positive correlation has been noted between the decreases in palmitic acid-oleic ratios (5), lecithin-sphingomyelin ratios (6), and the decrease in permeability of various mammalian erythrocytes toward penetrating nonelectrolytes such as glycerol and urea (7).

Several workers (8–10) have shown that changes in the fatty acid pattern of the rat erythrocytes can be induced by the type and quantity of fat in the diet. The red cells of rats fed fat-free or hydrogenated coconut oil diets exhibit, as readily and positively as does the liver tissue, the fatty acid pattern characteristic of essential fatty acid (EFA) deficiency.

Work in a number of laboratories (11, 12) has suggested that the differences in membrane permeability in the presence of cupric ion might be due primarily to alteration of the fatty acid pattern of the phospholipids.

EXPERIMENTAL METHOD

Male albino rats (Holtzman strain) were weaned at 18 days and divided at random into 2 groups. The normal groups received a diet consisting of: (in %) corn oil, 5; dextrose, 53; "vitamin-free" casein, 21; cellulose, 16; salt mix, USP XIV, 4; and vitamin fortification mixture,[2] 1. The EFA-deficient group received the same diet in which hydrogenated coconut oil [3] replaced the corn oil. Food and water were presented ad libitum and the animals were used from the second to the tenth month on the regimen.

Mitochondrial swelling studies. Rat liver mitochondria were prepared from normal and EFA-deficient rats as described by Ito and Johnson (13) and suspended in 0.3 M D-mannitol-0.001 M tris buffer, pH 7.4.

Received for publication June 30, 1967.

[1] L. E. Trojan is the recipient of an Institute of Nutrition and Food Technology predoctoral traineeship. Supported in part by National Institutes of Health, U.S. Public Health Service, Training Grant no. 5 T1 ES-17, and U.S. Public Health Service Grant no. HE-05473.

[2] Contained: (in g/kg vitamin mix) vitamin A conc (200,000 USP units/g), 9.93; vitamin D conc (400,000 USP units/g), 0.55; α-tocopherol, 11.05; ascorbic acid, 99.3; *i*-inositol, 11.05; choline chloride, 165.6; menadione, 4.96; *p*-aminobenzoic acid, 11.05; niacin, 9.93; riboflavin, 2.2; pyridoxine·HCl, 2.2; thiamine·HCl, 2.2; Ca pantothenate, 6.63; biotin, 0.044; folic acid, 0.198; vitamin B_{12} (with mannitol 0.1%) 2.98; (Vitamin Diet Fortification Mixture in Dextrose, Nutritional Biochemicals Corporation, Cleveland).

[3] Hydrol, Durkee Famous Foods, Cleveland.

The volumes were adjusted to give approximately equal initial light absorbance at 520 mμ. Appropriate aliquots of these suspensions were added to 3.0 ml (final volume) of 0.154 M KCl-0.02 M tris buffer, pH 7.4. Dilute solutions of $CuSO_4$ and recrystallized (14) digitonin (Fisher certified reagent) were prepared before use and added to the test systems in amounts of 2 to 20 μliters. Swelling of the mitochondria was followed by measuring the change in light absorption at 520 mμ, using a Cary Model 14 recording spectrophotometer (1).

Erythrocyte lysis studies. Blood was obtained from a tail cut, shaken gently with glass beads, and filtered through gauze. The defibrinated blood was diluted with 9 volumes of 0.90% NaCl and centrifuged for 5 minutes in an International clinical centrifuge.

The supernatant and buffy coat were removed by gentle suction and the erythrocytes were washed twice with isotonic saline. A 1:120 dilution of red blood cells in 0.90% NaCl was used routinely.

Hypotonic lysis studies were performed by adding 1.0 ml of the dilute red cell suspension to 9.0 ml of NaCl solution (final concentrations of NaCl ranged from 0.09 to 0.90%). For the cupric ion and digitonin studies the lytic agent was added to 9.0 ml of isotonic saline and the reaction was started by the addition of the dilute red cell suspension.

The samples were incubated for one hour, centrifuged, and aliquots of the supernatant fluid were transferred to colorimeter tubes. The light transmittance was measured at 415 mμ in a Coleman Jr. spectrophotometer. The supernatant from erythrocytes incubated in 0.90% NaCl was used as the blank and designated as 0% hemolysis, and that from the red cells incubated in 0.09% NaCl was set equal to 100% hemolysis.

All reagents used throughout this study were prepared with demineralized, Pyrex-distilled water.

RESULTS

Mitochondrial swelling studies

When EFA-deficient rat liver mitochondria were isolated in a D-mannitol medium the rate and extent of spontaneous swelling were essentially the same as that of the normal ones. However, when the mitochondria were added to the 0.154 M KCl-0.02 M tris medium containing cupric ion, differences were observed between the 2 types of mitochondria. Although both preparations swelled rapidly when cupric ion was present in the incubation media, the swelling rate of the normal mitochondria was greater than that observed in the EFA-deficient ones at all concentrations of Cu^{2+} tested (fig. 1).

To permit a further comparison of these differences, the net change in optical density at a given time (t), in the presence of copper, was calculated according to the following:

$$\%\ Cu^{2+}\text{-induced swelling} = \frac{[OD_t\ (\text{in absence of } Cu^{2+}) - OD_t\ (\text{in presence of } Cu^{2+})]}{OD_t\ (\text{in absence of } Cu^{2+})}$$

Figure 2 illustrates the time required to attain an optical density decrease of 50% in the presence of increasing concentrations of copper ion. At each concentration of Cu^{2+}, normal mitochondria required less time for a 50% decrease in absorbancy than the EFA-deficient mitochondria. A 50% decrease in absorbency was not observed with EFA-deficient mitochondria over the 20-minute swelling period when 1 μg of Cu^{2+} was present in the media. Normal mitochondria, however, exhibited this degree of swelling in about 12 minutes. The time required for a net 50% reduction in optical density decreased rapidly until the cupric ion concentration in the incubation mixture reached about 4 μg/3 ml. The swelling rates of neither preparation changed significantly when the cupric ion was increased from 4 to 10 μg. This possibly reflects the loading of immediately available electronegative sites on the mitochondrial membrane.

It had been shown previously (1) that ATP could reverse markedly the spontaneous swelling of EFA-deficient mitochondria that had been prepared in sucrose, whereas its only action on normal mitochondria was to inhibit further swelling. Addition of ATP [4] either 5 or 10 minutes after the initiation of copper-induced swelling protected

[4] Sigma Chemical Company, St. Louis.

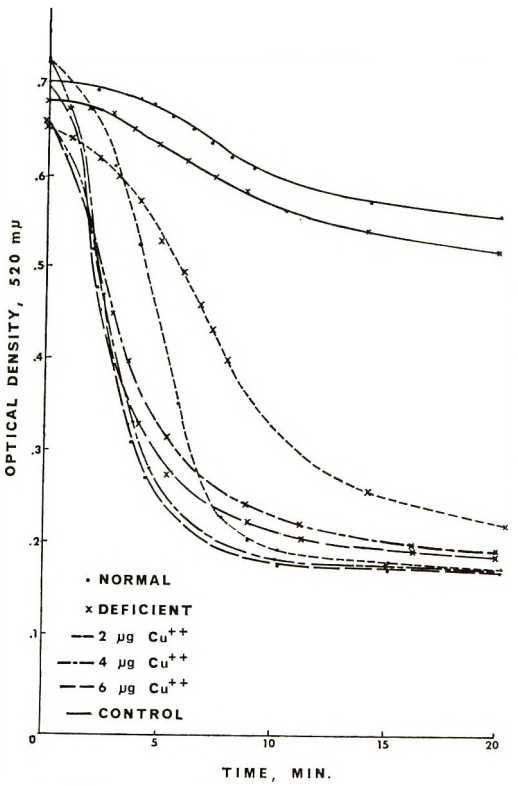

Fig. 1 Copper-induced swelling of rat liver mitochondria. Mitochondria were added to 0.154 M KCl–0.02 M tris buffer, pH 7.4 containing 0–6 μg cupric ion/3 ml. The course of swelling was followed at 520 mμ at room temperature.

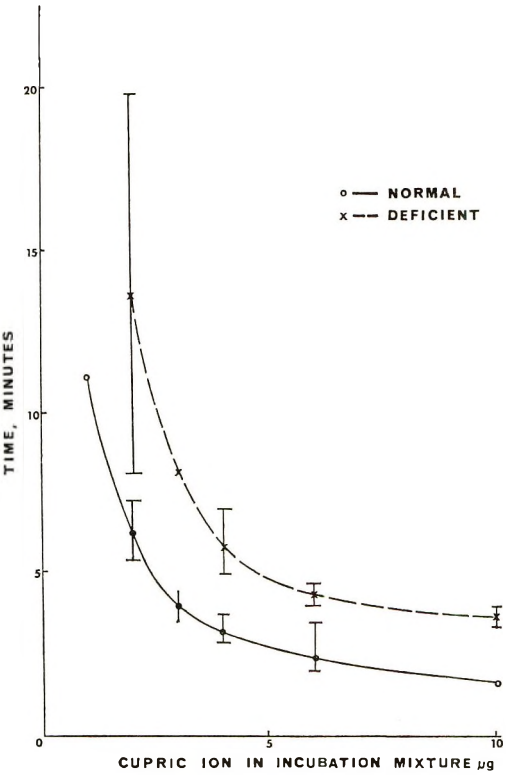

Fig. 2 Time required for 50% decrease in absorbancy by mitochondria swollen in the presence of copper. The vertical bars represent the range of values obtained at a given concentration of cupric ion. Abscissa values are μg/3.0 ml.

only those mitochondrial suspensions in which the optical density had not decreased to a value less than 50% that of the original.

ATP did not reverse the swelling process in either preparation. However, Cu^{2+}-induced swelling was partially inhibited when normal or EFA-deficient mitochondria were suspended in KCl-tris-containing ATP (fig. 3). ATP afforded greater protection to the EFA-deficient tissues at all concentrations of cupric ion tested (compare figs. 1 and 3).

Preliminary studies in our laboratory indicate normal mitochondria to be more susceptible to cupric ion titration than EFA-deficient mitochondria, when respiratory control, as measured with oxygen electrode, is used as a criterion of comparison. In normal mitochondria, the respiratory control was half-maximally inhibited (13) at a concentration of 2.2 μg Cu^{2+}/mg mitochondrial protein with succinate, and at 2.6 μg Cu^{2+} with α-hydroxybutyrate as the electron donor. Half-maximal respiratory control occurred in the EFA-deficient preparations at a concentration of 5.3 μg Cu^{2+}/mg mitochondrial protein with succinate as substrate and at a level of 4.6 μg Cu^{2+}, with α-hydroxybutyrate as substrate.

When digitonin was used to induce swelling, the EFA-deficient mitochondria swelled more rapidly and to a greater extent than the normal mitochondria. Figure 4 illustrates the effects of 0.1 and 0.2 mg of digitonin on mitochondrial swelling. After 5 minutes the action of 0.1 mg of digitonin/3.0 ml, had resulted in a net optical density decrease of 14% in the normal and 32% in the EFA-deficient mitochondria; 0.2 mg of digitonin enhanced swelling,

[5] Unpublished data, L. E. Trojan and R. M. Johnson.

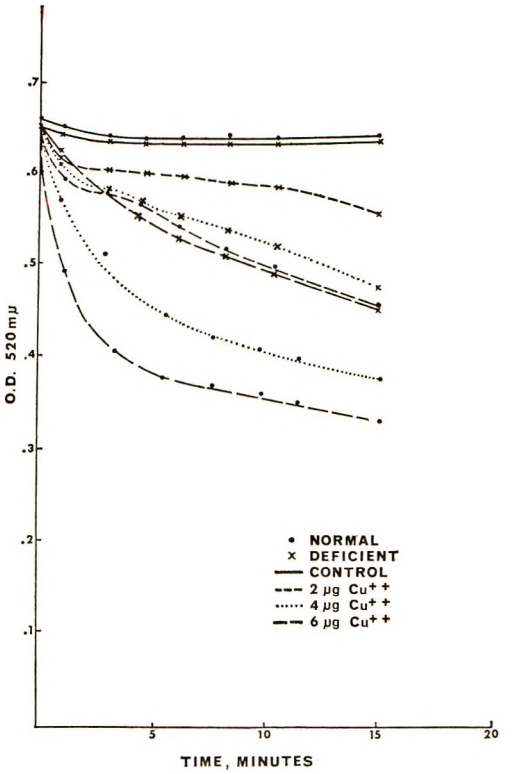

Fig. 3 Effect of ATP on copper-induced swelling of mitochondria. Mitochondria were added to 0.154 M KCl–0.02 tris buffer, pH 7.4, containing 0.01 M ATP, and 0–6 μg cupric ion/3.0 ml. The course of swelling was followed at 520 mμ at room temperature.

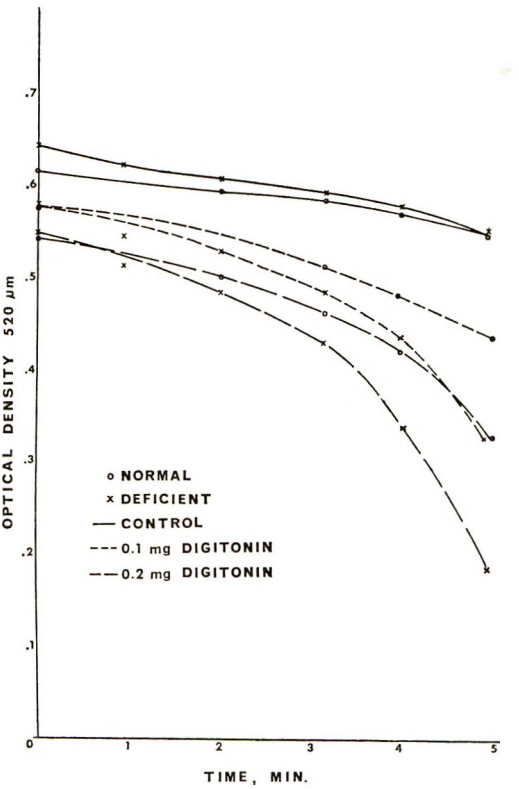

Fig. 4 Digitonin-induced swelling of rat liver mitochondria. Mitochondria were added to 0.154 M KCl–0.02 M tris buffer, pH 7.4, containing 0–0.2 mg digitonin in 3.0 ml. The course of swelling was followed at 520 mμ at room temperature.

over that of the control values, by 29% in the normal and 52% in the EFA-deficient mitochondria. The twofold, or greater, swelling of the EFA-deficient mitochondria could be observed with a digitonin concentration as high as approximately 0.5 mg/3.0 ml. Above this concentration, mitochondrial lysis occurred so rapidly that differences between the preparations were obliterated.

Erythrocyte lysis studies

When EFA-deficient erythrocytes were suspended in saline solutions of decreasing tonicity, they were significantly more susceptible to osmotic change than the normal red cell (fig. 5). Fifty percent hemolysis occurred at a saline concentration of 0.515% ± 0.032 in the EFA-deficient red blood cells, as compared with 0.470% ± 0.008 for the normals ($P < 0.05$). These results confirm the report of a highly significant increase in the fragility of fat-deficient red cells in 0.50% saline (8).

Preliminary experiments with cupric ion indicated that no appreciable hemolysis occurred at 25° for time-periods up to 3 hours. Hemolysis was noted when the erythrocytes were incubated at 37° for 1 hour. Figure 6 describes the results of copper lysis studies on erythrocytes from 12 normal and 10 EFA-deficient rats. Lysis was observed in normal red cells at approximately 4 μg Cu^{2+}/10 ml, whereas hemolysis was not noted in the deficient suspension until the Cu^{2+} concentration was between 6 and 7 μg/10 ml. Fifty percent hemolysis occurred at 8.0 μg cupric ion (range 6.3–11.1μg) for the normal cells

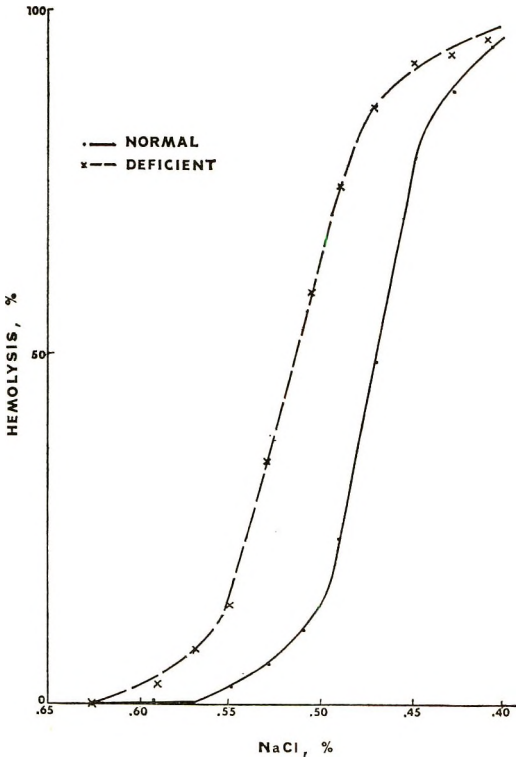

Fig. 5 Hypotonic lysis of erythrocytes. The erythrocytes were added to saline solution of varying tonicity and incubated for 1 hour at 20°. Each curve represents the mean hemolysis value obtained with the red blood cells from 6 rats.

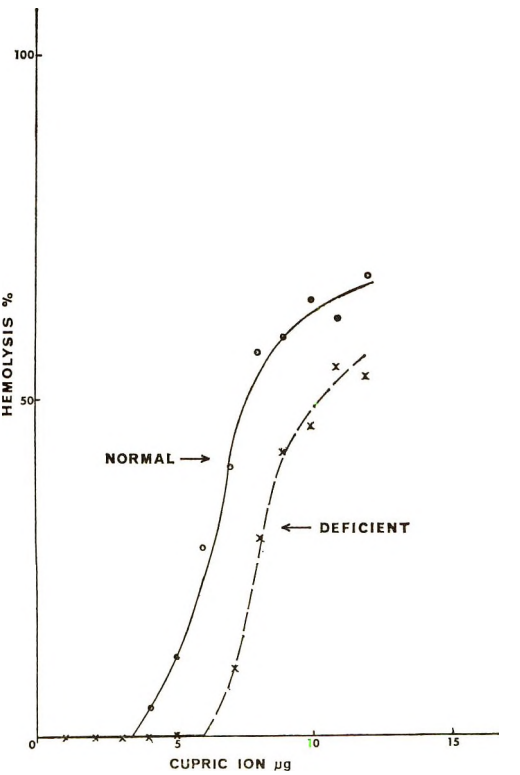

Fig. 6 Copper-induced lysis of erythrocytes. The erythrocytes were added to 0.90% NaCl containing varying amounts of cupric ion in 10 ml, and incubated for 1 hour at 37°. The curves represent the mean hemolysis values obtained with the erythrocytes from 12 normal and 10 EFA-deficient rats.

and 10.6 μg Cu^{2+} (range 8.0–14.7 μg) for the deficient ones. The values obtained did not lend themselves to statistical analysis due to wide daily variations. To eliminate the possibility that the very dilute working solution of $CuSO_4$ changed concentration on standing, the order of testing the red cells was alternated daily. The results were always the same, qualitatively; the EFA-deficient erythrocytes were more resistant to disruption by cupric ion than the normal ones.

The lytic effect of digitonin was tested in the range of 1 to 12 μg/10 ml isotonic saline suspension of red blood cells. Erythrocytes from 16 normal and 16 EFA-deficient rats were used in these studies. A typical experiment is illustrated in figure 7. Contrary to the differences noted when mitochondria were swollen in the presence of digitonin, the curves describing lysis of both normal and EFA-deficient erythrocytes were essentially identical. The average digitonin concentration at which 50% hemolysis occurred was 8.9 μg (range 7.2–10.6 μg)/10 ml of red blood cell suspension for the normal; and 9.2 μg (range 7.9–10.8 μg) for the deficient red blood cells.

An attempt was made to establish a difference in the rates at which the 2 types of red cells were lysed by digitonin. One milliliter of the dilute red blood cell suspension was added to 9.0 ml of isotonic saline containing 9 μg of digitonin—the mean concentration at which 50% lysis occurred. The tubes were mixed rapidly, incubated at 22° for zero to 30 minutes, and samples were centrifuged at 2-minute intervals. Fifty percent lysis was achieved within 5 minutes and no differences in the rate of

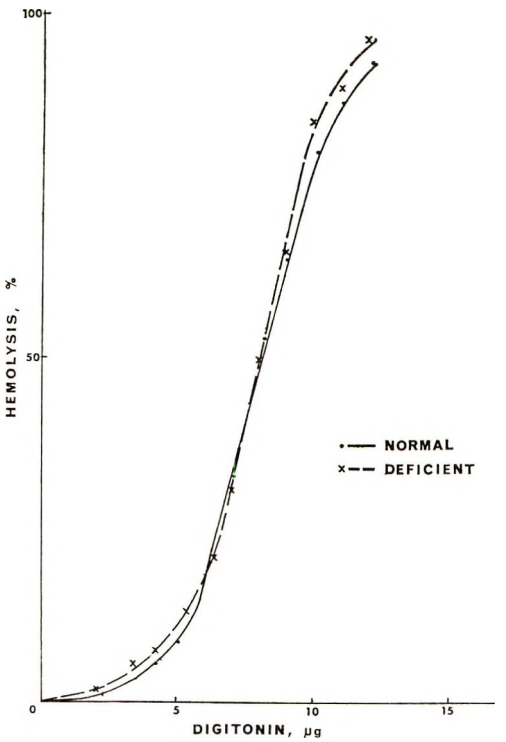

Fig. 7 Digitonin-induced lysis of erythrocytes. The erythrocytes were added to 0.90% NaCl containing varying amounts of digitonin in 10 ml and incubated for 1 hour at 22°.

lysis were observed between normal and EFA-deficient erythrocytes.

DISCUSSION

Earlier work suggested that deficient mitochondria possessed relatively unstable membrane structures, and that EFA-deficient erythrocytes are less resistant to osmotic change than normal ones (1, 8, 13). Therefore, the observation was unexpected that both mitochondria and the erythrocytes from deficient animals were more resistant to Cu^{2+}-induced swelling and lysis than the normal preparations.

To explain this apparent anomaly, it may be recalled that Lehninger (15) suggested that metal ions induce swelling by combining with mitochondrial sulfhydryl groups. The change in membrane permeability was presumably due to a disulfide-thiol reaction, followed by alterations in the tertiary or quaternary structures of membrane proteins. If the phospholipid fatty acid changes in an EFA deficiency result in an altered reaction between lamellar phospholipid and membrane protein, such alterations could lead to a relatively decreased accessibility of protein sulfhydryl groups to metal ions, that is, Cu^{2+}. Under these circumstances membranes of EFA-deficient animals might be expected to be more resistant to Cu^{2+}-induced swelling and lysis than normal ones, and the present observations need not be viewed as contradictory to earlier studies suggesting an increased labile membrane in EFA-deficient mitochondria.

The respiratory control of EFA-deficient mitochondria has been demonstrated in this laboratory to be more labile than that of normal mitochondria, in the presence of swelling agents such as digitonin or oligomycin (13), presumably because of easily altered respiratory chain-linked reactions. The action of digitonin is presumed to be primarily on lipid (lipoprotein) structures, and it was assumed, from the work cited above, that these structures in EFA-deficient mitochondria are more susceptible to the action of digitonin. The fact that, in the present study, the EFA-deficient mitochondria were more resistant to Cu^{2+} than normal ones, suggests that a site of action was involved which was not linked to respiratory chain-linked reactions in a way that either digitonin or oligomycin were. Nevertheless, it seems pertinent that the levels of Cu^{2+} which inhibited respiratory control of the normal versus the EFA-deficient mitochondria are in approximately the same proportion as those of digitonin that produced similar swelling rates in the 2 preparations. This might suggest that, in fact, the action of Cu^{2+} is respiration-linked, and that elucidation of the mechanism of its action may reveal vital information concerning a biochemical aberration occurring in EFA deficiency.

The effects of ATP described here are reminiscent of those observed in earlier experiments (1), in which sucrose was studied as a swelling agent. If, as has been presumed (16, 17), some of the effects of sucrose on swelling result from an effect on the coupling mechanism of oxidation to phosphorylation which controls con-

tractibility of the mitochondrial membrane, a similar explanation may exist for the action of Cu^{2+}. Obviously this is a tenuous conclusion, and is of value only in suggesting additional experimentation. However, the comparison with earlier experiments, indicated above, appears to provide some substantiation for the suggestion that the action of Cu^{2+} is respiration-linked.

Studies with lipid bilayers (18) suggested that the mode of action of saponifying agents could be explained as a modification of the lamellar membrane structure to that of localized "micellar" arrangements. If one assumes that the phospholipid molecules of the EFA-deficient membrane are interacting to a lesser degree due to lack of the structural stability afforded by arachidonic acid (19), then the phase transition to a micellar form, due to the presence of digitonin, would require less energy. Thus, the EFA-deficient membrane would be expected to be more susceptible to this agent than the normal membrane. In a study to be reported it was found that EFA-deficient mitochondria react differently than normal tissues to fragmentation by digitonin.[6]

The lack of differentiation between the normal and EFA-deficient red blood cells to digitonin hemolysis would not necessarily invalidate the above premise. Measurements of the course of swelling or of respiratory control changes are more sensitive than those of hemolysis, and more discerning methods of investigation may reveal a difference in erythrocyte sensitivity.

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[6] See footnote 5.

Effects of Dietary Fat Level on Pantothenate Depletion and Liver Fatty Acid Composition in the Rat [1]

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ABSTRACT Weanling male rats were fed a 6% or an 18% cottonseed oil diet, lacking pantothenate or supplemented with graded levels of pantothenate, to determine whether a high fat diet would accelerate the development of pantothenate deficiency or increase the pantothenate requirement for growth. The fatty acid composition of liver lipid fractions was also determined. Increasing the level of fat did not increase the pantothenate requirement for maximal growth or accelerate the development of the deficiency as indicated by body weight, adrenal weight, or onset and incidence of greying. Pantothenate deficiency with either the 6% fat or the 18% fat diet significantly lowered the relative proportions of phospholipid arachidonate and stearate. Liver CoA values in both the control and deficient groups fed the 18% fat diet were significantly lower than in the rats fed the 6% fat diet.

The accepted pantothenate requirement for growth of young rats is based on experiments with diets containing 5% corn oil (1, 2). Recent evidence has indicated, however, that a high level of dietary fat may accelerate the development of pantothenate deficiency and increase the pantothenate requirement for growth. Sewell et al.[2] reported that pantothenate deficiency developed more rapidly in pigs fed a 12% fat diet than in those fed a 2% fat diet. In addition, the pantothenate requirement for growth was higher with the 12% fat diet. Myszkowska (3) reported that symptoms of pantothenate deficiency appeared more rapidly in rats fed a 12% fat diet than in those fed a 6% fat diet, in contrast with an earlier report by Carter and Hockaday (4). Hatano et al.[3] suggested that increased dependence on fat for energy may alter pantothenate metabolism in rats.

The present study was conducted to compare in rats the effects of a high fat (18%) and a low fat (6%) diet on the following: (a) the development of pantothenate deficiency, (b) liver CoA, and (c) the growth response to graded levels of pantothenate. In addition, the fatty acid composition of liver lipid fractions was measured to determine whether the level of dietary fat might have some effect on the changes reported by Fidanza et al. (5, 6) in liver fatty acids of pantothenate-deficient rats fed a 10% corn oil diet. These changes included a decrease in the relative proportions of arachidonate and stearate and an increase in the proportions of palmitate and oleate in total liver fatty acids, in comparison with either ad libitum-fed or pair-fed controls.

MATERIALS AND METHODS

Male rats of the Long-Evans strain [4] were caged individually in suspended, wire-bottom, galvanized cages. They were fed ad libitum a casein-sucrose diet containing 6% or 18% cottonseed oil,[5] with-

Received for publication October 16, 1967.

[1] Supported in part by Public Health Service Research Grant no. AM-7753 from the National Institute of Arthritis and Metabolic Diseases, and the U. S. Department of Agriculture (RRF Project W-57).

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[4] Obtained from Simonsen Farms, Gilroy, California.

[5] Composition of the diets: 6% fat diet: (g/100 g diet) casein (vitamin-free), 20.0; cottonseed oil, 5.0; salts (UCB-1Rb), 3.5; choline bitartrate, 0.18; B vitamin premix in sucrose, 2.0; vitamin A, D, E, mix in cottonseed oil, 1.0; and powdered sucrose, 68.32. The 18% fat diet contained (g/100 g diet) casein (vitamin-free), 22.6; cottonseed oil, 17.0; salts, 3.94; choline bitartrate, 0.22; B vitamin premix in sucrose, 2.26; vitamin A, D, E, mix in cottonseed oil, 1.13; and sucrose 52.85.

The B-vitamin premix, when fed as 2% of the diet, provided the following levels of vitamins: (μg/g diet) thiamine·HCl, 10; riboflavin, 20; niacinamide, 120; pyridoxine·HCl, 20; folic acid, 4; biotin, 2; vitamin B_{12}, 0.04; menadione, 0.8; Ca D-pantothenate, as indicated.

The vitamin *A, D, E* mix provided, when fed as 1% of the diet, the following levels of vitamins per g diet: vitamin A acetate (crystalline), 10 IU; vitamin D_3 (crystalline), 1 IU; and *dl*-α-tocopheryl acetate, 0.22 IU.

Salts *UCB-1Rb* provided the following, when fed as 3.5% of the diet: (g/100 g diet) $CaCO_3$, 0.725; $CaHPO_4$, 1.13; Na_2HPO_4, 0.651; KCl, 0.730; $MgSO_4$, 0.230; $MnSO_4 \cdot H_2O$, 0.0154; $CuSO_4$, 0.0013; ferric citrate (16.7% Fe), 0.0151; $ZnCO_3$, 0.0021; and KIO_3, 0.0001.

out pantothenate, or supplemented with pantothenate. The protein, mineral and vitamin content of the 18% fat diet was adjusted so that ratios of these components to calories were the same as in the 6% fat diet. The level of pantothenate fed in experiments 1 and 2 was 3000 μg/100 g of the 6% fat diet and 3400 μg/100 g of the 18% fat diet. The levels fed in experiment 3 ranged from zero to 3000 μg/100 g diet. Tap water was given ad libitum. Body weights and food intakes were measured two or three times weekly. The average initial weight of the rats was 58 g in experiment 1 and 43 to 48 g in experiments 2 and 3. The rats were given access to food until the time of killing, when they were decapitated without anesthesia.

CoA of the liver was determined in the rats from experiment 1 and liver lipids in the rats from experiment 2. The livers were frozen in liquid nitrogen, weighed, lyophilyzed, and the lipids determined by methods used previously (7). Acid-soluble CoA (free CoA and acetyl CoA) and long-chain acyl CoA were extracted by a modification of the method of Williamson (8). An aliquot of the lyophilized liver powder (equivalent to 1.5 g wet weight) was homogenized with 4 volumes of cold 8% $HClO_4$, then centrifuged (0°) for 15 minutes at 14500 × *g*. The precipitate was resuspended in 3 volumes of 6% $HClO_4$ and centrifuged. The combined supernatants were adjusted to pH 5.5 with 3 M K_2CO_3 and aliquots used for determination of "acid-soluble" CoA. The residue was washed with 0.6% $HClO_4$ and distilled water, and then suspended in 3 volumes of 5 mM dithiothreitol (DTT), made to pH 10.5–11, with 1 N KOH-5 mM DDT, and let stand 20 minutes at 30°, with occasional stirring. $HClO_4$ (6%) was then added to pH 5.0, and the suspension centrifuged (0°) at 32000 × *g* for 15 minutes. Both the $HClO_4$ and the KOH supernatants were adjusted to pH 7 before CoA assay by the phosphotransacetylase method (9), with DTT (0.01 M) replacing cysteine·HCl (0.1 M) in the assay. Total CoA is the sum of the acid-soluble and long-chain acyl values.

RESULTS

Growth. Increasing the level of dietary fat from 6% to 18% had no significant effect on the development of pantothenate deficiency as indicated by body weight after 6 weeks of depletion in the first 2 experiments (fig. 1). The onset and incidence of greying in the deficient groups were similar with both levels of fat. However, the weights of the deficient groups fed the 18% fat diet were somewhat lower than those of the deficient groups fed the

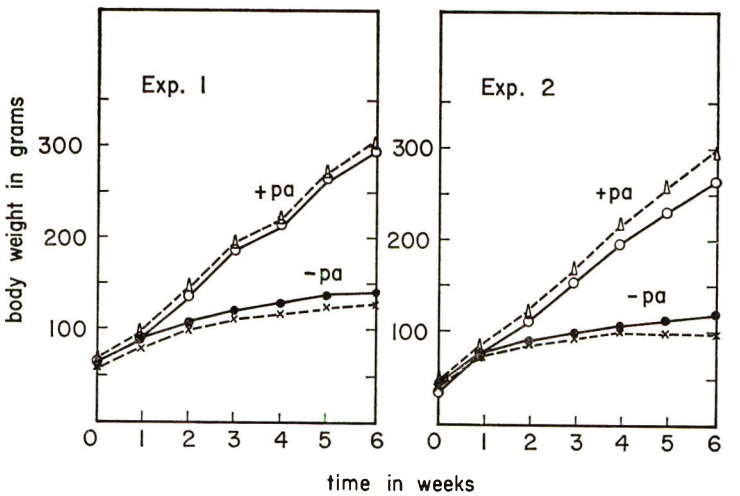

Fig. 1 Growth rates of male rats fed diets containing 6% or 18% cottonseed oil, with or without pantothenate. ●—●, −pa, 6% fat; x- - - -x, −pa, 18% fat; O—O, +pa, 6% fat; Δ- - -Δ, +pa, 18% fat.

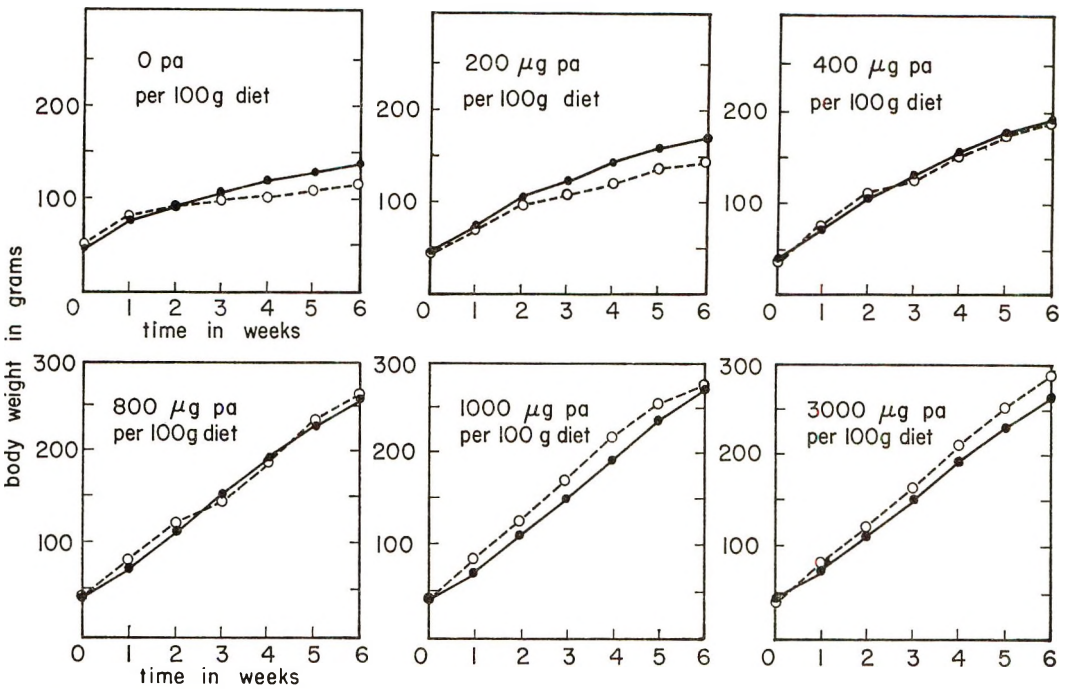

Fig. 2 Growth rates of male rats fed graded levels of pantothenate with diets containing 6% or 18% cottonseed oil. ●—●, 6% fat; O- - -O, 18% fat. The level of pantothenate on the chart is the level for the 6% fat diet. The level of pantothenate in the 18% fat diet (μg/100 g diet) is 1.13 times more, so that the amount of pantothenate per kilocalorie of diet is the same with both levels of fat.

6% fat diet, with a significant decrease ($P = 0.05$) in the third experiment (fig. 2). Mortality and variation in body weight were somewhat greater at 6 weeks in the deficient groups fed the high fat diet.

The effect of dietary fat level on the growth response to marginal levels of pantothenate (exp. 3) is shown in figure 2. The amount of pantothenate in the 18% fat diet was 1.13 times that of the 6% fat diet, so that the pantothenate level per kilocalorie was the same with both diets. The similar growth rates for both levels of fat at all intakes of pantothenate tested show that under our conditions, the pantothenate requirement for growth was not significantly increased by the increase in fat. The occurrence of maximal weight gain at the 800-μg level agrees with the conclusion of Barboriak et al. (2) that a pantothenate level of 800 μg/100 of a 5% fat diet will allow maximal growth in young male rats.

Liver CoA and adrenal weights. These were determined on the rats from experiment 1 (table 1). Increasing the level of

TABLE 1

Liver CoA and adrenal weights

Diet	Pantothenate	CoA: Total	CoA: Acid-soluble	CoA: Long-chain acyl	Long-chain acyl CoA / Total CoA × 100	Adrenal wt
			mμmoles/g wet wt		%	*mg/100 g body wt*
6% CSO [1]	—	235 ± 26 [2](5) [3]	157 ± 25	78 ± 3	33	27.4 ± 1.6(7)
6% CSO [1]	+	353 ± 39(5)	226 ± 38	128 ± 9	36	13.8 ± 1.5(5)
18% CSO [1]	—	140 ± 8(5)	79 ± 12	61 ± 8	43	21.6 ± 0.9(9)
18% CSO [1]	+	175 ± 22(5)	102 ± 11	72 ± 12	41	12.7 ± 0.6(5)

[1] CSO indicates cottonseed oil.
[2] Mean ± SE.
[3] Numbers in parentheses indicate number of rats/group.

TABLE 2

Proportions of fatty acids in liver phospholipids

Diet	Panto-thenate	Liver wt	Phospholipid	16:0	16:1	18:0	18:1	18:2	20:4
		g	*mg/g*			*wt %*			
6% CSO [1](8) [2]	−	4.94 ± 0.36 [3]	32.0 ± 1.1	23.1 ± 0.8	1.2 ± 0.2	19.4 ± 1.2	9.8 ± 0.6	13.8 ± 0.6	30.9 ± 0.5
6% CSO(8)	+	11.29 ± 0.43	31.0 ± 0.8	17.2 ± 0.4	0.5 ± 0.1	26.5 ± 0.5	5.4 ± 0.1	14.1 ± 0.5	35.0 ± 0.6
18% CSO(8)	−	4.61 ± 0.46	35.2 ± 1.0	20.5 ± 1.0	0.1 ± 0.1	23.3 ± 1.2	4.7 ± 0.8	19.5 ± 1.0	31.0 ± 1.1
18% CSO(7)	+	12.16 ± 0.42	32.1 ± 0.9	15.9 ± 0.4	0.2 ± 0.1	29.0 ± 0.4	3.2 ± 0.1	15.0 ± 0.6	34.8 ± 0.5

[1] CSO indicates cottonseed oil.
[2] Numbers in parentheses indicate number of rats/group.
[3] Mean ± SE.

TABLE 3

Proportions of fatty acids in liver triglycerides

Diet	Panto-thenate	Liver wt	Fatty acid	14:0	16:0	16:1	18:0	18:1	18:2	20:4
		g	*mg/g*				*wt %*			
6% CSO [1](8) [2]	−	4.94 ± 0.36 [3]	4.69 ± 1.4	1.2 ± 0.2	31.5 ± 1.3	5.3 ± 1.2	3.8 ± 0.7	37.8 ± 2.6	17.8 ± 3.7	1.6 ± 0.6
6% CSO(8)	+	11.29 ± 0.43	9.10 ± 1.0	1.3 ± 0.1	29.0 ± 0.4	3.3 ± 0.2	4.1 ± 0.3	31.8 ± 0.9	28.1 ± 1.0	2.0 ± 0.2
18% CSO(8)	−	4.61 ± 0.46	2.14 ± 0.8	0.5 ± 0.2	27.8 ± 1.6	0.8 ± 0.1	5.9 ± 1.1	18.2 ± 0.7	40.1 ± 2.4	4.4 ± 0.6
18% CSO(7)	+	12.16 ± 0.42	29.5 ± 0.4	0.6 ± 0.1	23.4 ± 0.3	0.6 ± 0.1	2.9 ± 0.2	18.4 ± 0.5	48.0 ± 0.5	3.8 ± 0.4

[1] CSO indicates cottonseed oil.
[2] Numbers in parentheses indicate number of rats/group.
[3] Mean ± SE.

TABLE 4

Proportions of fatty acids in liver sterol esters

Diet	Panto-thenate	Free sterol	Fatty acid	14:0	16:0	16:1	18:0	18:1	18:2	20:4
		mg/g	*mg/g*				*wt %*			
6% CSO [1](8) [2]	−	1.84 ± 0.04 [3]	0.41 ± 0.06	0.5 ± 0.1	31.5 ± 1.2	4.4 ± 0.6	24.4 ± 2.2	17.6 ± 1.9	13.5 ± 2.3	7.1 ± 0.5
6% CSO(8)	+	1.82 ± 0.03	0.67 ± 0.03	0.8 ± 0.1	25.2 ± 0.6	3.8 ± 0.4	22.0 ± 1.9	20.4 ± 1.0	17.2 ± 1.1	10.7 ± 0.6
18% CSO(8)	−	2.02 ± 0.08	0.56 ± 0.05	0.4 ± 1.1	30.7 ± 1.1	1.7 ± 0.2	34.1 ± 2.9	12.1 ± 0.6	24.7 ± 2.2	9.4 ± 0.8
18% CSO(7)	+	1.84 ± 0.04	1.42 ± 0.05	0.4 ± 1.1	19.2 ± 0.8	1.2 ± 0.1	13.8 ± 0.3	20.2 ± 0.6	32.0 ± 1.0	12.4 ± 0.8

[1] CSO indicates cottonseed oil.
[2] Numbers in parentheses indicate number of rats/group.
[3] Mean ± SE.

fat from 6% to 18% significantly reduced total and acid-soluble CoA and increased the proportion of long-chain acyl CoA in both the deficient and control groups, in comparison with the corresponding groups fed the 6% fat diet. With the 6% fat diet, the levels of total and long-chain acyl CoA were significantly lower in the deficient group than in the control. With the 18% fat diet, however, there were no significant differences between the deficient and control groups in total, acid-soluble or long-chain acyl CoA, although the values for the deficient group were lower.

Adrenal weights, expressed as mg/100 g body weight, were significantly higher in the deficient groups. The values for both control groups were similar, but the value for the deficient group fed the 6% fat diet was significantly greater than that for the deficient group fed the 18% fat diet.

Liver fatty acids. The changes in the relative proportions of phospholipid fatty acids in the deficient groups (table 2) follow the pattern reported by Constable et al. (5) for total liver fatty acids. With either level of dietary fat, pantothenate deficiency resulted in a significantly lower level of stearate and arachidonate and a significantly higher level of palmitate. Oleate, however, was significantly higher only in the deficient group fed the 6% fat diet.

The effects of the deficiency on the fatty acid composition of the triglyceride fraction (table 3) differed from the changes in phospholipid fatty acids. Arachidonate did not change, but the proportion of linoleate was lower in both deficient groups. Oleate was higher only with the 6% fat diet.

In sterol esters (table 4) in both deficient groups, the relative proportions of arachidonate and linoleate were significantly lower and that of palmitate significantly higher than in the controls. The concentration of esterified sterol was significantly increased with the high fat diet in both the deficient and control groups, but the increase was much greater in the control group.

DISCUSSION

The results of the growth studies provide no evidence that a high level of dietary fat reduced the growth response to limiting intakes of pantothenate or increased the pantothenate requirement for maximal growth of young male rats. Likewise, we obtained no strong evidence to show that the high fat diet accelerated the development of the deficiency except for a slightly greater mortality and variation in body weight after 6 weeks of depletion in rats fed the high fat, pantothenate-free diets. One might consider that the lower CoA values of the deficient rats fed the high fat diet indicated greater depletion. However, CoA values for the 18% fat control group were also lower than those of the 6% fat controls. The pantothenate level of the control 18% fat diet was 3400 μg/100 g or 4 times the estimated requirement (1), and fresh diet was made every week. Thus, it seems unlikely that the lower CoA values in the control group fed the 18% fat diet resulted from inadequate pantothenate intake unless the absorption of dietary pantothenate was reduced because of the increased fat content.

The lower values for liver CoA in both groups fed the high fat diet could be explained by the suggestion of Hatano et al.[6] that increased dependence on fat for energy may alter pantothenate metabolism. Their suggestion is based on the observation that diabetes in rats increased the urinary excretion and altered the tissue distribution of injected ^{14}C-pantothenate. A comparison of the excretion and tissue distribution of ^{14}C-pantothenate in rats fed low or high fat diets would indicate whether an increase in dietary energy supplied from fat would produce changes in pantothenate metabolism similar to those observed in diabetic rats.

It has been suggested that the ratio of long-chain acyl CoA to total CoA may be a determining factor in fatty acid synthesis in the liver (10–12). In the deficient rats fed the 6% fat diet, the ratio of long-chain acyl CoA to total CoA was the same as in the control group, and the reduction in total CoA was about 33%. We have been unable to observe a decrease in the incorporation of acetate-2-^{14}C or pyruvate-3-^{14}C into fatty acids by liver slices from rats fed the 6%

[6] See footnote 3.

fat diet, even though $^{14}CO_2$ production was reduced.[7] Klein and Lipmann (13) had reported a decrease in acetate-1-^{14}C incorporation into fatty acids by liver slices from pantothenate-deficient rats when the CoA level was reduced approximately 50%, but not when the reduction was less than 50%. The maintenance of a "normal" ratio, as well as a smaller reduction in total CoA, under our conditions, may explain why we have found no decrease in fatty acid synthesis by slices from pantothenate-deficient rats.

Decreases in the relative proportion of phospholipid arachidonate in liver have been reported in deficiencies of pyridoxine (14), protein (15), or lysine and threonine (16), in addition to pantothenate deficiency (5). As discussed by Viviani et al. (17), a decrease in arachidonate in a given experimental condition could be the net result of a very large number of metabolic changes, not only a change in the conversion of linoleate to arachidonate. Although there is no obvious explanation for the reduction of phospholipid arachidonate in pantothenate deficiency, the decrease could not be prevented by increasing the linoleate intake since similar decreases occurred in the deficient groups fed either the 6% or the 18% fat diet.

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ADDENDUM

After this paper had been submitted, we learned of the work of Fidanza et al. (Chem. Abstr., 67 (no. 10): 8331, '67) who found that the decrease in arachidonate and stearate in liver of pantothenate-deficient rats fed ω-methylpantothenic acid was chiefly in the phospholipid fraction.

[7] Unpublished results, M. A. Williams and M. Igelstrom.

Relation of Tissue Triglycerides to Dietary Saturated Medium- and Long-chain Triglycerides and Linoleic Acid Level[1]

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ABSTRACT It has been shown that the linoleate level of diets containing saturated medium- or long-chain triglycerides influences the extent to which these dietary acids are deposited in the epididymal adipose tissue of rats. It has not been shown whether triglycerides from other tissues are similarly affected and to what extent such diets influence the formation of triglycerides having specific structural patterns. Therefore, pure triglycerides (TG) were isolated, by preparative thin-layer chromatography, from total lipid extracts from liver, heart, and epididymal and retroperitoneal adipose tissues of rats fed diets containing no fat or 20% of saturated medium-chain triglycerides (MCT) or long-chain triglycerides (LCT) and supplemented with 0.1 or 2% of linoleic acid. These tissue triglycerides were analyzed for their total fatty acid composition and for that of the 2-position of the glyceride (by pancreatic lipase hydrolysis) and for the percentages of TG molecules having different degrees of unsaturation (by argentation chromatography). The higher linoleic acid supplement favored greater deposition of dietary fatty acids in TG of both adipose tissues but not in those of heart and liver. The heart had higher stearate levels in total TG and higher percentages of palmitate in the 2-position. Saturated TG (S_3) were higher in adipose tissues than in liver and heart. Higher linoleate levels left S_3 unchanged in the group fed no fat, increased it in those fed MCT and decreased it in the LCT-fed group. The data suggest that differences in triglyceride pattern were brought about by dietary fat and linoleate level. Furthermore, it appears that accumulation of S_3 containing high levels of medium-chain acids was beneficial whereas accumulation of S_3 made up mainly of palmitate and stearate was disadvantageous to the animal.

In earlier studies, dietary linoleic acid was found to have a regulatory effect on the fatty acid composition of rat epididymal adipose tissue. Inclusion of 2% of linoleic acid in purified diets containing 20% of saturated medium- or long-chain triglycerides led to increased deposition of the dietary fatty acids (1). However, these studies did not permit any conclusions as to whether any specific triglyceride types were preferentially formed from these dietary saturated fatty acids. Insofar as argentation chromatography and lipase studies have made it possible to examine some aspects of triglyceride structure, the present paper reports analyses of triglycerides from epididymal and retroperitoneal adipose tissues, liver, and heart of rats fed diets containing 0.1 or 2% of linoleic acid [2] and no fat or with 20% of either medium- or long-chain saturated triglycerides.

MATERIALS AND METHODS

The fatty acid compositions of the medium-chain (MCT) and long-chain (LCT) triglycerides used in these studies are shown in table 1; the fatty acids were distributed at random over the molecules. The preparation of these randomized triglycerides from coconut and other palm kernel oils has been described previously (2). The composition of the experimental diet is given in table 2.

Each diet was fed to 15 weanling male rats of the Columbia-Sherman strain for 8 weeks. At the end of this period, the survivors (table 3) were anesthetized with chloroform, blood was drawn from the heart, and their tissues were weighed and frozen on dry ice. Tissues from each group were pooled. The frozen tissues were cut on a freezing microtome and homogenized in chloroform:methanol (3) in a glass tube in dry ice by means of a homogenizer equipped with a motor-driven Teflon pestle.[3] Particle size did not exceed 40 μ.

Received for publication September 15, 1967.
[1] Aided by Grant U-1510 from the Health Research Council of the City of New York.
[2] Nutritional Biochemicals Corporation, Cleveland. This is a linoleic acid concentrate containing 75% of the acid and 25% of oleic acid.
[3] Tri-R Instruments, Inc., Jamaica, New York.

TABLE 1

Fatty acid composition of medium-chain (MCT) and long-chain triglycerides (LCT) fed to male rats

Fatty acid	6:0	8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	20:0
						% of fatty acids					
MCT	0.1	61.2	31.5	2.5	0.6	2.2	0.2	1.7	—	—	—
		92.7									
LCT				0.5	1.0	36.9	—	55.5	5.2	0.7	0.2
						92.4					

TABLE 2

Composition of diet

	%
Casein, alcohol-washed	30
Dextrose	44
Triglycerides or additional dextrose [1]	20
Salt mixture USP XIII	4
Cellulose [2]	2
	mg/kg
Choline dihydrogen citrate	1000
Inositol	1000
p-Aminobenzoic acid	300
Nicotinamide	100
Vitamin K[3]	10
Ca pantothenate	10
Vitamin B_{12} (0.1% trituration in mannitol)	5
Thiamine·HCl	2
Pyridoxine·HCl	4
Riboflavin	4
Folic acid	2.5
Biotin	0.025
Ascorbic acid	25
α-Tocopheryl acetate	50
Free α-tocopherol	10
β-Carotene	5
Vitamin D_2	0.5

[1] In the fat-free diets.
[2] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[3] Synkayvite, Hoffmann-LaRoche, Inc., Nutley, New Jersey.

Triglycerides (TG) were prepared from the ether-soluble fraction of the total lipid extract by preparative thin-layer chromatography. About 150 mg ether-soluble extract were applied to each of four 20 cm × 20 cm plates coated with a 250-μ layer of silica gel G, and the plates were developed in 80:20 petroleum ether:diethyl ether. The TG were scraped off, eluted with ether and rechromatographed for purification. Purity was checked by TLC. The TG were stored at −20° in N_2-flushed, evacuated, sealed ampules. All analytical procedures were carried out in subdued light, with freshly distilled solvents, and under a blanket of N_2 where possible.

Gas-liquid chromatography was carried out on a Barber-Colman Model 5000 instrument equipped with a 183-cm glass column packed with 12% diethylene glycol succinate on Anakrom ABS, a hydrogen flame detector, and a disc integrator. Analyses were carried out at a column temperature of 175° with an injector temperature of 245°, a detector temperature of 210°, and

TABLE 3

Survival rates and body and organ weights of rats fed diets containing no fat or 20% of medium-chain (MCT) or long-chain triglycerides (LCT) and supplemented with 0.1 or 2% of linoleic acid

	Survival rate	Avg body wt	Avg liver wt	Avg heart wt	Avg wt of epididymal fat	Avg wt of retroperitoneal fat
		g	*g*	*g*	*g*	*g*
Fat-free diet +						
0.1% linoleic acid	14/15	257 ± 7.2 [1]	7.3 ± 0.22	0.78 ± 0.070	2.8 ± 0.63	3.1 ± 0.78
2% linoleic acid	15/15	282 ± 4.9	7.9 ± 0.17	0.83 ± 0.055	2.8 ± 0.21	3.8 ± 0.36
20% MCT +						
0.1% linoleic acid	13/15	271 ± 7.7	7.7 ± 0.26	0.78 ± 0.091	2.7 ± 0.23	3.7 ± 0.23
2% linoleic acid	13/15	288 ± 9.1	8.3 ± 0.23	0.84 ± 0.079	2.2 ± 0.21	3.9 ± 0.49
20% LCT +						
0.1% linoleic acid	7/15	206 ± 16.8	7.4 ± 0.62	0.59 ± 0.099	1.2 ± 0.18	1.3 ± 0.31
2% linoleic acid	7/15	218 ± 15.3	7.5 ± 0.44	0.62 ± 0.072	1.1 ± 0.16	0.9 ± 0.24

[1] SEM.

a nitrogen flow of 100 cm^3/minute. Quantitative analyses of standards KD and K107 [4] agreed with the stated composition with an error of less than 4% for major components (more than 10% of the mixture) and less than 5% for minor components.

Structural analyses by argentation TLC were carried out essentially according to the method of Barrett (4) as modified by Blank et al. (5). We used 20 cm × 40 cm plates in a sandwich technique, and the plates were developed at an angle of about 30° from the horizontal surrounded by filter paper saturated with the solvent mixture (1.5% methanol in chloroform followed by 3% methanol in chloroform). The resulting bands, formed more or less by separation of the TG into types having different numbers of double bonds in the molecule, were scraped off individually and eluted with 5% methanol in pentane. As an internal standard, methyl pentadecanoate was added to each band immediately after the eluting solvent. The triglycerides were transmethylated with sodium methylate in methanol. As a check, the weights of the main constituent fatty acids in the various bands were added and expressed as percentages of the combined weight of the bands. These percentages were compared with the fatty acid composition of the total TG mixture. The results of the analysis were accepted only if the percentages for each acid as derived by the 2 methods agreed with a relative error of not more than 10%. In general, the value for linoleate derived from adding up the linoleate in the various bands was somewhat lower than its percentage in the total triglycerides.

In the calculation of the structural types present in a given band from GLC data, more than one possibility was sometimes suggested. In deciding which structural pattern to select, we based our judgment on Morris's discussion of the relative affinity of monoenes and dienes for silver (6). For example, a triglyceride containing 2 double bonds in the form of 2 monoenoic acids (O) will be somewhat less polar than one containing a dienoic acid (L) such as linoleic acid. Thus, from the top of the plate downward, the types would occur in the following order (with overlapping): S_3 (S = saturated acids), S_2O, SO_2, S_2L, O_3, SOL, O_2L, SL_2, OL_2, and L_3; in a band where overlapping occurred, the most likely combination, based on this order, was assumed.

Pancreatic lipase hydrolysis was carried out according to Luddy et al. (7) on 10 to 20 mg of triglyceride. Monoglycerides were isolated by TLC, and methyl pentadecanoate was added as an internal standard to permit estimation of the amount of monoglyceride and the extent of hydrolysis. Hydrolysis was usually limited to the conversion of approximately 20% of triglyceride to monoglyceride.

The reproducibility of structural analysis by argentation chomatography was examined in duplicate analyses carried out on the total TG from 2 human hearts. Duplicate values for the major structural types agreed with a relative error of <10% in almost all instances:

	S_3	S_2O	SO_2
No. 209 a,b	3.4,3.6	21.6,22.7	31.9,34.8
No. 292 a,b	2.7,2.5	13.7,12.8	34.7,36.8
		O_3	SOL
No. 209 a,b		16.2,14.8	15.4,15.4
No. 292 a,b		16.5,18.4	18.9,18.2

This same degree of precision was shown when the gas chromatographic analyses of the individual bands were checked against that of the total TG sample (see above). Although only one analysis was carried out on each sample in the present study, individual variation appears not to be an important factor inasmuch as each sample represented pooled tissue from all survivors in a group (7–15 animals). Furthermore, epididymal and retroperitoneal adipose tissues showed great similarity in the same group and differed markedly with different diets, whereas heart and liver exhibited tissue specificity in their structural patterns despite differences in diet (to be discussed below). Therefore, it seems permissible to rely on such conclusions from single analyses as are drawn from consistent patterns or differences which are so large as to be beyond the limits of error.

[4] Applied Science Laboratories, State College, Pennsylvania.

RESULTS

The survival rate and average weights of the rats and their livers, hearts, and epididymal and retroperitoneal adipose tissues are shown in table 3. There were fewer survivors among those fed LCT, which would be in agreement with observations of Gottenbos and Thomasson on rats fed hard fats (8). However, fatty acid analyses of adipose tissue TG showed that significant amounts of LCT had been absorbed.

The fatty acid compositions of adipose tissue, liver, and heart TG are shown in table 4. All tissue TG reflected the differences in dietary linoleate level, and both adipose tissues showed the previously described influence of linoleate on the deposition of other dietary fatty acids. Rats fed MCT and 2% of linoleic acid deposited

TABLE 4

Fatty acid composition of triglycerides from tissues of rats fed fat-free, medium-chain triglyceride (MCT), and long-chain triglyceride (LCT) diets supplemented with 0.1% and 2% of linoleic acid

	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2
				% of fatty acids				
Fat-Free diet +								
				Retroperitoneal adipose tissue				
0.1% linoleic acid	0.1	0.2	2.2	31.9	14.4	5.0	44.0	1.1
2% linoleic acid	tr	0.2	2.3	31.0	11.4	4.8	33.4	15.6
				Epididymal adipose tissue				
0.1% linoleic acid	tr	0.1	2.1	30.7	15.6	4.3	44.0	2.0
2% linoleic acid	0.1	0.1	2.3	29.8	12.8	3.6	33.6	16.2
				Liver				
0.1% linoleic acid	—	0.1	1.6	30.2	11.8	3.2	48.1	2.0
2% linoleic acid	tr	0.1	1.1	26.5	7.4	2.3	30.7	21.0
				Heart				
0.1% linoleic acid	—	0.2	2.2	31.2	8.9	6.1	45.2	2.5
MCT diet +								
				Retroperitoneal adipose tissue				
0.1% linoleic acid	2.5	0.9	3.4	35.7	11.3	6.9	36.7	1.2
2% linoleic acid	6.7	1.7	3.4	30.9	6.4	6.8	24.8	13.5
				Epididymal adipose tissue				
0.1% linoleic acid	1.2	1.2	3.6	35.8	11.9	5.5	37.3	1.5
2% linoleic acid	7.4	2.1	3.6	31.1	7.1	5.4	22.9	16.2
				Liver				
0.1% linoleic acid	0.8	0.4	2.1	33.8	8.0	5.2	40.7	3.0
2% linoleic acid	1.1	0.5	1.9	29.3	4.2	4.4	28.6	23.7
				Heart				
0.1% linoleic acid	—	0.4	2.2	34.7	5.1	7.2	42.4	3.0
2% linoleic acid	0.8	0.8	2.8	30.9	4.6	8.2	27.3	17.9
LCT diet +								
				Retroperitoneal adipose tissue				
0.1% linoleic acid	0.2	1.1	3.0	30.7	8.5	10.5	40.1	5.6
2% linoleic acid	0.1	0.9	2.4	26.9	4.4	13.2	29.6	22.0
				Epididymal adipose tissue				
0.1% linoleic acid	0.1	0.8	2.6	29.6	9.1	10.0	41.0	6.2
2% linoleic acid	0.1	0.6	2.3	25.8	6.1	12.2	30.3	21.6
				Liver				
0.1% linoleic acid	—	0.1	1.3	30.3	5.2	12.9	42.2	5.7
2% linoleic acid	—	0.2	0.9	26.7	1.1	12.3	20.7	28.4
				Heart				
0.1% linoleic acid	—	0.3	1.4	32.6	2.6	21.1	33.6	4.8
2% linoleic acid	—	tr	0.8	34.9	1.3	23.9	22.1	16.7

more C_{10} to C_{14} and the corresponding group fed LCT deposited more stearate than the groups given the lower linoleic acid supplements. The elevation in stearate level with increased dietary linoleate was probably real since it occurred in both kinds of adipose tissue although the markedly higher linoleate levels could have resulted in an apparent lowering of all other percentages; furthermore, these observations are in agreement with previous studies (1). The fatty acid compositions of retroperitoneal and epididymal adipose tissues were strikingly similar. This was somewhat unexpected in view of many reports in the literature on variations in fatty acid composition of adipose tissues from different sites in other species and in view of known differences in response to fat-mobilizing hormones of these 2 adipose tissues (9). These and our previous adipose tissue values generally agree with the recent data of Leveille et al. (10) except for their low C_{10} values.

TABLE 5

Percentages of molecules containing various combinations of saturated (S), monoenoic (O), and dienoic (L) acids in triglycerides from tissues of rats fed diets containing no fat or 20% of medium-chain (MCT) or long-chain triglycerides (LCT) supplemented with 0.1 or 2% of linoleic acid

	S_3	S_2O	SO_2	S_2L	O_3	SOL	O_2L	SL_2
Fat-free diet +								
				Retroperitoneal adipose tissue				
0.1% linoleic acid	3.0	24.4	43.5	2.1	22.2	3.4	0.6	—
2% linoleic acid	4.1	20.1	21.6	7.9	12.4	16.7	10.7	5.5
				Epididymal adipose tissue				
0.1% linoleic acid	3.9	24.1	42.1	2.2	21.4	5.1	1.2	—
2% linoleic acid	3.6	13.1	23.9	6.8	14.4	18.7	10.4	9.1
				Liver				
0.1% linoleic acid	2.2	14.1	57.3	0.8	17.7	7.6	—	—
2% linoleic acid	2.4	8.8	24.6	5.4	8.8	29.6	9.6	10.7
				Heart				
0.1% linoleic acid	2.8	36.2	36.4	2.5	16.2	4.9	—	1.0
MCT diet +								
				Retroperitoneal adipose tissue				
0.1% linoleic acid	10.7	36.8	40.3	3.1	7.0	2.1	—	—
2% linoleic acid	16.2	23.1	11.0	19.5	9.6	13.3	4.5	2.7
				Epididymal adipose tissue				
0.1% linoleic acid	11.7	32.6	41.0	1.0	10.5	3.2	—	—
2% linoleic acid	14.6	20.8	9.4	20.2	10.7	14.7	5.0	5.1
				Liver				
0.1% linoleic acid	3.8	23.8	45.0	0.9	7.4	16.1	0.9	—
2% linoleic acid	4.9	14.3	14.7	9.4	8.1	26.0	10.4	12.2
				Heart				
0.1% linoleic acid	7.7	27.4	34.9	9.8	14.9	7.2	1.2	—
2% linoleic acid	8.9	18.3	10.0	15.5	7.9	15.8	5.9	3.8
LCT diet +								
				Retroperitoneal adipose tissue				
0.1% linoleic acid	8.6	28.0	28.5	6.7	16.1	10.1	2.0	—
2% linoleic acid	6.6	16.5	13.8	18.2	6.6	21.2	8.3	8.3
				Epididymal adipose tissue				
0.1% linoleic acid	8.2	28.5	29.5	5.0	16.2	10.1	1.3	1.2
2% linoleic acid	5.9	16.0	13.1	15.4	8.1	21.6	9.5	7.9
				Liver				
0.1% linoleic acid	5.8	25.8	39.6	0.3	6.0	17.1	0.4	—
2% linoleic acid	4.4	11.4	6.6	16.7	2.2	31.1	1.0	15.4
				Heart				
2% linoleic acid	14.0	22.4	11.2	28.1	2.4	17.8	—	3.1

Liver and heart TG showed some tissue specificity in their response to dietary influences. Liver TG had the highest linoleate levels with both dietary levels of this acid, and heart TG had the highest stearate levels with all dietary regimes.

Table 5 summarizes the results of structural analyses. The higher levels of types containing linoleate associated with higher levels of dietary linoleic acid did not have the same pattern with all diets, nor were the compensatory lower levels of other types the same. The decreased level of oleate with increased tissue linoleate was associated with lower levels of O_3 in the rats fed the fat-free and LCT diets but not in those fed MCT (except in the heart), although the latter showed the greatest decline in oleate. This group had the greatest decline in SO_2.

Adipose tissue levels of the completely saturated type, S_3, increased with increased dietary linoleate in the group fed MCT, decreased in those fed LCT and remained the same in those fed the fat-free diet. Liver S_3 followed the same pattern to a much lesser degree. Table 6 compares the fatty acid compositions of the S_3 bands with the saturated fatty acid composition of the total TG (on the basis of 100% for saturated acids). There was no evidence of a selective process in the adipose tissue in the distribution of fatty acids in the S_3 band compared with the total TG of that tissue. However, in the liver, there was a marked tendency for less C_{16} and more C_{14} and C_{18} to occur in the S_3 band than in the total TG regardless of diet and linoleate supplement; increased dietary linoleate enhanced this distribution in 2 instances. In

TABLE 6

Saturated fatty acid compositions of total triglycerides (corrected to S = 100%) and their saturated fractions (obtained by argentation chromatography) from tissues of rats fed fat-free, medium-chain triglyceride (MCT), and long-chain triglyceride (LCT) diets supplemented with 0.1% and 2% of linoleic acid

	10:0		12:0		14:0		16:0		18:0	
	Total	S_3	Total	S_3	Total	S_3	Total	S_3	Total	S_3
Retroperitoneal adipose tissue										
Fat-free + 0.1% linoleic acid	0.3	0.6	0.5	0.9	5.6	6.9	81.0	74.0	12.7	16.6
Fat-free + 2% linoleic acid	0.1	0.8	0.5	0.8	6.0	7.2	81.0	77.8	12.5	12.8
MCT + 0.1% linoleic acid	5.1	0.8	1.8	0.6	6.9	3.9	72.2	77.5	13.9	17.0
MCT + 2% linoleic acid	13.5	13.5	3.4	4.1	6.9	8.2	62.3	60.2	13.7	11.0
LCT + 0.1% linoleic acid	0.4	0.4	2.4	1.8	6.6	6.7	65.3	65.0	23.1	26.4
LCT + 2% linoleic acid	0.2	0.1	2.1	1.7	5.5	6.4	61.9	63.6	30.4	28.3
Epididymal adipose tissue										
Fat-free + 0.1% linoleic acid	0.2	—	0.3	—	5.6	7.1	82.6	81.4	11.6	11.4
Fat-free + 2% linoleic acid	0.3	0.9	0.3	1.1	6.4	7.5	83.0	79.7	10.0	11.1
MCT + 0.1% linoleic acid	2.5	0.9	2.5	2.5	7.6	9.2	75.7	74.2	11.6	13.2
MCT + 2% linoleic acid	14.9	11.0	4.2	4.9	7.2	8.9	62.7	63.8	10.9	9.9
LCT + 0.1% linoleic acid	0.2	—	1.9	2.0	6.0	7.2	68.7	68.6	23.2	22.2
LCT + 2% linoleic acid	0.2	—	1.5	1.9	5.1	6.4	62.8	61.2	29.7	30.5
Liver										
Fat-free + 0.1% linoleic acid	—	—	0.3	—	4.6	8.8	86.2	78.3	9.1	12.9
Fat-free + 2% linoleic acid	tr	1.1	0.3	0.7	3.7	12.5	88.2	68.4	7.6	16.2
MCT + 0.1% linoleic acid	1.9	4.1	0.9	2.0	5.0	8.4	80.5	67.5	12.3	16.6
MCT + 2% linoleic acid	3.0	4.7	1.3	2.1	5.1	7.8	78.6	70.2	11.8	16.4
LCT + 0.1% linoleic acid	—	—	0.2	0.3	2.9	3.3	68.0	62.8	28.9	32.8
LCT + 2% linoleic acid	—	—	0.5	—	2.3	2.9	66.6	46.6	30.6	45.5
Heart										
Fat-free + 0.1% linoleic acid	—	0.3	0.5	0.7	5.5	5.7	78.5	80.2	15.4	12.6
MCT + 0.1% linoleic acid	—	2.6	0.9	1.9	5.0	7.5	78.0	73.0	16.2	15.0
MCT + 2% linoleic acid	1.8	3.2	1.8	2.5	6.4	7.6	71.0	71.4	18.9	14.2

TABLE 7

Fatty acid composition of the 2-position in triglycerides from tissues of rats fed different levels of linoleic acid in diets containing no fat or 20% of medium-chain (MCT) or long-chain (LCT) saturated triglycerides

	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2
				% of fatty acids				
Fat-free diet +								
				Retroperitoneal adipose tissue				
0.1% linoleic acid	0.4	0.4	1.6	11.1	15.6	2.0	53.3	3.0
2% linoleic acid	0.1	0.1	1.1	10.4	11.6	1.9	46.5	26.5
				Epididymal adipose tissue				
0.1% linoleic acid	—	—	1.7	12.9	17.6	1.4	62.9	3.2
2% linoleic acid	—	—	1.2	10.3	13.2	1.4	42.3	27.8
				Liver				
0.1% linoleic acid	—	—	1.0	7.6	13.6	1.7	71.8	3.3
2% linoleic acid	—	—	2.3	5.4	8.6	tr	44.5	34.8
MCT diet +								
				Retroperitoneal adipose tissue				
0.1% linoleic acid	0.3	0.4	2.6	17.3	13.2	2.8	55.3	3.0
2% linoleic acid	1.4	0.7	3.2	17.3	8.9	1.9	35.6	27.3
				Epididymal adipose tissue				
0.1% linoleic acid	0.4	0.4	2.8	18.0	14.9	3.0	55.0	4.1
2% linoleic acid	1.0	0.6	2.6	15.5	7.3	2.2	29.2	26.4
				Liver				
0.1% linoleic acid	—	—	1.8	10.8	11.5	1.5	62.3	4.5
2% linoleic acid	—	—	2.1	9.1	8.0	2.5	37.6	36.8
				Heart				
0.1% linoleic acid	—	—	2.4	22.6	6.5	1.4	44.8	4.8
LCT diet +								
				Retroperitoneal adipose tissue				
0.1% linoleic acid	tr	0.1	1.4	13.4	7.5	3.2	52.8	11.4
2% linoleic acid	0.1	0.2	0.9	10.2	4.1	3.3	35.4	36.8
				Epididymal adipose tissue				
0.1% linoleic acid	—	—	1.6	14.0	10.0	3.2	58.9	11.4
2% linoleic acid	—	—	1.1	11.1	5.3	3.1	37.5	41.6
				Liver				
0.1% linoleic acid	—	—	1.0	12.3	5.2	7.9	60.2	9.1
2% linoleic acid	—	—	0.8	6.1	2.7	5.2	28.3	50.4

the 3 heart samples, as in the adipose tissues, this type of fatty acid distribution was not noticeable.

The fatty acid compositions of the 2-position of the TG are given in table 7. In general, the fatty acid distribution corresponded to what one would expect: high oleate and linoleate and low palmitate and stearate values. C_{14} (and even more, C_{10}) were predominantly incorporated in the outer positions; this was in contrast with TG of human adipose tissue and hearts, where nearly 50% of the total myristate was esterified in the 2-position (11). Palmitate in 2-position, however, was highest in heart TG of both rats and humans.

DISCUSSION

The results of these studies confirmed the regulatory effect of linoleic acid on the composition of adipose tissue triglycerides. Liver and heart TG did not show an increased deposition of characteristic dietary saturated acids with higher linoleate levels. The high LA content of TG from the animals fed LCT in conjunction with the previously noted increased dietary requirements for LA when such a fat was fed (12), may give a clue as to how LCT interferes with the utilization of linoleic acid.

Analysis of the data as to whether the results support the existence of organ-spe-

cific TG is not easy, because of the strong influence of the dietary TG and on account of mobilization and transport phenomena. However, existence of organ-specific triglycerides was supported by the occurrence, regardless of the feeding of different diets, of high stearate levels in heart TG, low percentages of S_3 in heart and liver compared with adipose tissues, the characteristic fatty acid composition of the S_3 band of the liver, the high SO_2 values in liver, and the high percentage of palmitate in the 2-position of heart TG.

In discussing the significance of the changes of TG types under the influence of the various diets we are assuming that the biochemical changes associated with the correction of a defiicency state are of biological advantage to the animal; therefore the conditions in the animals fed the high LA diets represent a more desirable state than those in the group fed the low LA diets. This then suggests that the increase in the saturated TG in the animals fed MCT and the decrease of this fraction in the groups fed LCT was advantageous. This apparent paradox can be explained by the difference in the composition of the respective TG. Accumulation of TG containing larger amounts of medium-chain acids and relatively smaller amounts of palmitate and stearate was beneficial whereas the accumulation of TG consisting mainly of palmitate and stearate probably was harmful to the animal.

ACKNOWLEDGMENTS

The authors thank Alec Robertson of Drew Chemical Corporation, Boonton, New Jersey for the MCT and LCT and Priscilla Knapp of Hoffmann-La Roche, Inc., Nutley, New Jersey for the vitamins used in the diets.

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^{65}Zn and Stable Zinc Absorption, Excretion and Tissue Concentrations as Affected by Type of Diet and Level of Zinc in Normal Calves[1]

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ABSTRACT The effects of dietary zinc level (2 vs. 38 ppm) in a purified diet and of a practical versus a purified diet with the same zinc level (38 ppm) on ^{65}Zn metabolism were determined following a single oral dosing in 2.5- and 4.5-month-old male Holstein calves. The 12 animals were killed 14 days after ^{65}Zn dosing and before those given the zinc-deficient diet developed any clinical indication of a deficiency. Calves fed the low zinc purified diet absorbed considerably more ^{65}Zn, but there was little overall difference between groups fed the practical-type diet and the purified diet with added zinc. Those given the practical diet had a higher fecal endogenous ^{65}Zn excretion the second week after dosing. Younger calves in the first experiment absorbed a much higher percentage of ^{65}Zn from diets with adequate zinc than older ones in the later experiment. There was no difference in net ^{65}Zn retention due to age among those given the zinc-deficient diet, suggesting that differences associated with age were not caused by reduced ability of animals to absorb zinc but rather to effects of other factors on the homeostatic control mechanism. Tissue samples from calves given the deficient diet retained much more of the administered ^{65}Zn dose in all tissues than those given zinc-adequate diets. When results were calculated relative to net absorbed dose, the more active metabolic tissues including liver, heart, lung, kidney, spleen, testicles and digestive tract sections from calves fed the zinc-deficient diet still retained much more ^{65}Zn than comparable tissues of the other calves: this effect was greater in the younger calves. As percentage of absorbed dose, diet did not have marked effects on ^{65}Zn in tissues which metabolize ^{65}Zn more slowly, including hair, scrotum, muscle and bone. Most tissues from calves fed the zinc-deficient diet contained somewhat less stable zinc than those from animals fed adequate zinc. Dry matter content of tissues was not affected by the diets.

The importance of zinc in the health of animals and the many serious consequences of a deficiency are well-established, including several studies with ruminants (1–8). ^{65}Zn has been used to investigate some aspects of zinc metabolism in ruminants (9–13). When the same zinc-deficient diet was used, beginning one week before ^{65}Zn dosing, zinc-deficient ruminants had a higher net absorption of ^{65}Zn than normal animals (10). Retention of ^{65}Zn in more active tissues tended to be larger and ^{65}Zn turnover rate reduced in deficient animals (10). Zinc-deficient ruminants also had a lower endogenous fecal excretion of ^{65}Zn (9). In normal animals, feeding a diet with a higher zinc content resulted in increased endogenous excretion (9). However, effects of level of dietary zinc on zinc absorption apparently have not been studied previously in normal ruminants.

The present study was conducted to investigate the effects on zinc metabolism of adding a nutritionally adequate amount of zinc to a zinc-deficient purified diet when all animals were normal. ^{65}Zn metabolism also was investigated in comparable animals fed a practical-type diet containing approximately the same zinc level as the zinc-supplemented purified diet. The effects of these dietary treatments were studied in 2.5- and 4.5-month-old calves in separate experiments.

EXPERIMENTAL PROCEDURE

Three experimental diets[2] were used: a) a zinc-deficient purified diet; b) the same purified diet plus 36 ppm of supplemental zinc as ZnO; and c) a practical-type diet. The zinc-deficient purified diet contained 2 ppm zinc by analysis and was the same

Received for publication September 29, 1967.
[1] University of Georgia, College of Agriculture Experiment Stations Journal Series Paper no. 151, College Station, Athens, and Institute of Comparative Medicine Paper no. 686. Supported in part by Public Health Service Research Grant no. AM-07367-NTN from the National Institute of Arthritis and Metabolic Diseases.
[2] See footnote 2, next page.

TABLE 1

Age, body weight, feed consumption and fecal ^{65}Zn excretion of calves used in experiments A and B

Calf no.	Diet	Exp.	Age at dosing	Body wt avg during experiments	Feed consumption	^{65}Zn fecal excretion 13-day total [1]
			days	*kg*	*kg/day*	
1	Purified + Zn	A	87	81	1.70	56.4
6	Purified + Zn	A	63	60	1.36	42.2
	Avg		75	70	1.53	49.3
9	Purified + Zn	B	139	146	2.72	84.5
12	Purified + Zn	B	129	125	2.49	88.7
	Avg		134	136	2.60	86.6
2	Purified deficient	A	77	62	1.42	24.9
4	Purified deficient	A	74	75	1.51	32.8
	Avg		76	68	1.46	28.9
7	Purified deficient	B	141	134	2.61	32.3
10	Purified deficient	B	138	152	2.95	19.5
	Avg		140	143	2.78	25.9
3	Practical	A	76	76	1.59	55.6
5	Practical	A	69	69	1.51	66.6
	Avg		72	73	1.55	61.1
8	Practical	B	140	147	2.84	72.7
14	Practical	B	118	107	2.15	83.3
	Avg		129	127	2.50	78.0

[1] As percentage of single oral dose.

as that described previously (9), except the following were used per 100 kg: riboflavin (50%), 2.0 g; Ca DL-pantothenate (equivalent to 45% D-pantothenic acid), 3.3 g; vitamin A palmitate (325,000 IU/g), 17.6 g; and oxytetracycline (55 g/kg), 88 g.

The practical-type diet consisted of the following per 100 kg: ground corn grain, 65 kg; soybean meal (44% protein), 21 kg; dried citrus pulp, 10 kg; $CaCO_3$ (marble dust), 1.43 kg; dicalcium phosphate (anhydrous, food grade), 1.0 kg; NaCl, 444 g; $Fe_2(SO_4)_3 \cdot xH_2O$, (20% Fe by assay) 7.8 g; $MnSO_4 \cdot H_2O$, 1.4g; $CuSO_4 \cdot 5H_2O$, 0.98 g; $CoCO_3$ (45–50% Co by assay), 0.012 g; KI, 0.008 g; ZnO, 1.87 g; vitamin A palmitate (20,000 IU/g), 220 g; vitamin D_3 (200,000 IU/g), 2.2 g; and oxytetracycline (55 g/kg), 88 g. It contained 38 ppm zinc.

Twelve uncastrated male Holstein calves were fed a practical-type diet including milk replacer and calf starter before the experiment. The calves were adjusted to metabolism crates and given one of the three experimental diets for 7 days before a single oral dose of 330 μCi of ^{65}Zn, which was administered via gelatin capsules as described previously (10). The ^{65}Zn contained 1711 μCi of ^{65}Zn/mg of zinc. The study was conducted in 2 parts, experiment A and experiment B, with 2 animals per diet in each experiment. The animals used in experiment A averaged 2.5 months of age and those in experiment B, 4.5 months (table 1).

Total fecal collections were made daily for 13 days following ^{65}Zn dosing. In experiment B, total urinary excretion was also determined. Blood samples were obtained at specified intervals. Fourteen days following ^{65}Zn dosing, the animals were anesthetized by injection of sodium pentobarbital and killed by cannulation of the carotid artery to remove blood from the organs and tissues. The tissue samples were frozen until analyzed for stable zinc and ^{65}Zn. The tissues were sampled and analyses made as described previously (11). In experiment B, the contents were washed out of the sections of the gastrointestinal tract and section samples taken as follows: (a) rumen-reticulum, whole organ; (b) abomasum, whole organ; (c) duodenum,

[2] Appreciation is extended to the Kraft Foods Company, Garland, Texas, for dried whole whey; to the Chas. Pfizer Company, Terre Haute, Indiana, for antibiotics and vitamins; to Dawe's Laboratories, Inc., Chicago, for vitamin A; to Hoffmann-LaRoche, Inc., Nutley, New Jersey, for the biotin; to the Commercial Solvents Company, New York, for choline; to Merck and Company, Rahway, New Jersey, for pyridoxine and vitamin B_{12}; to Distillation Products Industries, Rochester, New York, for *d*-α-tocopheryl acetate; to American Cyanamid Company, Princeton, New Jersey, for folic acid; to Abbott Laboratories, North Chicago, Illinois, for menadione; to Basic, Incorporated, Cleveland, for magnesium oxide; and to the Allied Chemical Company, Atlanta, Georgia, for urea.

first 1.83 m of the small intestine; (d) small intestine 2, second 1.83 m of small intestine; (e) small intestine 3, 3.6 m from the approximate center of the remaining small intestine; (f) cecum, whole organ; and (g) large intestine, whole organ.

Contents of the last 2 sections also were taken in experiment A. The various samples of feed, feces, urine and tissues were analyzed for stable zinc by atomic absorption spectrophotometry following nitric-perchloric-sulfuric acid wet ashing of samples (14). The ^{65}Zn analyses on feces, blood, and urine samples were made with an automatic gamma test tube changer system with a NaI (TI) well crystal.[3] ^{65}Zn content of the various organs and tissues was determined with a whole-body counter designed for small samples and a 400 channel multichannel analyzer.[4]

The tissue and blood ^{65}Zn concentration data were adjusted to the average body size for all calves within experiments A and B (10). This has the effect of calculating the data to a uniform dosing level per kilogram of body weight. Thus for experiment A, level of ^{65}Zn was 4.7 μCi/kg, and for experiment B, 2.44 μCi/kg of body weight.

As determined by frequent clinical examination, there was never any indication of a zinc deficiency in any animal. The clinical examination by a trained clinician is still regarded as the most reliable way to determine whether a given animal has a zinc deficiency (6).

RESULTS

The daily rate and accumulated total fecal excretion of ^{65}Zn by the calves are presented in figures 1A and 1B. The rate reached a peak on the second day after dosing and declined sharply thereafter. For the first 5 days the animals fed the zinc-deficient purified diet excreted considerably less ^{65}Zn than either of the groups fed a normal amount of zinc. During this time and for the total period there was not a clearly defined difference between groups fed the purified diet with added zinc and those given the practical-type diet with a comparable level of zinc.

Presumably, ^{65}Zn excreted via feces after the first week following the single oral dosing represents that of endogenous origin, as essentially all the undigested feed consumed at time of dosing is excreted before this time (15). When calculated in terms of that retained by the animals after 7 days, those fed the zinc-deficient diet had a much lower (significant at 1% level) endogenous loss of ^{65}Zn than either of the other groups (fig. 1A and table 2). Both totally and as a percentage of retained ^{65}Zn, calves fed the practical-type diet excreted appreciably more endogenous ^{65}Zn than either of the other groups (fig. 1A and table 2). When fed the zinc-deficient purified diet, there was little difference in endogenous fecal loss between the

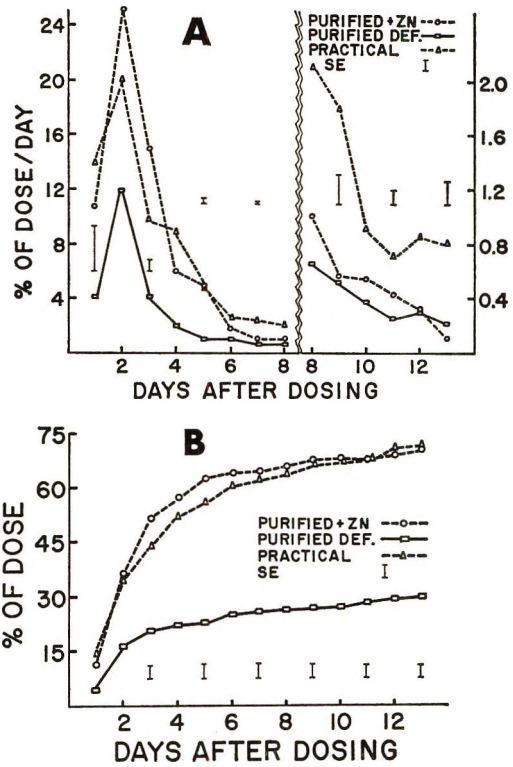

Fig. 1 Effect of dietary zinc level and type of diet on fecal ^{65}Zn excretion from a single oral dose by normal calves: (A) daily rate, and (B) accumulated total. The SE is 1 standard error of a treatment mean calculated from error mean square. Four animals/treatment.

[3] Manufactured by Baird Atomic, Cambridge, Massachusetts.

[4] The whole-body counter was manufactured by Metrix, Inc., Deerfield, Illinois, and the multichannel analyzer by Technical Measurements, Inc., North Haven, Connecticut.

TABLE 2
Endogenous fecal ^{65}Zn excretion for days 8 to 13 calculated as a percentage of net ^{65}Zn absorption after 7 days

	Fecal ^{65}Zn retention			
	Purified + Zn diet	Purified deficient diet	Practical diet	SE [1]
	% of absorbed dose/calf			
Exp. A	4.6[a] [2] (2)[3]	3.7[a] (2)	14.6[b] (2)	
Exp. B	22.8[c] (2)	2.6[a] (2)	25.9[c] (2)	
Avg	13.7[x] (4)	3.1[y] (4)	20.3[z] (4)	1.1

[1] SE is standard error of a treatment mean for 4 calves.
[2] Values not followed by the same letter are significantly different at the 1% level. Those followed by *a, b, c* tested separately from those with *x, y, z*.
[3] Numbers in parentheses indicate number of calves/group.

younger and older calves (exp. A vs. B) (table 2). However, when adequate zinc was fed, endogenous ^{65}Zn excretion was much higher in older calves.

In groups fed the zinc-deficient purified diet, there was little difference between the two age groups in total fecal ^{65}Zn excretion for the 13-day period (table 1). However, the younger calves (exp. A) fed the other 2 diets excreted much less ^{65}Zn via feces than the older ones. Likewise, difference between age groups was less with the practical diet than with the zinc-supplemented purified diet.

Specific activity data of the fecal samples are presented in figure 2. These values are presented as the percentage of the ^{65}Zn dose administered which was excreted per milligram of zinc, rather than in terms of absolute activity, because of the greater usefulness in biological interpretation of the data (10). Throughout the 13-day collection period feces from calves fed the zinc-deficient purified diet had much higher specific activities than those from the other groups (fig. 2). This was opposite to the ^{65}Zn excretion values as a percentage of the dose, and was due to the much larger effect of the zinc-deficient purified diet on stable zinc excretion (table 3) in comparison with ^{65}Zn levels. For a few days after dosing, there was comparatively little difference in specific activity values for animals fed the practical diet and the purified + zinc diet (fig. 2). However, for the last several days, specific activity was higher in feces of those given the practical diet with a comparable level of zinc. This effect is largely a reflection of the lower ^{65}Zn excretion by the group given the purified + zinc diet during this time (fig. 1A).

Fecal zinc levels were many times higher for animals fed diets containing normal amounts of zinc relative to those fed the deficient diet (table 3). These data are in agreement with previous work in which diets with comparable levels of zinc have been used (1, 16, 17). No clearly defined difference in fecal zinc levels was noted between animals given purified plus zinc and the practical-type diets. However, when fed diets with adequate zinc younger animals (exp. A) excreted feces with a lower content of zinc (table 3). This age effect was not observed in those given the zinc-deficient diet. Level of zinc in urine and total zinc excreted per day in urine were not significantly influenced ($P = 0.05$) by dietary treatments (table 3).

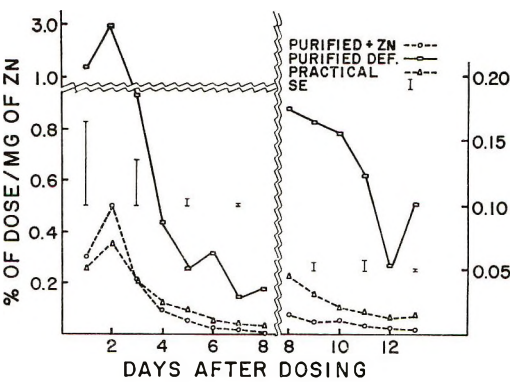

Fig. 2 Effect of dietary zinc level and type of diet on specific activity of zinc in feces of normal calves following a single oral dosing. SE is 1 standard error of a treatment mean calculated from error mean square. Four animals/treatment.

TABLE 3

Effects of dietary zinc level and type of diet on average zinc content of feces, urine, and serum of normal calves

	Purified + Zn diet			Purified-deficient diet			Practical diet			
	Exp. A (2) [1]	Exp. B (2)	Avg (4)	Exp. A (2)	Exp. B (2)	Avg (4)	Exp. A (2)	Exp. B (2)	Avg (4)	SE [2]
Feces, μg Zn/g dry matter	130.8	201.2	166.0[a] [3]	11.9	12.2	12.0[b]	161.8	216.2	189.0[a]	12.3
Feces, mg Zn/day	31.0	83.4	57.2[a]	2.7	5.2	4.0[b]	34.8	84.2	59.5[a]	3.5
Urine, μg Zn/ml	—	0.048	0.048 [1]	—	0.051	0.051 [1]	—	0.056	0.056 [1]	—
Urine, mg Zn/day	—	0.49	0.49[a]	—	0.48	0.48[a]	—	0.91	0.91[a]	—
Serum, μg/Zn/ml	1.12	1.44	1.28[a]	0.84	0.78	0.81[b]	1.24	1.36	1.30[a]	0.07

[1] Numbers in parentheses indicate number of calves/group. Average for urine is for 2 animals/treatment.

[2] SE is standard error of a treatment mean for 4 animals/treatment.

[3] Average values followed by the same letter are not significantly different at the 5% probability level.

Whole blood levels of ^{65}Zn are presented in figure 3. Within 24 hours after dosing, approximate peak values had been obtained in most cases. After about 2 days, there was a moderate decline. When calculated as a percentage of the administered dose, ^{65}Zn content was appreciably higher in whole blood of calves fed the zinc-deficient purified diet (fig. 3A). However, when the data were calculated relative to the net absorption after 13 days, no large differences were noted among the groups (fig. 3B). Toward the end of the period, those given the purified diet with added zinc tended to have a moderately lower level of ^{65}Zn in the blood than the others.

The ^{65}Zn level and specific activity in blood serum was higher at 2 days after dosing than at later times (only specific activity values are shown) (fig. 4). The rate of decline in ^{65}Zn and specific activity levels in serum became slower as time progressed in general agreement with previous work in ruminants following a single oral dosing of ^{65}Zn (10, 11). When calculated as a percentage of the administered dose, calves fed the zinc-deficient purified diet had higher serum ^{65}Zn and specific activity levels than the others (fig. 4A). However, when calculated as a percentage of dose not excreted via feces during the 13-day collection period after dosing, serum ^{65}Zn levels were lower for calves fed the deficient diet than for the others. Relative to net absorbed dose, the effects of the zinc-deficient diet on serum specific activity largely disappeared (fig. 4B).

The amounts of ^{65}Zn retained in various tissues 14 days after the single oral dose are presented in table 4. Liver contained the highest concentration followed by spleen, heart, lung, kidney, testicles and rib bone. The rib always showed an appreciably higher ^{65}Zn concentration than the tibia. As a percentage of the administered dose, all the tissues from the calves fed the zinc-deficient diet contained much more ^{65}Zn than those from calves fed an adequate amount of zinc (table 4). Since their total body burden of ^{65}Zn was also much higher, the amounts were calculated as a percentage of that which had not been excreted via feces (table 5). This gives an approximate percentage of the total body

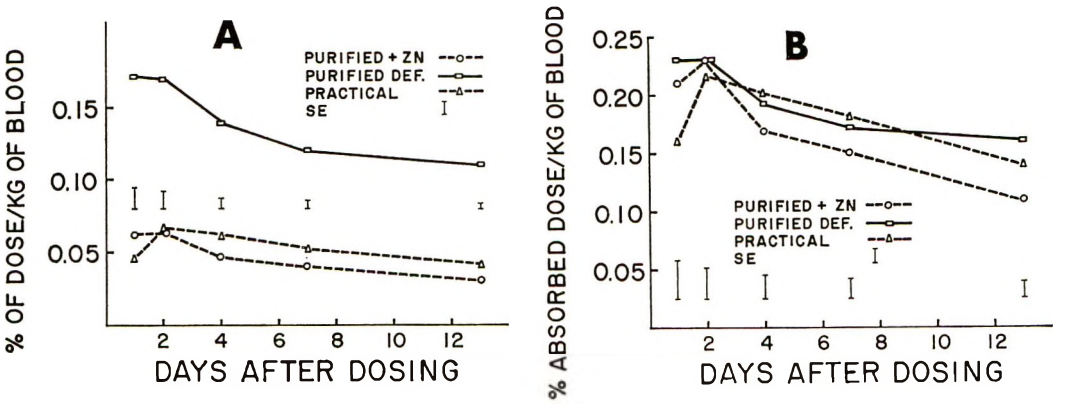

Fig. 3 Effect of dietary zinc level and type of diet on ^{65}Zn content of whole blood from normal calves following a single oral dosing: (A) calculated as percentage of administered dose per kg of blood (5- or 10-g sample counted), and (B) calculated as percentage of absorbed dose. The SE is 1 standard error of a treatment mean calculated from error mean square. Four animals/treatment.

burden, as very little is excreted via urine or other routes. As a percentage of the net absorbed dose, liver, heart, lung, kidney, spleen, testicles, and skin from zinc-deficient animals contained much more ^{65}Zn than comparable tissues from animals fed either of the zinc-adequate diets. This effect was much more pronounced in the younger animals (exp. A) than in the older ones (exp. B) (table 5). Level of zinc in the diet had little effect on these ^{65}Zn values in bone and hair. Muscles of zinc-deficient animals had somewhat more ^{65}Zn (not significant at 5% level), relative to net absorbed dose, than those from animals fed the purified diet plus zinc, but not more than those from animals fed the practical-type diet. The ^{65}Zn content of most tissues was essentially the same in animals fed the purified diet with adequate zinc and the practical-type diet (tables 4 and 5). However, ^{65}Zn level was higher in hair from animals fed the practical-type diet.

In general the specific activity data of tissues reflect the same phenomena as the ^{65}Zn content data (table 6). After adjusting

TABLE 4

Effect of dietary zinc level and type of diet on ^{65}Zn retention relative to administered dose in tissues of normal calves

Tissue	^{65}Zn retention			
	Purified + Zn diet (4)[1]	Purified deficient diet (4)	Practical diet (4)	SE [2]
	% of dose/kg fresh tissue			
Liver	0.47[a] [3]	2.73[b]	0.60[a]	0.19
Heart	0.19[a]	1.59[b]	0.25[a]	0.06
Lung	0.24[a]	1.59[b]	0.30[a]	0.06
Kidney	0.21[a]	1.52[b]	0.28[a]	0.07
Spleen	0.28[a]	2.18[b]	0.39[a]	0.11
Testicles	0.13[a]	1.05[b]	0.17[a]	0.04
Scrotum (unshaved)	0.10[a]	0.39[b]	0.16[a]	0.04
Skin (clipped)	0.09[a]	0.47[b]	0.11[a]	0.02
Hair	0.14[a]	0.38[b]	0.28[c]	0.02
Muscle (round)	0.16[a]	0.62[b]	0.24[a]	0.04
Rib	0.37[a]	0.86[b]	0.46[a]	0.07
Tibia, whole	0.20[a]	0.45[b]	0.27[a]	0.04

[1] Numbers in parentheses indicate number of calves/group.
[2] SE is standard error of a treatment mean for 4 animals.
[3] Within each tissue values not followed by the same letter are significantly different at the 5% probability level.

TABLE 5

Influence of dietary zinc level and type of diet on distribution of net absorbed ^{65}Zn in tissues in 2.5-month-old (exp. A) and 4.5-month-old (exp. B) calves

Tissue [1]	Net absorbed ^{65}Zn in tissues									
	Purified + Zn diet			Purified-deficient diet			Practical diet			
	Exp. A (2)[2]	Exp. B (2)	Avg (4)	Exp. A (2)	Exp. B (2)	Avg (4)	Exp. A (2)	Exp. B (2)	Avg (4)	SE [3]
	% of absorbed ^{65}Zn dose/g of Zn									
Liver	1.50	1.59	1.54[a 4]	4.54	2.95	3.75[b]	1.92	2.09	2.01[a]	0.23
Heart	0.55	0.79	0.67[a]	2.50	1.89	2.19[b]	0.82	0.86	0.84[a]	0.06
Lung	0.70	1.01	0.85[a]	2.68	1.72	2.20[b]	1.04	0.94	0.99[a]	0.08
Kidney	0.61	0.85	0.73[a]	2.70	1.50	2.10[b]	0.90	0.96	0.93[a]	0.06
Spleen	0.83	1.18	1.00[a]	3.61	2.40	3.01[b]	1.27	1.33	1.30[a]	0.11
Testicles	0.38	0.53	0.46[a]	1.84	1.06	1.45[b]	0.57	0.58	0.58[a]	0.05
Scrotum (unshaved)	0.32	0.34	0.33[a]	0.70	0.39	0.55[a]	0.67	0.35	0.51[a]	0.10
Skin (clipped)	0.30	0.29	0.29[a]	0.86	0.44	0.65[b]	0.37	0.31	0.34[a]	0.05
Hair	0.43	0.51	0.47[a]	0.65	0.39	0.52[a]	1.12	0.59	0.85[b]	0.08
Muscle (round)	0.49	0.53	0.51[a]	1.10	0.64	0.87[a]	0.92	0.63	0.77[a]	0.12
Rib	1.03	1.60	1.31[a]	1.30	1.12	1.21[a]	1.44	1.66	1.55[a]	0.16
Tibia, whole	0.57	0.82	0.70[a]	0.76	0.52	0.64[a]	0.91	0.89	0.90[a]	0.09

[1] The interaction between dietary treatments and experiments was significant at the 1% level for heart, lung, kidney, spleen and testicles, and at the 10% level for liver and skin. This interaction was not significant at the 10% level for the other tissues.

[2] Numbers in parentheses indicate number of calves/group.

[3] SE is standard error of a treatment mean for 4 animals.

[4] Within each tissue values for dietary treatments not followed by the same letter are significantly different at the 1% level except for hair which was significant at the 5% level.

TABLE 6

Influence of dietary zinc level and type of diet on the ^{65}Zn specific activity of tissues from 2.5-month-old (exp. A) and 4.5-month-old (exp. B) calves

Tissue [1]	^{65}Zn specific activity of tissues								
	Purified + Zn diet			Purified-deficient diet			Practical diet		
	Exp. A (2)[2]	Exp. B (2)	Avg (4)	Exp. A (2)	Exp. B (2)	Avg (4)	Exp. A (2)	Exp. B (2)	Avg (4)	SE [3]
				% of absorbed ^{65}Zn dose/g of Zn						
Liver	49.3	64.3	56.8[a] [4]	213.2	130.7	172.0[b]	72.7	65.2	69.0[a]	4.8
Lung	49.4	64.8	57.1[a]	203.3	131.5	167.4[b]	81.9	62.5	72.2[a]	6.7
Kidney	36.8	70.6	53.7[a]	162.9	114.7	138.8[b]	46.8	62.0	54.4[a]	7.4
Testicles	28.6	48.6	38.6[a]	151.8	103.0	127.4[b]	39.8	51.1	45.5[a]	4.8
Hair	3.1	5.3	4.2[a]	5.5	4.8	5.2[ab]	9.1	6.6	7.8[b]	0.8
Muscle (round)	28.0	28.3	28.2[a]	58.8	42.6	50.7[b]	25.0	30.7	27.8[a]	5.3
Rib	16.0	36.2	26.1[a]	26.7	29.9	28.3[a]	22.7	37.6	30.2[a]	3.7
Tibia (joint)	10.6	16.4	13.5[a]	16.3	16.8	16.5[a]	15.5	18.8	17.1[a]	3.4
Tibia (shaft)	9.3	13.7	11.5[a]	14.8	8.8	11.8[a]	13.3	16.7	15.0[a]	2.0

[1] The interaction between dietary treatments and experiments was significant at the 1% level for testicles and liver, and at the 5% level for the kidney and lung, but it was not significant at the 10% level for the other tissues.

[2] Numbers in parentheses indicate number of calves/group.

[3] SE is standard error of a treatment mean for 4 animals.

[4] Within each tissue values for dietary treatments not followed by the same letter are significantly different at the 1% level except for muscle which was significant at the 5% level.

for net absorption, most tissues (except for hair, bone and muscle) of calves fed the zinc-deficient diet had much higher specific activities than those from comparable animals fed adequate zinc. Again this effect was much greater in the younger animals (exp. A). Hair from animals fed the practical-type diet had a higher specific activity than that from those fed the zinc-adequate purified diet.

Total zinc and dry matter content of many of the tissues are presented in table 7. Most tissues from animals fed the zinc-deficient diet contained less zinc than those from animals given an adequate amount of zinc in the diet. This was true even though there was no clinical indication of a zinc deficiency for any animal receiving this diet. No consistent trend was noted in zinc levels of tissues from animals fed the 2 types of diets with adequate zinc. Dry matter content of the tissues was not materially affected by any of the diets (table 7).

The ^{65}Zn content of the various sections of the gastrointestinal tract as percentages of the net absorbed dose are presented in table 8. In every tissue from the abomasum through the large intestine, there was a higher level of ^{65}Zn in animals fed the zinc-deficient purified diet than in those fed the other diets.

In table 8, data are presented on the ^{65}Zn level in the dry matter found in the contents of the several sections of the gastrointestinal tract 14 days after the ^{65}Zn dosing. Generally these quantities are variable within animals fed the same diet, as shown by the relative size of the standard errors. Those fed the zinc-deficient purified diet had a higher level of ^{65}Zn in contents of the second section of the small intestine than the others. There was more ^{65}Zn in the contents of the cecum and large intestine of animals fed the practical diet than in those given either of the purified diets. This much higher level is in agreement with the larger endogenous fecal loss for the same animals just before the time the samples were collected.

DISCUSSION

These results show quite conclusively that level of dietary zinc has a large effect

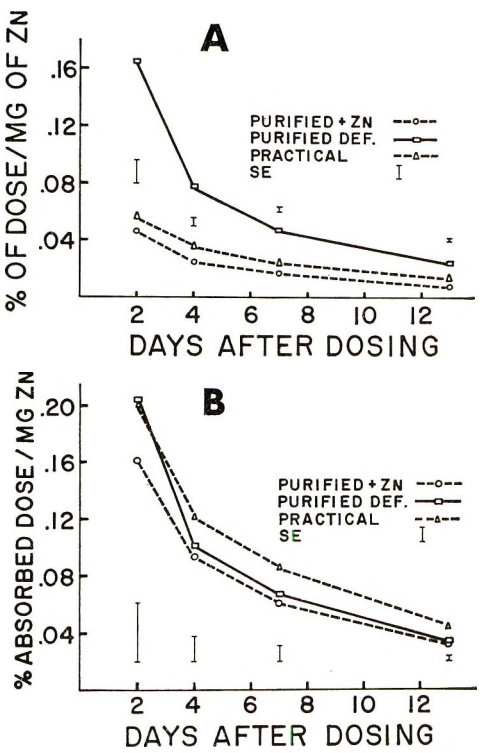

Fig. 4 Effect of dietary zinc level and type of diet on specific activity of zinc in blood serum from normal calves following a single oral dosing: (A) specific activity of zinc calculated as percentage of administered dose, and (B) specific activity of zinc calculated as percentage of absorbed dose. The SE is 1 standard error of a treatment mean calculated from error mean square. Four animals/treatment. The average serum zinc levels for animals fed the 3 diets were as follows: (in μg of zinc/ml) a) purified + Zn, 1.28; purified-deficient, 0.80; and practical, 1.30. Average concentration for each treatment was relatively uniform throughout the study.

on percentage of zinc absorbed when the calves have been fed the different dietary levels for a week before the ^{65}Zn test dose. Also, endogenous losses are reduced when level of zinc in the diet is low. A clinical zinc deficiency, independent of dietary zinc content, increases zinc absorption (10) and reduces endogenous excretion (9). Thus, it is evident that calves have a homeostatic control mechanism for zinc which operates both through changes in zinc absorption and endogenous losses. This conclusion is in agreement with that of Cotzias et al. (18) from mouse data. It seems probable that changes at the tissue level are responsible for the homeostatic control changes which occur in zinc absorption and endogenous excretion. Although these and previous results (9, 10, 11) give some insight into the homeostatic control mechanism for zinc in cattle, there are still many more questions than answers. The speed with which the mechanism is effective has not been established fully; moreover, the modifying effects or lack of them by other dietary factors is largely unknown; nor has the biochemical nature of the mechanism been established. There appear to be many parallels in the homeostatic control of zinc and iron (19).

The rate of decline in fecal endogenous ^{65}Zn excretion during the 8- to 13-day period (fig. 1A) was much more rapid than that observed previously when a single intravenous dose was given (9). This difference in rate of decline suggests that some ^{65}Zn from the oral dose may be metabolized somewhat differently from that given intravenously. Possibly this might result from greater retention from the oral dose in certain parts of the intestinal tract mucosa which is later sloughed and becomes a part of the endogenous excretion.

A much larger proportion of the ^{65}Zn body burden was retained by calves fed the zinc-deficient diet in the tissues which metabolize zinc more rapidly. This probably indicates that many binding sites for zinc were depleted within the 7-day period this diet was fed before ^{65}Zn dosing. These sites are perhaps the ones which have a lower tenacity for zinc than those which are responsible for the symptoms of a zinc deficiency. The effects of the deficient diet were exhibited more quickly on ^{65}Zn distribution in tissues than are the clinical manifestations of a deficiency (6).

The higher ^{65}Zn absorption, when fed diets with a normal level of zinc, by the younger calves in experiment A, compared with experiment B is in agreement with previous findings (12). That the effect of age was not present when the animals were given the zinc-deficient diet could be interpreted to suggest that the age influence was an indirect one. The ability of the older animals, fed the zinc-deficient diet, to absorb as much ^{65}Zn as the younger ones given the same diet suggests that absorp-

TABLE 7

Effect of dietary zinc level and type of diet on stable zinc and dry matter content in tissues of normal calves

Tissue	Stable zinc				Dry matter			
	Purified + Zn diet (4)[1]	Purified deficient diet (4)	Practical diet (4)	SE[2]	Purified + Zn diet (4)	Purified deficient diet (4)	Practical diet (4)	SE
	μg Zn/g dry tissue				%	%	%	
Liver	101.4[a 3]	83.7[a]	111.1[a]	11.7	26.6[a]	26.2[a]	26.9[a]	0.71
Lung	81.1[a]	72.5[a]	79.6[a]	10.6	18.6[a]	18.2[a]	17.4[a]	0.91
Kidney	73.2[a]	76.5[a]	84.5[a]	5.8	19.7[a]	19.6[a]	20.9[b]	0.28
Testicles	78.9[a]	69.7[a]	82.3[a]	4.5	15.8[a]	16.2[a]	15.6[a]	0.43
Hair	128.7[a]	111.0[b]	114.2[b]	3.0	91.0[a]	89.6[a]	91.9[a]	1.00
Muscle	86.4[a]	77.8[a]	129.0[a]	16.1	21.0[a]	22.0[ab]	22.2[b]	0.30
Tibia (joint)	84.9[a]	62.2[a]	80.4[a]	7.0	62.0[a]	68.6[a]	68.4[a]	2.61
Tibia (shaft)	71.4[a]	64.0[a]	76.3[a]	6.6	85.5[a]	85.0[a]	84.1[a]	1.32
Rib	78.8[a]	61.8[b]	81.4[a]	4.2	69.1[a]	70.2[a]	68.0[a]	1.61

[1] Numbers in parentheses indicate number of calves/group.
[2] SE is standard error of a treatment mean for 4 animals.
[3] Within each tissue values not followed by the same letter are significantly different at the 5% level.

TABLE 8

Influence of dietary zinc level and type of diet on ^{65}Zn retention in digestive tract tissues and contents of normal calves in experiment B

Section	^{65}Zn retention in digestive tract tissues				^{65}Zn retention in digestive tract dry contents			
	Purified + Zn (2)[1]	Purified deficient (2)	Practical (2)	SE[2]	Purified + Zn (2)	Purified deficient (2)	Practical (2)	SE
	% of absorbed dose/kg fresh tissue				*% of absorbed dose/kg dry contents*			
Rumen-reticulum	1.06[3]	—	1.10[3]	—	2.96[a 4]	0.56[a]	4.85[a]	1.36
Abomasum	0.77[a 4]	0.89[b]	0.80[ab]	0.02	6.06[a]	0.28[a]	1.54[a]	1.54
Duodenum	0.78[a]	1.42[b]	0.87[a]	0.06	3.57[a]	3.24[a]	3.03[a]	1.23
Small intestine, no. 2	0.81[a]	1.66[b]	0.90[a]	0.05	2.06[a]	4.43[b]	3.07[c]	0.20
Small intestine, no. 3	0.78[a]	1.52[b]	0.85[a]	0.04	2.27[a]	2.86[a]	2.94[a]	0.38
Cecum[5]	0.94[a]	1.28[b]	1.01[a]	0.03	1.98[a]	1.16[a]	5.11[b]	0.70
Large intestine[5]	0.86[a]	1.21[b]	0.91[ab]	0.08	2.10[a]	1.51[a]	5.72[b]	0.80

[1] Numbers in parentheses indicate number of calves per treatment.
[2] SE is standard error of a treatment mean for 2 animals except 4 for contents of cecum and large intestine.
[3] One animal.
[4] Average values not followed by the same letter are significantly different at the 5% level.
[5] Four animals/treatment for these sections of contents but not of tissues.

tion ability per se is not reduced with age in the ranges involved here. The much larger effect of the deficient diet on ^{65}Zn retention in the metabolically more active tissues of younger calves suggests a greater tissue affinity for zinc in these animals (tables 5 and 6). However, in interpreting these results it is essential to consider that experiments A and B were not conducted concurrently. Thus, the effects of age are confounded with any unknown effects associated with time, such as different batches of diets and other possible variables.

The influence of a zinc deficiency on stable zinc content of tissues from animals that had received the same diet for at least several days before sampling were studied recently (16). Effects of the deficiency on tissue zinc content were not as large as those in most earlier reports in which dietary zinc level at time of sampling was confounded with the effects of the clinical deficiency (1, 16). The data in the present study indicate that level of zinc in the diet has some effect on zinc content in tissues even when there are no indications of a deficiency. Apparently, the effects of a zinc-deficient diet on tissue zinc levels have 2 components, with one occurring quickly and the other much more slowly. As shown in this study, within one week after the

deficient diet is given there is a reduction in zinc content of many tissues. Following this time, level of zinc does not change rapidly (16). Subsequently, when animals develop a clinical deficiency a further reduction occurs in zinc content of some tissues (16). However, in most tissues of ruminants, the reduction in tissue zinc is not really large. Thus, when the small influence of the zinc-deficient diet on total zinc content of tissues and the very large effect on ^{65}Zn distribution from a single oral dose are considered together, it appears that some zinc in these tissues may be much more labile and rapidly metabolized than other fractions. It seems probable that depletion or lack of depletion at the more labile binding sites is somehow reflected back to the intestine resulting in increased or decreased absorption of zinc.

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Vitamin A Activity of β-Apo-Carotenals in Coturnix Coturnix Japonica[1,2]

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ABSTRACT A study was made to determine the vitamin A activity of β-apo-carotenals for Coturnix coturnix japonica (Japanese quail). β-Apo-8′-carotenal, β-apo-10′-carotenal and β-apo-12′-carotenal prepared from β-carotene and synthetic β-apo-8′-carotenal and β-apo-12′-carotenal were used. Growth, viability, egg production, fertility, hatchability of eggs, and viability and growth of progeny demonstrate that these β-apo-carotenals are vitamin A-active compounds. When the β-apo-carotenals were fed, substantial quantities of vitamin A were found in blood serum, liver and egg yolk, which are further evidence of the vitamin activity of β-apo-carotenals. Vitamin A content of blood serum and liver of male quail was higher than that of females. The data indicate that these β-apo-carotenals can be used as a sole source of vitamin A for Japanese quail.

β-Apo-8′-carotenal and β-apo-12′-carotenal are vitamin A-active for rats (1,2)[4]; and β-apo-10′-carotenal also is reported to have vitamin A activity (2).[5] When β-apo-8′-carotenal was the sole source of vitamin A activity it was equivalent to 1.20×10^6 USP units vitamin A/g, based on growth of rats (3). β-Apo-8′-carotenal has vitamin A value for chickens and is a coloring agent for egg yolks (4); however, reports indicate activity too low for use as a vitamin A source (5).[6] The present study with quail as the test animal was to help clarify the role of β-apo-carotenals as vitamin A sources for animals.

EXPERIMENTAL METHODS

Newly hatched Coturnix coturnix japonica[7] (Japanese quail) were fed a 28% protein, carotenoid-free, vitamin A-deficient diet (table 1) for 12 days. At 12 days of age the quail were distributed at random into 5 test groups of 10 birds each and a control group of 20 birds. The chicks were raised in wire-floor batteries in a room at 23°, using higher brooder temperatures (30–33°) for the first 2 weeks. Feed and water were available at all times.

Three β-apo-carotenals were prepared by oxidation of β-carotene, separated chromatographically and the purity was determined (6). Two synthetic β-apo-carotenals also were used (table 2). The supplements were dissolved in diethyl ether and added to 20 ml cottonseed oil. The ether was evaporated under reduced pressure and temperature of 50°. Each compound (approximately 7 mg dissolved in oil) was mixed with 1 kg of basal diet (table 1). Feeds containing the supplements were stored in the dark below 0°, and only enough for a 24-hour feeding was removed each day. When the birds were about 30 days old, they were changed from the starter to a carotenal-supplemented layer diet, similar to the starter diet except for lower protein (20%) and higher calcium and energy content. Fresh feed was prepared weekly, and the test lasted 8 weeks. Quail were weighed at the start of the test period and at weekly intervals (fig. 1). Egg production is reported as weekly averages per hen per group (table 2). Livers and eggs were obtained from birds selected at random from each group for determination of vitamin A content.

To determine hatchability of eggs from hens raised with the experimental diets,

Received for publication August 8, 1967.

[1] Contribution no. 75, Department of Biochemistry, Agricultural Experiment Station, Manhattan, Kansas 66502.

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[5] See footnote 4.

[6] Haslach, H. 1964 The biological activity of 13-mono-*cis* vitamin A and β-apo-8′-carotenal in the growth test with chickens. Master's thesis, Ludwig Maximibians University, Munich, Germany.

[7] Eggs obtained from flock maintained in this laboratory.

TABLE 1

Carotenoid and vitamin A-free starter diet

	g
Soybean oil meal, reground (44%)	14,400
Sorghum grain, ground	6,300
White corn, ground	7,500
Cottonseed oil	300
Premix A	4,545
Premix B	216.2

Premix A:	g	Premix B:	g
Non-fat dried milk	300	Vitamin D_3 (15,000 ICU/g)	5.4
Dried brewer's yeast	600	B-vitamin mix [2]	8.4
Extracted fish meal (50%)	1200	Vitamin B_{12} conc [3]	10.5
Distillers dried solubles	600	Methionine	17.4
Steamed bonemeal	675	Chlorotetracycline conc [4]	8.4
Salt	180	Zinc bacitracin conc [5]	18.0
$MnSO_4 \cdot H_2O$	12	4-nitrophenylarsonic acid conc [6]	18.0
Trace mineral mix [1]	18	Amprolium conc [7]	18.0
		Soybean oil meal	120.0
			mg
		Menadione	60.0

[1] CCC trace mineral mix; contained: (in %) Mn, 10; Fe, 10; Ca, 14; Cu, 1; Zn, 5; I, 0.3; and Co, 0.1; Calcium Carbonate Company, Quincy, Illinois.
[2] Merck 1233; contained: (in g/kg) riboflavin, 17.6; Ca pantothenate, 35.2; niacin, 63.8; and choline chloride, 176; Merck and Company, Inc., Rahway, New Jersey.
[3] Proferm 20; 44 mg B_{12}/kg. Commercial Solvents Corporation, Terre Haute, Indiana.
[4] Aurofac 10; 22 g chlorotetracycline/kg. American Cyanamid, Princeton, New Jersey.
[5] Baciferm; 22 g zinc bacitracin/kg. Commercial Solvents Corporation.
[6] Histostat; 0.025% 4-nitrophenylarsonic acid; Dr. Salsbury's Laboratories, Charles City, Iowa.
[7] Amprol; 0.0125% amprolium; Merck and Company, Inc.

TABLE 2

Number of females in experimental groups, and number of eggs laid

Group	Supplement [1]		No. females	No. eggs laid/wk/female, exp. week no.					
	Type carotenal	Amount [2]		6	7	8	9	10	Total
		mg/kg diet							
1	β-apo-8′	6.8	5	0.6	4.8	3.2 [3]	6.2	6.4	21.2
2	β-apo-8′	7.0	2	0.0	5.0	2.5 [3]	6.5	6.0	19.0
3	β-apo-12′	6.7	8	0.5	4.2	5.6	5.2	5.7	21.2
4	β-apo-12′	7.0	6	0.5	3.5	6.0	4.1	3.3	17.4
5	β-apo-10′	6.9	5	0.0	2.2	6.4	4.8	5.4	18.8
6	None	—	no egg production, high mortality						

[1] Supplements to groups 1, 3 and 5 prepared by oxidation of β-carotene (6); supplements to groups 2 and 4 were synthetic products furnished by Hoffmann-LaRoche, Inc., Nutley, New Jersey.
[2] Calculated from $E^{1\%}_{1\ cm}$, 2160, 2190, and 2640 for β-apo-8′-, β-apo-10′-, and β-apo-12′-carotenals, respectively (7).
[3] Groups 1 and 2 were accidentally left without water for a day at the beginning of the week, which may have affected egg production for a few days.

TABLE 3

Number of eggs set, fertile eggs, infertile eggs, percentage hatchability, average weight of progeny at 12 days of age, and percentage mortality

Group no.	No. eggs incubated	No. fertile eggs	No. infertile eggs	Hatchability [1]	Mortality to 12 days	Avg wt/bird at 12 days
				%	%	g
1 [2]	20	19	1	89.4	17.6	30.4
2	10 [3]	6	3	66.6	0.0	28.0
3	20	18	2	78.8	7.1	30.1
4	20	11	9	72.7	50.0	30.0
5	20	17	1	82.3	7.1	29.0

[1] Based on number of fertile uncracked eggs.
[2] See table 2 for kinds and quantities of carotenals given.
[3] Only 10 eggs were available; only 2 females in group 2.

20 eggs collected from each group during the last 2 weeks of the experimental period were incubated, except that only 10 eggs were available from group 2. Quail chicks that hatched from eggs of each test group were given a commercial starter diet for 12 days; then they were weighed (table 3), examined for abnormalities, and the experiment was terminated.

Vitamin A content of serum was estimated by the Kimble method (8). Vitamin A was extracted from liver and egg yolk by a method described by Parrish et al. (9), except that extraction was by shaking instead of use of a blender. Vitamin A content was estimated by the Carr-Price technique (8). In these methods the vitamin A determined included retinol, retinyl esters, and retinal.

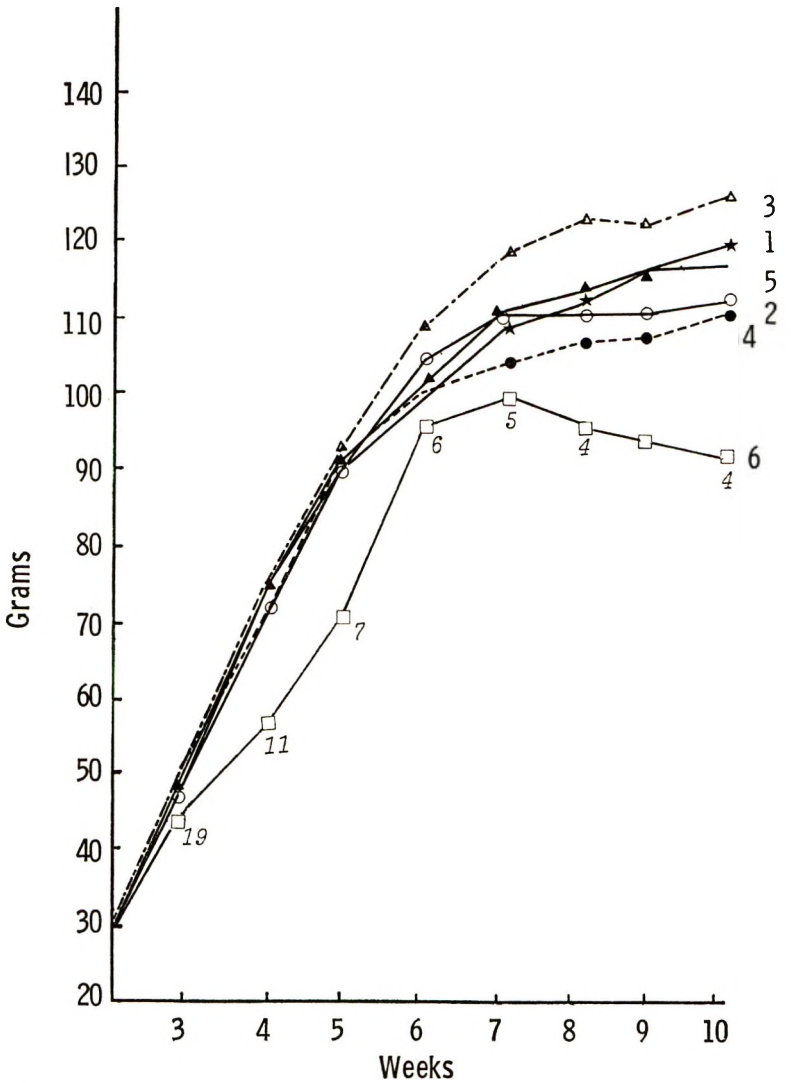

Fig. 1 Growth of quail fed test diets following use of a basal vitamin A and carotenal-free diet for first 2 weeks after hatching. Groups 1, 3, and 5 received β-apo-8'-, β-apo-12'- and β-apo-10' carotenals, respectively, prepared by oxidation β-carotene. Groups 2 and 4 received synthetic β-apo-8'- and β-apo-12'-carotenals, respectively. Group 6 was fed basal diet only; numbers in italics are number of survivors at each period in group 6. No birds were lost in any other group.

RESULTS AND DISCUSSION

Quail (groups 1 to 5), which received the β-apo-carotenals, consistently performed better than those of group 6 getting the basal diet only (fig. 1). During the first 5 weeks there was essentially no difference in average weights of quail receiving β-apo-carotenal supplements. After that time, egg laying and the uneven numbers of the 2 sexes in various groups probably caused part of the weight differences observed. Quail grew better with β-apo-carotenals prepared by oxidation of β-carotene (groups 1, 3 and 5) than with synthetic β-apo-carotenals (groups 2 and 4), but there is no apparent explanation for this. The tendency for weights to reach a plateau at 7 weeks of age is normal, since the birds mature and start laying when about 5 to 6 weeks old.

No deaths occurred among groups fed the supplemented diets, but a high mortality (40% the second week) occurred in the control group. Before death the birds were unable to walk normally and had a mild-to-moderate swelling of the eyes, accompanied in some cases by mild infection and "cheesy" exudate. Uric acid accumulations were found in the ureters of dead birds. These abnormalities are associated with vitamin A deficiency in chickens.

Egg production started at 6 weeks of age and increased in all supplemented groups to 5 or more eggs per hen per week (table 2). Although laying by hens of group 4 decreased toward the end of the test, that of group 3, also receiving β-apo-12'-carotenal, was as high as that for any group.

Eggs collected during weeks 9 and 10 were incubated. They hatched on day 17. The number of fertile eggs, infertile eggs, percentage hatchability, and growth to 12 days of age are recorded in table 3.

Growth, viability, egg production, fertility, hatchability of eggs and viability and growth of progeny indicated that β-apo-carotenals can serve as a sole source of vitamin A activity for quail. In general, data on these foregoing factors are within the range obtained in this laboratory when normal vitamin A-supplemented diets are fed.

Significant quantities of vitamin A were formed from β-apo-carotenals; this vitamin A was carried in the serum, stored in the liver and transferred to the egg (table 4). Irrespective of the β-apo-carotenal given, blood serum and liver of males contained larger quantities of vitamin A than those of females. Storage of vitamin A by quail receiving β-apo-carotenals as the only vitamin A source, which has not been reported previously, demonstrated a substantial vitamin A activity of β-apo-carotenals. These results confirmed earlier reports on vitamin A activity of β-apo-carotenals (1–4) [8] and make questionable the reports (5)[9] that only traces of vitamin A were found in the liver of chickens fed β-apo-8'-carotenal, and that it had vitamin A activity too low for use as a source of vitamin A.

[8] See footnote 4.
[9] See footnote 6.

TABLE 4

Vitamin A content of blood serum, liver and egg yolk of quail receiving β-apo-carotenals

Group [1]	Sex	Blood serum [2]	Liver [3]	Egg yolk [3]
		μg/100 ml	*μg/g*	*μg/g*
1	F	59.0	34.4	17.5
1	M	120.5	65.6	—
2	F	108.9	25.9	14.4
2	M	125.6	30.0	—
3	F	50.6	21.2	13.5
3	M	— [4]	41.6	—
4	F	56.0	11.2	13.0
4	M	104.9	16.0	—
5	F	102.3	20.4	19.4
5	M	139.3	24.4	—

[1] See table 2 for kinds and quantities of supplements given.
[2] Pooled serum sample, all birds of group.
[3] Average of samples from 2 birds selected at random from each group.
[4] Insufficient blood serum available, since there were only 2 males in the group.

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Iron Utilization and Metabolism in the Chick

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ABSTRACT Experiments were conducted with chicks to determine whether the iron-containing tissues and tissue enzymes show iron deficiency before a reduction in hemoglobin and packed cell volume is obtained. The results indicate that these latter parameters were reduced earlier than myoglobin level, ferritin and hemosiderin iron levels and cytochrome c and succinic dehydrogenase activities. Iron deficiency in the chick was characterized by poor growth, and reduction in hemoglobin, packed cell volume, myoglobin, ferritin, hemosiderin and succinic dehydrogenase activity. The amount of hemosiderin iron exceeded the amount of ferritin iron in both deficient and normal chicks. The ratio of hemoglobin to ferritin plus hemosiderin indicated that after 3 weeks the iron stored in the liver was being utilized for hemoglobin formation by the iron-deficient chick. Nitrilotriacetic acid (NTA) and ethylenediaminetetraacetic acid (EDTA), combined equimolarly with iron, had little if any effect on body weight, hemoglobin, packed cell volume, myoglobin and ferritin and hemosiderin-iron in the liver or succinic dehydrogenase activity. When NTA and EDTA were fed in the uncomplexed form in the basal diet or in the diet with 10 mg/kg of added iron, the values for hemoglobin and packed cell volume indicated that the former compound improved iron utilization slightly and EDTA interfered with it somewhat. When body weight, hemoglobin and packed cell volume were used as the criteria, the chick's requirement for iron was found to be 79.0, 77.5 and 77.0 mg/kg of diet, respectively. A realistic estimate of the chick's requirement for iron is, therefore, 75 to 80 mg iron/kg of diet. No evidence was obtained that the soybean protein basal diet reduced the utilization of iron. Thus, the possibility that the phytic acid in the soybean protein interfered with the availability of iron was ruled out.

The iron status of an animal body is routinely expressed by the concentration of hemoglobin in the blood. However, Beutler et al. (1) observed sideropenia without anemia in humans by examination of the sternal bone marrow for stainable iron. Furthermore, Waldenström (2, 3) showed that many patients suffered from definite symptoms of iron deficiency without showing anemia, and that these symptoms were rapidly alleviated by administration of iron. Possibly alteration in metabolism and changes in the iron levels of other iron-containing compounds occur in tissues at a time different from the changes occurring in the blood.

Hahn and Whipple (4) reported that there was no decrease in the myoglobin content of the muscles of anemic dogs. However, Gubler et al. (5) found that the myoglobin levels were lowered in the muscles of iron-deficient pigs. Beutler et al. (6) and Davidson and Jennison (7) have demonstrated that the storage of labile-pool iron compounds, ferritin and hemosiderin, are depleted in iron-deficient subjects. Hahn and Whipple (4) observed that tissue enzymes containing iron did not reflect a deficient state. However, Cohen and Elvehjem (8) had previously shown that the absorption bands of cytochrome *b* and c were reduced in intensity in iron- and copper-deficient rats, and were not restored by treatment with copper alone. Furthermore, Beutler (9) and Kampschmidt et al. (10) observed that in experiments with rats fed iron-deficient diets, the reduction of hemoglobin was equalled and even exceeded by the reduction in the level of cytochrome c in the liver. In addition, Beutler and Blaisdell (11) reported that the activity of succinic dehydrogenases in iron-deficient rats showed only intermediate degrees of depletion.

Along with the problem concerning the rate at which iron-containing tissues reflect a deficient state, the factors affecting the availability of iron to the organism are of

Received for publication July 3, 1967.

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[2] This paper represents a part of a thesis submitted by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of the University of California, Davis.

nutritional importance. Davis et al. (12) reported that 700 mg/kg of the disodium dihydrogen salt of ethylenediaminetetraacetic acid (EDTA), when fed with a soybean protein diet, decreased the chick's requirement for zinc, manganese and copper but failed to decrease the iron requirement. Hawkins et al. (13) reported that 100 mg/kg EDTA fed with a low iron diet to rats increased serum iron, but the average difference was not statistically significant, and there was no effect upon the liver iron or hemoglobin level in the blood. Rubin and Princiotto (14) observed that the absorption of EDTA iron was enhanced, but that the increased urinary output more than compensated for the increased absorption, with the net result that lesser amounts of the chelated iron were retained by these animals than by those receiving ferric chloride. In contrast, the nitrilotriacetic acid-iron (NTA-iron) chelate did not cause increased excretion of oral iron in the urine. They also reported that anemic animals can utilize iron prepared in a chelated form as well as in the ionized form of ferric chloride.

Davis et al. (12) observed that isolated soybean protein increased the requirement of chicks for zinc, manganese and copper, but the chick's requirement for iron was not affected. Bhattacharya and Esh (15) fed rats diets containing various proteins at 3 levels and noted that as the percentage protein decreased, the amount of iron deposited in the liver or spleen also decreased, but with the exception of gelatin, the kind of protein did not influence the amount of iron deposited. These authors (16) later suggested that the peptides and amino acids resulting from protein digestion act as vehicles for iron transport, and only a deficiency of protein reduces the amount of iron absorbed.

In view of these findings, studies were undertaken with chicks to determine whether a decrease in the iron levels of myoglobin, ferritin, hemosiderin, cytochrome c and succinic dehydrogenase occurred before a reduction in the content of hemoglobin or the packed cell volume would be observed. The effect of the chelating agents, NTA and EDTA, on the utilization of iron was also studied. In addition, the effect that isolated soybean protein and a set mixture of casein and gelatin might have upon the levels and activities of iron-containing compounds was investigated. Finally, a more precise study on the quantity of iron required by the chicks was conducted. The results of these studies are presented in this report.

METHODS AND MATERIALS

One-day-old male chicks were selected by weight, identified with numbered wingbands and weighed individually at the start and each day or week thereafter. The chicks were housed in galvanized, electrically heated battery brooders with raised wire-mesh floors. All parts of the battery were coated with an epoxy resin to prevent trace mineral contamination.

Two soybean protein basal diets (diets 1 and 3) and 2 casein-gelatin basal diets (diets 2 and 4) were used in these studies. The composition of these diets is presented in table 1. The isolated soybean protein was washed with EDTA according to the procedure developed by Davis et al. (17, 18). Vitamin-free casein and USP grade gelatin were used in the other basal diets. The ingredients of the basal diets were analyzed for iron and copper by the atomic absorption method, and the content of the basal diets, presented in table 2, was calculated from the assay results. The acid digests were supplemented with a known amount of iron solution so that the low iron content of the ingredients could be determined with greater accuracy. All basal diets contained a complete mineral mixture, except for iron. Feed and distilled water were provided ad libitum.

In experiment 1, 15 New Hampshire chicks per treatment were selected. Each treatment was replicated 6 times, and 20 chicks per treatment were decapitated at weekly intervals for 4 weeks, when blood, liver and heart tissues were removed for analyses. The values in the tables are the averages of the individual analyses. In experiment 2, 11 New Hampshire chicks per treatment were selected, and at 4 weeks of age, the chicks were killed and blood and tissues were collected. In experiment 3, 15 White Plymouth Rock chicks per treatment were selected, and the experiment was ter-

TABLE 1

Composition of basal diets

	Diet 1 (Exps. 1 and 2)	Diet 2 (Exp. 3)	Diet 3 (Exp. 4)	Diet (Exp. 4)
	%	%	%	%
Soybean protein [1]	25.8	—	27.5	—
Casein [2]	—	20.0	—	19.25
Gelatin	—	10.0	—	9.25
Soybean oil	3.0	10.0	8.0	8.0
Vitamin mix [3]	3.15	3.15	3.15	3.15
Mineral mix [4]	2.25	2.21	2.21	2.21
DL-Methionine	0.5	0.4	0.5	0.4
Glycine	0.3	—	0.3	—
L-Arginine	—	0.3	—	0.3
Dicalcium phosphate, hydrated	2.25	1.86	2.69	1.86
Calcium carbonate	1.40	1.99	1.51	1.99
Cellulose [5]	2.0	3.0	2.0	3.0
Butylated hydroxytoluene	0.025	—	—	—
Ethoxyquin [6]	—	0.0125	0.125	0.125
Cornstarch	59.35	47.59	52.14	50.62
Metabolizable energy, kcal/kg	137.9	137.9	140.4	140.1

[1] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Cincinnati; washed 4 times with 0.5% Na_2EDTA.
[2] Vitamin-Free Casein, Nutritional Biochemicals Corporation, Cleveland.
[3] When included in the diet at a level of 3.15% the vitamin mixture contributed the following vitamins/kg of diet: (in milligrams) riboflavin, 10; thiamine·HCl, 10; pyridoxine·HCl, 10; Ca pantothenate, 30; niacin, 100; folic acid, 5; 2-methyl-1,4-naphthoquinone, 10; choline chloride (25%), 2000; biotin, 0.2; and (in micrograms) vitamin B_{12}, 10.0; and (in grams) vitamin A palmitate (10,000 IU/g), 1; vitamin D_3 (1500 ICU/g), 1; and vitamin E conc as *dl*-α-tocopheryl acetate (Distillation Products Industries) (44 IU/g), 2.0.
[4] When included in the diet at the level of 2.25% in experiments 1 and 2, diet 1, and 2.21% in experiments 3 and 4, diets 2, 3, and 4 (values in parentheses), the mineral mixture contributed the following minerals in grams/kg of diet: NaCl, 7.0 (5.0); $MnSO_4 \cdot H_2O$, 0.154, (0.308); $CuSO_4 \cdot 5H_2O$, 0.024 (0.024); ZnO, 0.07 (0.07); $Co(C_2H_3O_2O_2)_2 \cdot 4H_2O$, 0.02 (0.005); KI, 0.01 (0.005); $Al_2(SO_4)_3 \cdot 18 H_2O$, 0.25 (0.25); $MgSO_4 \cdot 7 H_2O$, 4.0 (3.0); KCl, 2.0 (0.0); K_2HPO_4, 9.0 (12.0); $Na_2MoO_4 \cdot 2H_2O$, 0.01 (0.005); $NaHCO_3$, 0.0 (1.44); and $Na_2SiO_3 \cdot 5 H_2O$, 0.0 (0.004).
[5] Solka Floc, Brown Company, Berlin, New Hampshire.
[6] Santoquin, Monsanto Company, St. Louis.

TABLE 2

Iron and copper content of ingredients and basal diets

Used in diet no.	Ingredient or diet	Fe	Cu
		mg/kg	*mg/kg*
1 and 3	EDTA-washed isolated soybean protein	27	16.0
2 and 4	Vitamin-free casein	23	3.2
2 and 4	Gelatin	33	7.5
1, 2, 3 and 4	Cellulose	132	5.0
1, 2, 3 and 4	Soybean oil	64	0.5
1, 2 and 3	Cornstarch [1]	12	2.0
4	Cornstarch [2]	16	2.5
1	Soybean protein basal diet	18.64	11.55 [3]
2	Casein-gelatin basal diet	23.97	8.64 [3]
3	Soybean protein basal diet	23.55	11.98 [3]
4	Casein-gelatin basal diet	24.66	8.84 [3]

[1] Obtained from A. E. Staley Manufacturing Company, Decatur, Illinois.
[2] Obtained from Corn Products Company, Argo, Illinois.
[3] Includes 6.11 mg of added Cu/kg of diet.

minated at 4 weeks of age. At this time blood and feces were collected from each of 3 chicks per pen. In experiment 4, 38 White Plymouth Rock chicks per lot were selected for the lots receiving the soybean protein basal diet, and 10 chicks per lot were selected for the lots receiving the casein-gelatin basal diet. Each treatment was duplicated, and 8 chicks per treatment of those receiving the soybean protein basal diet were killed daily for one week and then again at 2 weeks along with 8 chicks

per treatment of those receiving the casein-gelatin basal diet. Blood and cardiac tissue were collected.

The soybean protein basal was fed with and without added iron in experiment 1. In experiment 2, iron chelates were prepared using 2 chelating agents which were fed in comparison with 3 levels of iron in an isolated soybean protein basal diet. Quantities of $Na_2EDTA \cdot 2H_2O$, and Na_2NTA were added to equimolar quantities of ferrous sulfate in demineralized water. After both compounds had dissolved, the excess solution was evaporated. The crystalline complexes were washed, recrystallized and dried. The iron chelates were added to the basal diet so that the respective diets contained the same level of iron as the basal diet supplemented with ferrous sulfate. The level of the NTA-iron chelated complex added to the diet was 0.013%, and that of the EDTA-iron complex was 0.014%. In experiment 3, two chelating agents were added to the soybean protein basal diets, with and without added iron. A lot receiving phytic acid was also included in the group receiving the latter diet. The amount of chelating agent used was 3 times the amount of iron, calculated on a molar basis. At the end of the experimental period, a gelatin capsule containing 0.25 ml of an ^{55}Fe solution having 367,500 cpm was fed to each of 3 chicks. The chicks were killed 24 hours later when the blood and feces were collected for the determination of radioactivity. The radioactivity of the blood plasma was determined in a liquid scintillation system after the plasma proteins had been dissolved by the addition of Hyamine hydroxide, heated to 60° for 30 minutes, and 0.5 ml of this solution added to 14.5 ml of the scintillation solution. The whole blood and feces were ashed, and the ash was dissolved in concentrated hydrochloric acid, before evaporating. The residue was dissolved in demineralized water, and an aliquot was added to the scintillation solution for counting. In experiment 4 graded levels of iron were fed with the treated isolated soybean protein and casein-gelatin basal diets.

The blood samples for the determination of hemoglobin concentration and packed cell volume in all experiments were obtained by cardiac puncture or decapitation and transferred immediately to heparinized tubes. The modified alkaline-acid hematin method of Bankowski (19) was used for hemoglobin determination. The values for packed cell volume were obtained using a microcapillary centrifuge.

The myoglobin content of cardiac tissue was determined by the method of Anthony et al. (20). The colorimetric determinations of ferritin and hemosiderin were performed on liver tissue by using an adaptation of the methods of Kaldor (21), Sandell (22) and Johnson and Ulrich (23). In the method used the iron was reduced to the ferrous state with hydroquinone in the presence of citrate, and then reacted with o-phenanthroline at pH 4 to form a stable complex. The optical density was determined with the Beckman spectrophotometer at 508 mμ.

Cytochrome c of liver tissue was determined by the method of Potter and DuBois (24), as modified by Beutler (9). The manometric method of Singer and Kearney (25) was used for the determination of succinic dehydrogenase in cardiac tissue.

The results presented in this report were subjected to statistical analyses according to procedures for analysis of variance and calculation of regression and correlation coefficients as described by Snedecor (26), and for comparisons involving 2 sample means as described by Steel and Torre (27).

RESULTS AND DISCUSSION

In experiment 1 the effect of iron deficiency on chick growth and body constituents containing iron was studied. The basal diet contained 19 mg Fe/kg, and the supplemented diet contained 99 mg Fe/kg of diet. The results of feeding these diets to chicks are presented in table 3. They showed that growth was increased significantly, with the addition of iron, by the second week ($P < 0.005$). The difference in the percentage packed cell volume of the blood of the supplemented chicks and the unsupplemented chicks was statistically significant by the third week ($P < 0.005$). Hemoglobin concentration in the blood of the chicks receiving dietary iron was greater than the hemoglobin concentration of the unsupplemented chicks after the first week,

TABLE 3

Effect on iron-containing compounds of chicks from feeding two levels of iron (exp. 1)

Measurements and calculated ratios [1]	(Total Fe in diet) (mg/kg)	Experimental period, weeks				
		0	1	2	3	4
Body wt, g	(19)	44.2	87.0	143.4	206.8	273.3
	(99)	44.2	88.6	166.7[a]	280.2[a]	438.5[a]
Vol of packed cells, %	(19)	25.5	17.5	16.5	18.9	19.5
	(99)	25.5	20.9	22.8	27.2[a]	27.9[a]
Hb, g/100 ml blood	(19)	10.7	7.5	5.2	5.6	5.8
	(99)	10.7	8.8	9.1[a]	10.2[a]	11.8[a]
Mean corpuscular Hb concn	(19)	0.42	0.43	0.31	0.30	0.30
	(99)	0.42	0.42	0.40	0.38	0.42
Myoglobin, mg/g dry tissue	(19)	0.20	0.26	0.15	0.09	0.13
	(99)	0.20	0.27	0.15	0.14	0.25[d]
Ferritin, μg Fe/g dry tissue	(19)	12.0	4.0	3.0	2.7	2.0
	(99)	12.0	4.0	4.6[b]	5.2[d]	6.8[a]
Hemosiderin, μg Fe/g dry tissue	(19)	15.8	10.2	6.8	5.9	5.3
	(99)	15.8	10.1	9.12	8.6[c]	12.1[c]
Ferritin:hemosiderin	(19)	0.76	0.39	0.45	0.45	0.38
	(99)	0.76	0.40	0.50	0.60	0.56
Hb:ferritin + hemosiderin	(19)	0.38	0.53	0.53	0.66	0.79
	(99)	0.38	0.62	0.66	0.76	0.63
Cytochrome c, $\times 10^{-5}$ μmoles/g dry tissue	(19)	27.0	86.2	83.4	86.0	82.7
	(99)	27.0	83.2	86.9	95.7	87.7
Succinic dehydrogenase, Q_{O_2}	(19)	9.7	5.9	2.5	1.9	4.2
	(99)	9.7	9.6[a]	9.0[a]	8.9[a]	9.2[a]

[1] When the mean of the group fed the supplemented diet was compared with the mean of the group fed the unsupplemented diet of the same time period, a superscript of *a* indicates a significance of $P < 0.005$; *b*, $P < 0.010$; c, $P < 0.025$; and *d*, $P < 0.050$.

and was statistically significant at the second week ($P < 0.005$). When the mean corpuscular hemoglobin concentration was calculated, the results indicated that the concentration of hemoglobin in the erythrocytes of the supplemented chicks remained approximately the same throughout the experimental period. This indicated the deficiency state was reflected in the erythrocytes at 2 weeks of age in the chicks fed the low iron diet.

Myoglobin concentration in cardiac tissue was reduced in both the deficient and control groups at 2 and 3 weeks, but the difference in concentration of myoglobin between these 2 groups did not become statistically significant until 4 weeks ($P < 0.050$). These results indicated myoglobin has a slower turnover rate in the chick than hemoglobin. Theorell et al. (28) reported that a specific activity of radioactive iron injected into guinea pigs significantly above zero was not achieved in myoglobin until one month. The specific activity of hemoglobin, however, was significant during the first week, indicating the turnover rate of hemoglobin iron is much more rapid than that for myoglobin. Hahn and Whipple (4), however, have reported that there was no decrease in myoglobin under conditions of iron depletion. It appears that the slower turnover rate and the length of the experimental period determined whether or not a reduction in the myoglobin level in iron-deficient tissue will be observed.

The data on ferritin and hemosiderin indicated these storage forms of iron were also decreased at approximately the same rate from one week of age when chicks were given an iron-deficient diet. When compared with the ferritin and hemosiderin of the chicks fed the control diet, the decrease of ferritin was statistically significant at 2 weeks ($P < 0.010$), and that of hemosiderin at 3 weeks ($P < 0.025$). The determination of ferritin and hemosiderin iron in the liver tissue of deficient and sufficient chicks of experiment 1 revealed that from the beginning until the termination of the experiment, the amount of iron in hemosiderin exceeded the amount in ferritin in both groups. As far as is known, this is the first observation that a greater

amount of iron is deposited in the liver as hemosiderin than as ferritin. Shoden et al. (29) reported that in human and rabbit tissues at physiological levels of iron, a greater amount of iron was determined as ferritin than as hemosiderin. Morgan and Walters (30) observed that when storage iron in the liver and spleen was less than 500 μg/g of tissue, iron was stored as ferritin, but when the level was above 1000 μg/g of tissue more iron was deposited as hemosiderin.

The results of calculating the ratio between the ferritin and hemosiderin iron showed that, in the supplemented group, a relatively greater increase in the ferritin iron level was obtained after the first week on experiment. Little change in the ferritin-to-hemosiderin ratio in the liver of deficient chicks receiving the basal diet was observed after the first week. This is in agreement with Morgan's (30) work with anemic rats. Ferritin and hemosiderin serve as labile pools of iron and it would be expected that, when a decrease in hemoglobin concentration occurs, iron stored in the liver as ferritin or hemosiderin would be utilized for hemoglobin formation. The ratio of hemoglobin to ferritin plus hemosiderin indicated that this, in fact, did occur during the last week of the experimental period.

The determination of cytochrome c in the liver of iron-deficient and supplemented chicks showed no statistical difference between the values obtained. However, Beutler (9) reported that iron-deficient rat liver tissue showed a fourfold reduction in the level of cytochrome c in 26 weeks. The lack of a depression in the level of cytochrome c in chick liver tissue may be due to inability to develop a basal diet lower than 19 mg Fe/kg and the relatively short experimental period.

Unlike cytochrome *c*, the succinic dehydrogenase activity in iron-deficient cardiac tissue showed a marked decrease which was statistically significant after the first week ($P < 0.005$). Beutler and Blaisdell (11) reported that the activity of this enzyme per gram of rat cardiac tissue decreased, but that the heart weight of the iron-deficient animals increased so that the total succinic dehydrogenase activity was approximately equal. Davis et al.[3] observed that in a study with iron-deficient and iron-supplemented chicks, fed a diet similar to the one used in this experiment, no difference was observed in the weights of the hearts.

In the second experiment the effect of iron chelated with NTA or EDTA on iron utilization was studied. The results of this experiment are presented in table 4. These compounds were included in the experimental diets at levels to supply 40 and 41 mg Fe/kg of diet, respectively. The basal diet contained 39 mg Fe/kg from ferrous sulfate. By setting the dietary iron at an intermediate level, it was thought that chelation might improve iron utilization to the point where 40 mg/kg would prove to be almost adequate. Two other diets, the unsupplemented basal diet, and one with a total of 119 mg Fe/kg were included so

[3] Unpublished data, P. N. Davis, L. C. Norris and F. H. Kratzer, 1961.

TABLE 4

Effect on iron-containing compounds of chicks from feeding iron complexes (exp. 2)[1]

Fe added as	None	$FeSO_4$	NTA-Fe	EDTA-Fe	$FeSO_4$
Quantity, mg/kg diet	—	20	22	21	100
Total Fe in diet, mg/kg	19	39	41	40	119
Body wt, g	257.3[a]	404.1[a]	406.8	416.2	464.0[a]
Vol packed cells, %	15.1[a,b]	21.9[b]	22.8	23.6	27.8[a,b]
Hb, g/100 ml blood	4.4[a,c]	7.0[b,c]	8.0	8.7	10.9[a,b]
Myoglobin, mg/g dry tissue	0.12	0.21	0.20	0.26	0.33
Ferritin, μg Fe/g dry tissue	3.3	3.6[c]	1.5[c]	3.6	5.8
Hemosiderin, μg Fe/g dry tissue	7.5	6.6	5.0	8.7	11.8
Cytochrome *c*, $\times 10^{-5}$ μmoles/g dry tissue	63.8	75.0[a]	70.3	115.4[a]	71.3
Succinic dehydrogenase, Q_{O_2}	11.6	15.1	11.0	13.2	14.8

[1] The values within a parameter with a superscript of *a* have a significant difference of $P < 0.001$; *b*, $P < 0.005$; *c*, $P < 0.010$; *d*, $P < 0.025$; and *e*, $P < 0.050$ when compared with the basal diet containing 39 mg Fe/kg of diet.

that the response to the chelated iron compounds could be evaluated. The same criteria were used in this experiment as in experiment 1. The amount of the chelated iron retained by the chick was not determined in this experiment. Iron chelated with EDTA in general improved the utilization of iron, but the differences were not statistically significant. Rubin and Princiotto (14) stated that EDTA-iron chelate enhances the absorption of iron to a slightly greater degree than oral ferric chloride, but that this was more than compensated for by an increased urinary iron output. Even though EDTA might have increased the urinary output of iron in this study, its presence did not apparently interfere with hemoglobin formation or with the other compounds studied that contained iron. The cytochrome c value for the EDTA-iron group was highly significant statistically. This value is unexplainable in the light of the lack of response obtained with the other cytochrome c determinations made in this work.

The presence of NTA-iron, as with EDTA-iron, did not affect the levels of iron in the iron-containing compounds with the exception of the significant decrease in the deposition of ferritin iron ($P < 0.010$). The NTA-iron complex may possibly have prevented the iron from being deposited as ferritin-iron, but in view of the values of the other parameters, this does not appear likely. The chicks receiving the basal diets containing the 3 levels of iron showed an increase in body weight, packed cell volume, hemoglobin concentration, myoglobin level and ferritin-iron content as the dietary iron level increased. The other parameters in general showed an increase as the level of iron increased. Except for the indication of the reduction of ferritin by NTA-iron it appears that the iron in the chelated complexes is available to chicks for tissues and enzymatic activities.

In experiment 3, the effect of NTA and EDTA on iron utilization was studied by adding them, independently of iron, to the diet at a level 3 times the quantity required to combine with the dietary iron on an equimolar basis. The effect of phytic acid on the iron utilization was also studied in this experiment. The iron content of the diets was 24.0 and 34.0 mg Fe/kg. The results are presented in table 5. The addition of the chelating agents to the basal diet containing 24 mg Fe/kg produced no statistically significant differences in body weight. However, NTA added to the basal diet containing 34 mg Fe/kg did produce a significant increase in body weight ($P < 0.050$). Packed cell volume and hemoglobin content were reduced slightly by EDTA. This is in agreement with the previous report by Davis et al. (12) in which 700 mg EDTA/kg were added to the diet. In contrast with EDTA a slight increase in hemoglobin content was noted with NTA. Radioactive iron was also fed to these chicks to measure the effect of chelating agents on iron retention. When NTA was

TABLE 5

Effect of feeding various chelating agents on the absorption of radioactive iron by the chick (exp. 3) [1]

Diet or chelating agent	Basal diet	NTA	EDTA	Phytic acid	Basal diet	NTA	EDTA	Basal diet
Quantity added, mg/kg diet	—	303	480	142	—	429	680	—
Total Fe in diet, mg/kg	24	24	24	24	34	34	34	124
Body wt, g	382.3	370.2	375.8	379.6	394.4[a,e]	424.1[e]	387.5	444.9[a]
Vol packed cells, %	15.9[c,d]	17.5	12.9	15.1	20.5[d,c]	19.3	18.1	23.5[c,e]
Hb, g/100 ml blood	4.3[d]	5.2	3.8	4.8	6.8	7.3	5.7	8.9[d]
^{55}Fe uptake in 1 ml blood, %	3.54[a,c]	3.34	1.56[a]	4.31[c]	0.40[a,b]	0.17[b]	1.15[b]	0.03[a]
^{55}Fe in feces,[2] %	0.97	1.72	2.68	0.58	5.75	5.29	5.43	5.23
^{55}Fe retained, %	99.03	98.28	97.32	99.42	94.25	94.71	94.57	94.77

[1] The values within a parameter with a superscript of *a* have a significant difference of $P < 0.001$; *b*, $P < 0.005$; c, $P < 0.010$; *d*, $P < 0.025$; and *e*, $P < 0.050$.

[2] Data could not be analyzed statistically because samples were composite of 3 chicks; hence no individual data are available.

fed with the basal diet containing 24 mg Fe/kg, less ^{55}Fe was found in the whole blood or plasma of the chicks than in the blood of chicks fed the basal diet, whereas more ^{55}Fe was excreted in the feces, and therefore, less was retained by the body. When NTA was fed with the basal diet containing 34 mg Fe/kg, the same response in the blood was noted, but there were no differences in the amount of ^{55}Fe excreted in the feces or retained by the body. EDTA produced the same general response that NTA did when it was added to a basal diet containing 24 mg Fe/kg. When it was added to the basal diet containing the additional 10 mg Fe/kg, there was a significant increase in the amount of ^{55}Fe contained in the whole blood and the amount excreted was slightly less than that in the group fed the basal diet. Phytic acid did not appear to affect the utilization of iron, and EDTA may have decreased slightly iron utilization.

The results indicated more radioactive iron was in the blood of the chicks fed the diets containing 24 mg Fe/kg than in the blood of the chicks fed the diets containing an additional 10 mg Fe/kg. As the amount of dietary iron increased, the percentage of radioactivity decreased. This is in agreement with Wheby et al. (31) who reported that with increasing iron doses, when everted gut sacs were used, increased uptake and transfer by the mucosa were observed, and in the iron-deficient animal the uptake and transfer were greater than in animals that had normal or large doses of iron. These results might also be explained by the effect of dilution of the radioactive iron with greater amounts of nonradioactive iron.

In the procedure for determining ^{55}Fe the counting proved difficult because of developing chemiluminescence in the scintillation solutions made up of the plasma solutions containing the Hyamine hydroxide, and the solutions prepared from the ashed blood and feces samples. This appeared to cause the radioactivity of the samples to increase day after day until a maximum was reached in approximately 13 days, after which it decreased. When this phenomenon was observed, only the counts made at 16 hours after preparation were used. This standardization allowed for comparison of all the data in the plasma, blood and feces series. The phenomenon appears to be similar to that reported by Herberg (32) and Vaughan et al. (33). Their radioactivity also increased in the samples over a short period and then declined. The period of increase in this study apparently was much larger than previously reported. The reasons for this effect are not understood.

The purposes of experiment 4 were to ascertain whether the chick's requirement for iron could be determined from criteria other than growth, and to compare a soybean basal diet with a casein-gelatin diet to determine whether the small amount of phytic acid contained in the soybean protein interferes with iron absorption. Graded levels of iron were fed with both the EDTA-washed soybean protein and casein-gelatin basal diets containing 23.6 mg and 24.7 mg Fe/kg of diet, respectively. The experimental period was 2 weeks. The results are presented in table 6. The body weight of the chicks receiving the soybean and casein-gelatin protein diets containing the supplemental iron were significantly greater ($P < 0.005$) than that for the groups receiving the basal diets. Significant differences were also obtained when packed cell volume and hemoglobin concentrations were determined. In contrast with the results obtained in experiment 1 no response to the iron levels was observed when the tissue was analyzed for succinic dehydrogenase at 2 weeks of age.

In experiment 4, observations were also made daily to determine whether this enzyme's activity decreased within the first 7 days and thus could be used to show an iron-deficient state before the concentration of hemoglobin was reduced. Within this time no significant indication of reduced succinic dehydrogenase activity was evident. The result of this experiment may have been influenced by a basal diet containing a higher level of iron than was contained in the basal diet used in experiment 1. The amount of iron required for the normal functioning of this enzyme is apparently so small that a 20% increase (19 to 24 mg/kg) in dietary iron evidently supplied the needed iron. Significant dif-

TABLE 6

Effect on iron-containing compounds of chicks from feeding diets containing graded levels of iron (exp. 4)[1]

Measurement	Soybean basal		Casein-gelatin basal	
	(Total Fe in diet) (mg/kg)	14-day experimental period	(Total Fe in diet) (mg/kg)	14-day experimental period
Body wt, g	(24)	136.2[e]	(25)	148.2[b,c,d,e]
	(44)	155.4	(45)	166.2[c]
	(64)	150.9	(65)	171.2[d]
	(84)	164.9[e]	(85)	182.4[b]
	(124)	163.7[e]	(125)	173.9[c]
	(144)	162.3[e]	(145)	170.9[d]
Vol packed cells, %	(24)	14.3[a,b,d]	(25)	14.7[a,b]
	(44)	19.8[d]	(45)	20.6[b]
	(64)	23.9[b]	(65)	21.8[a]
	(84)	25.7[b]	(85)	24.2[a]
	(124)	27.4[a]	(125)	24.1[a]
	(144)	25.5[b]	(145)	25.6[a]
Hb, g/100 ml blood	(24)	4.5[a,c,d]	(25)	4.5[a]
	(44)	6.6[d]	(45)	6.6[a]
	(64)	8.4[c]	(65)	7.9[a]
	(84)	9.3[c]	(85)	8.6[a]
	(124)	9.6[a]	(125)	8.9[a]
	(144)	9.0[c]	(145)	9.2[a]
Succinic dehydrogenase, Q_{O_2}	(24)	13.8	(25)	13.2
	(44)	11.1	(45)	13.4
	(64)	12.5	(65)	12.3
	(84)	12.5	(85)	11.9
	(124)	11.1	(125)	11.8
	(144)	12.8	(145)	11.0

[1] When the means of the supplemented groups with one diet were compared with the mean of the unsupplemented group fed the same diet, a superscript of *a* indicates a significance of $P < 0.001$; *b*, $P < 0.005$; *c*, $P < 0.010$; *d*, $P < 0.025$; and *e*, $P < 0.050$.

ferences were observed after 7 days in the hemoglobin and packed cell volume values. Under these conditions, it must be concluded that hemoglobin concentration reveals iron deficiency in the organism earlier than any of the other parameters used in these studies.

The data from the 3 significant criteria obtained from the chicks receiving the 2 diets fed in experiment 4 were plotted against the logarithm of the iron levels according to the method discussed by Hegsted (34). The requirement of the chick for iron was taken to be the point where the regression lines intercepted the plateau lines of maximal response. This procedure is illustrated in figure 1. The point of interception for body weight response was 79 mg Fe/kg of diet, 77.5 mg Fe/kg for packed cell volume, and 77 Fe/kg for hemoglobin concentration. A more realistic estimate of the requirement ranges perhaps between 75 and 80 Fe/kg of diet. This range appears valid because of the close agreement between the 3 determinations, and also because using a combination of the 3 parameters for an estimation of this requirement has greater validity than using one parameter alone.

The copper content of the soybean protein and the casein-gelatin basal diets used in experiment 4 was 12 and 9 mg Cu/kg of diet, respectively. Hill and Matrone (36) have reported that, as the iron level was increased, less copper was needed for a given concentration of hemoglobin and vice versa. The NRC (35) recommends a level of 40 mg Fe and 4 mg Cu/kg of diet. Hill and Matrone (36) suggested, on the basis of blood data alone, that a diet containing 50 mg Fe and 5 mg Cu/kg of diet would be closer to the chick's requirement. However, on the basis of body weight and blood data, Davis et al. (18) reported that

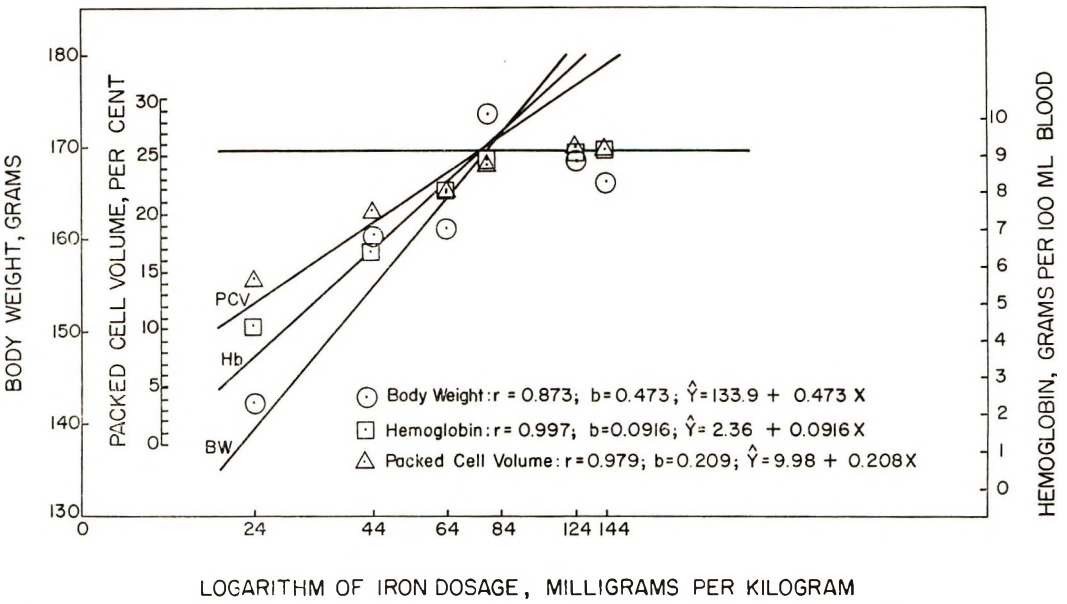

Fig. 1 Relationship of body weight, hemoglobin concentration, and packed cell volme to log-dosage of iron.

the requirement for 4-week-old chicks appeared to be between 65 and 105 mg Fe/kg of diet when the diet contained 10 mg Cu/kg of diet. The present study pinpoints the chick's requirement between these levels.

In experiment 4, there was no interaction between iron and the type of protein used in the 2 diets ($P > 0.250$), despite the fact that the growth of the chicks fed the diet containing the soybean protein were significantly smaller than those receiving the casein-gelatin diet ($P < 0.001$). It is possible, therefore, that the poorer growth response obtained with the soybean protein basal diet was due to lack of unidentified, growth-promoting substances that are contained in the other protein sources. The results showed that the level of phytic acid or other substances that might influence the utilization of iron, contained in the soy basal diet, did not impair the utilization of iron. O'Dell and Savage (34) and Widdowson and McCance (38) implicated phytic acid as a substance which reduces the absorption of iron. However, Sharp et al. (39), Fuhr and Steenbock (40), Nakamura and Mitchell (41) and Turnbull et al. (42) have presented evidence to show that the phytic acid contained in foods, and salts of phytic acid added to the diet, did not prevent the assimilation of dietary iron.

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