Influences of Dietary Carbohydrate-Fat Combinations on Various Functions Associated with Glycolysis and Lipogenesis in Rats

II. GLUCOSE VS. SUCROSE WITH CORN OIL AND TWO HYDROGENATED OILS[1]

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ABSTRACT Weanling rats were fed diets differing only in source of carbohydrate and fat for 2 to 4 weeks. Livers were assayed for glucose-6-phosphatase and fructose diphosphatase activities, and for content of glycogen and lipids. Effects on enzyme activities of substituting fructose for glucose were similar to those observed on substituting sucrose for rice starch (previous report). Feeding either hydrogenated coconut oil (HCO) or hydrogenated peanut oil (HPO) in place of corn oil (CO) modified the enzymatic responses to dietary fructose. Results with HPO were somewhat different than those with HCO. Labile phosphorus values were highest in groups fed sucrose or fructose with CO, and lowest in those fed rice starch or glucose with HPO. Effects of dietary carbohydrate on accumulation of lipid in liver appeared to be a function of the type of fat fed, namely, substitution of a fructose source for a direct glucose source resulted in the accumulation of less fat in livers of rats fed CO, but of more fat in livers of rats fed a hydrogenated oil. Proportions of phospholipid and cholesterol in liver lipid, and concentration of cholesterol in serum also varied with the combination of carbohydrate and fat fed.

The type of carbohydrate fed to rats influences the activity of several enzyme systems involved in carbohydrate metabolism (1–4). Freedland and Harper (1) noted that the stimulation of glucose-6-phosphatase activity resulting from the substitution of an indirect source of glucose (e.g., fructose) for a direct source (dextrin) appeared to be an adaptation of the enzyme system due to increased gluconeogenesis. Recent studies from this laboratory demonstrated that metabolic responses to the dietary source of carbohydrate are partially dependent on the type of fat in the diet (5). For example, adaptation in the activities of the glucose-6-phosphatase and fructose diphosphatase enzyme systems, and changes in glycogen content of the liver resulting from the substitution of sucrose for rice starch were modified by the type of fat fed (corn oil vs. hydrogenated coconut oil). Further evidence for an influence of the dietary source of fat on carbohydrate metabolism was the significantly greater glucose-6-phosphatase activity in livers from rats fed hydrogenated coconut oil with rice starch as compared with that in livers from rats fed corn oil with rice starch. Lipid content of the liver and cholesterol concentration of the serum also varied with the combination of carbohydrate and fat in the diet.

The experiments reported in the present paper were designed to gain more information about the above interrelationships by the following means: 1) Determining whether substitution of fructose for glucose with corn oil and with saturated fat would produce the same results as had the substitution of sucrose for rice starch; 2) comparing effects of feeding a hydrogenated fat containing long-chain fatty acids with those observed on feeding coconut oil; and 3) following the course of liver lipid variations for an additional 2 weeks.

Criteria for evaluating differences in carbohydrate and lipid metabolism were the same as used in the previous study (5) except that in the second part of the current study livers were also assayed for labile phosphorus from ADP and ATP.

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EXPERIMENTAL

Experimental animals, diets, and groups

Male, weanling rats [2] of the Sprague-Dawley strain, weighing approximately 50 g each at the beginning of the study, were fed different carbohydrate-fat combinations. Rations for all groups consisted of the following: (in per cent) protein, 20 (casein supplemented with 2% of DL-methionine); fat, 15; carbohydrate, 60.55; choline chloride, 0.2; salts (6),[3] 4; and vitamin mix,[4] 0.25. Variables were the type of carbohydrate and the type of fat.

Part 1. A total of 52 rats was divided into 8 groups (6 or 7 rats /group). Each group was fed a diet containing one of the following carbohydrate - fat combinations for 2 weeks: corn oil [5] (CO) with glucose, CO with fructose, hydrogenated coconut oil [6] (HCO) with glucose, HCO with fructose, and each of these 4 combinations with added cholesterol.[7]

Part 2. Since some differences were observed between effects of monosaccharides in part 1, and those of rice starch and sucrose in the previous study (5), part 2 was designed to investigate these differences by feeding the monosaccharides and the more complex carbohydrates simultaneously to different groups of rats. Also, hydrogenated peanut oil [8] (HPO) was used in place of HCO, to observe effects of providing more long-chain fatty acids. The approximate fatty acid content of the 2 fats, as supplied by the manufacturers, was as follows: for HPO (in per cent) palmitic, 12; stearic, 79; arachidic and longer saturated, 6; and mono-unsaturated, 3; as compared with HCO (in per cent) caprylic, 6; capric, 6; lauric, 44; myristic, 18; palmitic, 11; and stearic, 15. The iodine number of the HPO was approximately 4, and that of the HCO was 1 to 3. A total of 96 rats was divided into 8 groups of 12 rats each. Four rats from each group were fed the experimental diet for 2 weeks, four for 3 weeks, and four for 4 weeks. The 8 carbohydrate-fat combinations were: CO and HPO each with rice starch,[9] sucrose, glucose, and fructose.

PROCEDURES

After animals had been fed experimental diets for the times specified above, they were decapitated. Methods used for serum cholesterol determinations and for liver assays for activities of the glucose-6-phosphatase (G-6-Pase) and fructose diphosphatase (FDPase) enzyme systems, for glycogen, and for total lipid, phospholipid, and cholesterol are described in a previous paper (5). An additional assay carried out in part 2 was the determination of labile phosphorus from ADP + ATP. The adenosine phosphates from samples of liver homogenates were adsorbed onto charcoal, and labile phosphorus from ADP + ATP was hydrolyzed off by boiling in HCl (7). The inorganic phosphorus released was determined by the method of Fiske and Subbarow (8).

RESULTS

Growth rates and relative liver weights

The feeding of hydrogenated oil in place of corn oil resulted in a depression of the growth rate, more severe with HPO than with HCO. However, the average weekly weight gain for each group for the entire experimental period was at least 30 g, and did not fall below 26 g for any group in any single week (table 1).

Relative liver weights (liver weight/ 100 g of body weight) were significantly greater in all groups fed a source of fructose (sucrose or fructose) as compared with those of corresponding groups fed a direct source of glucose (rice starch or glucose) (table 1). Differences were significant at the 1 or 2% level [10] in all instances except one, sucrose vs. rice starch with CO at 4 weeks. This observation is consistent with previous reports from other laboratories that relative liver weight is increased by feeding fructose in place of glucose (9) or dextrin (1).

[2] Obtained from Hormone Assay Laboratories, Inc., Chicago.
[3] Salt Mixture-W, obtained from Nutritional Biochemicals Corporation, Cleveland.
[4] The vitamin mix provided the following: (in mg/ 100 g ration) thiamine·HCl, 0.08; riboflavin, 0.6; pyridoxine, 0.4; Ca pantothenate, 4.0; niacin, 5.0; inositol, 20.0; folic acid, 0.04; vitamin B_{12}, 0.004; biotin, 0.02; vitamin A powder, 10.0 (200 units); calciferol, 0.18 (150 units); DL-α-tocopherol powder, 30.0 (7.5 units); and menadione, 0.38.
[5] Mazola, Corn Products Company, Argo, Illinois.
[6] Hydrol, Durkee Famous Foods, Chicago.
[7] Cholesterol added at level of 1% of total ration at expense of dietary fat.
[8] Obtained from Procter and Gamble Company, Cincinnati.
[9] Obtained from Morningstar-Paisley, Inc., New York.
[10] Student's *t* test.

TABLE 1

Growth rates and relative liver weights of rats fed different combinations of carbohydrates and fats

Weeks fed diet	Direct glucose source with CO [1]	Fructose source with CO	Direct glucose source, saturated fat	Fructose source with saturated fat
	g	*g*	*g*	*g*
		Weight gain/week		
	G and CO	F and CO	G and HCO	F and HCO
1 (7) [2]	44 ± 1 [3]	41 ± 2	41 ± 1	37 ± 1
2 (7)	47 ± 2	49 ± 3	41 ± 2	41 ± 2
	G and CO + C	F and CO + C	G and HCO + C	F and HCO + C
1 (6)	44 ± 2	45 ± 1	41 ± 1	41 ± 2
2 (6)	47 ± 3	42 ± 3	39 ± 3	32 ± 3
	RS and CO	S and CO	RS and HPO	S and HPO
1 (12)	45 ± 1	46 ± 1	33 ± 2	26 ± 2
2 (12)	53 ± 1	53 ± 1	40 ± 2	45 ± 1
3 (8)	49 ± 1	44 ± 2	41 ± 1	44 ± 1
4 (4)	52 ± 3	53 ± 1	46 ± 1	42 ± 4
	G and CO	F and CO	G and HPO	F and HPO
1 (12)	43 ± 1	40 ± 1	33 ± 1	27 ± 2
2 (12)	48 ± 1	48 ± 1	37 ± 1	33 ± 1
3 (8)	46 ± 2	43 ± 1	34 ± 2	30 ± 2
4 (4)	50 ± 4	45 ± 6	33 ± 2	29 ± 2
		Relative liver weights [4]		
	G and CO	F and CO	G and HCO	F and HCO
2 (7)	4.81 ± 0.10	6.22 ± 0.22	5.05 ± 0.12	6.26 ± 0.15
	G and CO + C	F and CO + C	G and HCO + C	F and HCO + C
2 (6)	4.92 ± 0.14	6.44 ± 0.22	4.85 ± 0.23	5.92 ± 0.23
	RS and CO	S and CO	RS and HPO	S and HPO
2 (4)	4.92 ± 0.06	5.72 ± 0.10	4.40 ± 0.13	5.47 ± 0.19
3 (4)	4.77 ± 0.10	5.43 ± 0.13	4.54 ± 0.12	5.28 ± 0.07
4 (4)	4.54 ± 0.08	4.86 ± 0.18	4.04 ± 0.16	5.46 ± 0.33
	G and CO	F and CO	G and HPO	F and HPO
2 (4)	5.01 ± 0.07	6.33 ± 0.11	4.57 ± 0.22	5.50 ± 0.19
3 (4)	4.79 ± 0.05	5.85 ± 0.12	4.62 ± 0.12	5.70 ± 0.17
4 (4)	4.46 ± 0.09	5.99 ± 0.41	4.24 ± 0.08	5.52 ± 0.11

[1] Abbreviations: CO = corn oil, HCO = hydrogenated coconut oil, HPO = hydrogenated peanut oil, C = cholesterol (1% of ration), G = glucose, F = fructose, RS = rice starch, S = sucrose.
[2] Number of rats per group.
[3] SE of mean.
[4] Liver weight/100 g of body weight.

Enzyme activities and glycogen levels

The activities of the 2 enzyme systems were calculated as total activity (units/100 g of body weight) and as specific activity (units/100 mg of liver nitrogen). Qualitative relationships among groups were the same for both methods (fig. 1), although quantitative differences between the fructose-fed and glucose-fed groups were usually greater on the basis of total activity because of the larger livers in rats fed fructose. In evaluating various methods for expressing concentration of intracellular proteins, Knox (10) cites value "per 100 g of animal" as one means of relating protein content to the functional need of the whole animal, and comments that "such bases are particularly useful when great changes in size of the animal or organ have occurred during the experiment." Although activity of the enzyme system rather than concentration of the apoenzyme was measured in this experiment, the same reasoning would appear to be applicable and valid. Freedland and Harper expressed G-6-Pase and FDPase activities as total activity (1, 2), and

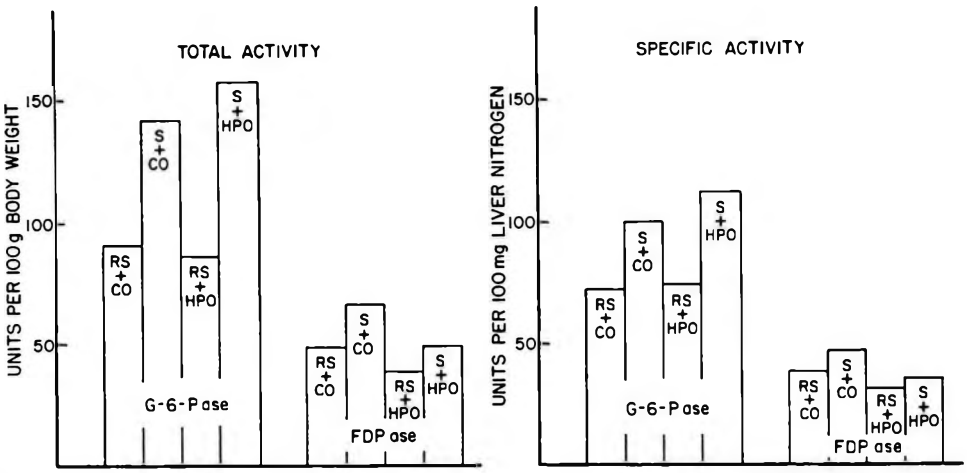

Fig. 1 Total activity and specific activity of the glucose-6-phosphatase and fructose diphosphatase enzyme systems in livers from rats fed different carbohydrate-fat combinations. Averages of values at 2, 3, and 4 weeks. RS = rice starch, S = sucrose, CO = corn oil, HPO = hydrogenated peanut oil.

pointed out that adaptation in G-6-Pase activity to fructose feeding was accomplished partly by an increase in specific activity of the enzyme and partly by an increase in total liver tissue (1). Since glucose released by the action of the G-6-Pase enzyme system of the liver is transported by the bloodstream to cells throughout the body, body weight was chosen as the more meaningful basis for evaluating functions closely associated with supply of glucose for body tissues. Therefore, as in the previous paper (5), activities of the 2 enzyme systems are expressed as total activity, and glycogen content of the liver is given in milligrams per 100 g of body weight (tables 2 and 3). Labile phosphorus was also calculated on the basis of body weight for comparative purposes (table 3).

Part 1. Substitution of glucose for fructose in CO diets resulted in a 60% increase in G-6-Pase activity ($P < 0.01$) (table 2). This degree of stimulation is almost identical with that obtained previously by the substitution of sucrose for rice starch in CO diets (5). A stimulatory effect of HCO on G-6-Pase activity was again observed, namely, activity of the enzyme system was significantly greater

TABLE 2

Enzyme activities and glycogen levels in livers from rats fed different combinations of carbohydrates and fats for 2 weeks (part 1)

Cholesterol added [1]	Glucose with CO [2]	Fructose with CO	Glucose with HCO	Fructose with HCO
	Glucose-6 phosphatase activity (units [3]/100 g of body weight)			
−	104 ± 2 [4]	166 ± 7	136 ± 12	175 ± 12
+	110 ± 6	164 ± 7	145 ± 16	177 ± 12
	Fructose diphosphatase activity (units/100 g of body weight)			
−	48 ± 3	79 ± 4	50 ± 3	63 ± 5
+	71 ± 3	90 ± 3	62 ± 5	71 ± 5
	Glycogen (mg/100 g of body weight)			
−	314 ± 20	476 ± 30	256 ± 18	443 ± 34
+	327 ± 65	553 ± 95	336 ± 57	454 ± 51

[1] Cholesterol added at level of 1% of total ration, at expense of fat.
[2] Abbreviations: CO = corn oil, HCO = hydrogenated coconut oil.
[3] One unit = activity catalyzing release of 1 μmole of inorganic phosphate/minute, at 37.5°C.
[4] SE of mean.

TABLE 3

Changes with time in enzyme activities, glycogen, and labile phosphorus (from ADP + ATP) in livers from rats fed different combinations of carbohydrates and fats (part 2)

Weeks fed diet	Direct glucose source with CO [1]	Fructose source with CO	Direct glucose source, with HPO	Fructose source with HPO
	Glucose-6 phosphatase activity (units [2]/100 g of body weight)			
	RS and CO	S and CO	RS and HPO	S and HPO
2	92 ± 8 [3]	158 ± 9	84 ± 7	168 ± 5
3	93 ± 6	148 ± 10	92 ± 6	155 ± 7
4	89 ± 3	120 ± 5	83 ± 8	148 ± 8
	G and CO	F and CO	G and HPO	F and HPO
2	131 ± 2	197 ± 20	123 ± 4	168 ± 13
3	112 ± 8	191 ± 9	120 ± 3	182 ± 9
4	100 ± 2	146 ± 8	100 ± 4	162 ± 4
	Fructose diphosphatase activity (units/100 g of body weight)			
	RS and CO	S and CO	RS and HPO	S and HPO
2	51 ± 2	72 ± 4	37 ± 4	54 ± 2
3	49 ± 1	66 ± 2	43 ± 2	50 ± 1
4	46 ± 3	63 ± 2	36 ± 3	46 ± 4
	G and CO	F and CO	G and HPO	F and HPO
2	52 ± 5	89 ± 12	45 ± 1	62 ± 6
3	56 ± 4	84 ± 6	42 ± 2	54 ± 3
4	51 ± 2	63 ± 5	38 ± 3	51 ± 3
	Glycogen (mg/100 g of body weight)			
	RS and CO	S and CO	RS and HPO	S and HPO
2	199 ± 19	325 ± 38	152 ± 35	266 ± 32
3	264 ± 45	258 ± 29	163 ± 7	214 ± 20
4	225 ± 17	268 ± 17	150 ± 16	245 ± 45
	G and CO	F and CO	G and HPO	F and HPO
2	256 ± 12	401 ± 43	228 ± 38	250 ± 45
3	239 ± 19	275 ± 43	209 ± 26	215 ± 13
4	208 ± 14	389 ± 49	159 ± 15	208 ± 29
	Labile phosphorus from ADP + ATP (μg/100 g of body weight)			
	RS and CO	S and CO	RS and HPO	S and HPO
2	463 ± 27	590 ± 82	327 ± 36	433 ± 47
3	419 ± 45	560 ± 67	314 ± 25	462 ± 66
4	384 ± 36	492 ± 29	269 ± 18	480 ± 46
	G and CO	F and CO	G and HPO	F and HPO
2	426 ± 37	625 ± 14	339 ± 21	502 ± 39
3	458 ± 50	643 ± 46	336 ± 28	624 ± 93
4	415 ± 50	693 ± 69	300 ± 8	478 ± 49

[1] Abbreviations: CO = corn oil, HPO = hydrogenated peanut oil, RS = rice starch, S = sucrose, G = glucose, F = fructose.
[2] One unit = activity catalyzing release of 1 μmole of inorganic phosphate/minute, at 37.5°C.
[3] SE of mean.

when glucose was fed with HCO than when the same carbohydrate was fed with CO ($P < 0.05$). This effect was observed both with and without incorporation of cholesterol into the diets. Highest values of G-6-Pase activity occurred when rats were fed fructose with HCO (table 2).

Using glucose and fructose as the direct and indirect sources of glucose (respectively) resulted in a pattern of FDPase responses similar to that observed with rice starch and sucrose in the previous study, namely, a substantial increase when a fructose source was substituted for a direct glucose source with CO ($P < 0.01$), no stimulation by HCO, and a small increase above the glucose-HCO group ($P < 0.05$) when fructose was fed with CO (table 2). Glycogen levels tended to parallel FDPase activity, except that the

feeding of fructose with HCO resulted in a marked increase in liver glycogen over the amount deposited with the glucose-HCO diet ($P < 0.01$).

The addition of cholesterol to the diets had no significant effect on G-6-Pase activity in any of the groups (table 2). On the other hand, FDPase activity was significantly increased by the addition of cholesterol to CO diets ($P < 0.01$ with glucose-CO diets and $P < 0.05$ with fructose-CO diets), but not by addition of cholesterol to HCO diets. No significant effect of dietary cholesterol on glycogen content of livers was evident, because of the very large variations among animals within each group.

Part 2. Absolute activities of the G-6-Pase enzyme system, and degree of stimulation of FDPase activity by a fructose source were greater in groups fed glucose or fructose than in corresponding groups fed rice starch or sucrose at 2 weeks (table 3). These differences tended to lessen with time, and had practically disappeared by the fourth week.

The effect of dietary HPO on G-6-Pase activity differed from that of HCO in part 1 (table 2) and in the previous study (5), namely, liver G-6-Pase activity in rats fed HPO with rice starch or glucose was not increased above the activity in rats fed CO with the same carbohydrate. Furthermore, the feeding of HPO with either rice starch or glucose depressed FDPase activity, glycogen levels, and labile phosphorus below corresponding values from groups fed CO (table 3). When data from determinations at 2, 3, and 4 weeks were combined, the extent of the depressing effect of HPO on FDPase activity was approximately 20% ($P < 0.01$), and on labile phosphorus about 25% ($P < 0.01$). The effect of HPO on glycogen content of livers was more variable, a 32% decrease ($P < 0.01$) with rice starch, and a barely significant decrease of 15% with glucose.

Lipid data

Part 1. The percentages of total lipid in the livers at 2 weeks varied significantly with the combination of carbohydrate and fat fed (table 4). With CO as the dietary fat, feeding fructose resulted in less total liver lipid than did feeding glucose ($P < 0.01$). But with HCO, feeding fructose resulted in more total liver lipid than did feeding glucose ($P < 0.01$). The addition of cholesterol to the diets greatly increased the deposition of fat in livers from all groups — by about 100% in CO-fed rats, and by approximately 70% in HCO-fed rats (table 4).

Percentages of phospholipid and cholesterol in liver fat of rats fed no added cholesterol showed no sharp differences among groups at 2 weeks (table 4), although percentages of both of these lipid

TABLE 4

Liver lipids and serum cholesterol in rats fed different combinations of carbohydrates and fats for 2 weeks (part 1)

Cholesterol added [1]	Glucose with CO [2]	Fructose with CO	Glucose with HCO	Fructose with HCO
	Total liver lipid (% of liver)			
−	3.35 ± 0.09 [3]	2.91 ± 0.08	3.40 ± 0.10	4.14 ± 0.16
+	6.53 ± 0.58	6.09 ± 0.46	5.64 ± 0.24	6.19 ± 0.25
	Phospholipid (% of total liver lipid)			
−	38.7 ± 1.4	42.0 ± 1.1	43.8 ± 1.4	36.7 ± 1.8
+	16.4 ± 1.2	17.3 ± 1.2	23.0 ± 2.0	21.8 ± 1.6
	Cholesterol (% of total liver lipid)			
−	13.2 ± 0.4	11.2 ± 0.6	10.0 ± 0.7	8.2 ± 0.3
+	17.9 ± 2.4	20.5 ± 2.5	24.0 ± 6.1	16.5 ± 1.6
	Serum cholesterol (mg/100 ml serum)			
−	155 ± 6	145 ± 8	123 ± 7	136 ± 5
+	108 ± 8	108 ± 11	155 ± 11	127 ± 8

[1] Cholesterol added at level of 1% of total ration, at expense of fat.
[2] Abbreviations: CO = corn oil, HCO = hydrogenated coconut oil.
[3] SE of mean.

fractions were lowest in livers containing the most fat (fructose-HCO group). The addition of cholesterol to the diets resulted in significant decreases ($P < 0.01$) in the proportion of phospholipid in total liver lipid with all carbohydrate-fat combinations (table 4). On the other hand, the proportion of cholesterol in liver lipid was significantly increased ($P < 0.01$) by addition of cholesterol to both fructose diets. Increases with glucose diets were more variable, namely, barely significant with glucose-CO and significant at the 5% level with glucose-HCO diets.

A clearer picture of overall changes in lipid content of the liver can be seen when total lipid, phospholipid, and cholesterol are expressed as milligrams per gram liver (fig. 2). Dietary cholesterol enhanced cholesterol accumulation about threefold with all carbohydrate-fat combinations. Phospholipid content of livers tended to decrease with administration of cholesterol, but the difference was significant at the 5% level only with the fructose-CO diets. When no cholesterol was fed, phospholipid content of liver was significantly

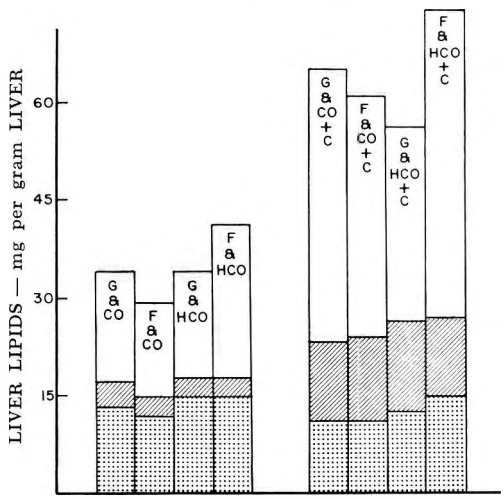

Fig. 2 Total lipid, cholesterol, and phospholipid (in mg per gram liver) in livers from rats fed different carbohydrate-fat combinations for 2 weeks (part 1). G = glucose, F = fructose, CO = corn oil, HCO = hydrogenated coconut oil, C = cholesterol. Complete bar represents total lipid, stippled portion represents phospholipid, and diagonally shaded portion represents cholesterol.

greater in rats fed HCO than in those fed CO, with either glucose ($P < 0.05$) or fructose ($P < 0.01$) as the dietary carbohydrate. When cholesterol was fed, differences between effects of fat were significant ($P < 0.02$) between fructose groups only (fig. 2).

The incorporation of 1% of cholesterol in the diets did not cause accumulation of cholesterol in the serum as it did in the liver (table 4). In fact, serum cholesterol concentrations were somewhat lower ($P < 0.02$) in the CO-fed groups when cholesterol was added to the diets. Other investigators have noted the tendency of dietary cholesterol to produce sharp increases in liver cholesterol accompanied by small, variable fluctuations in serum cholesterol when the diet contains an appreciable amount of fat and linoleic acid (11, 12).

Part 2. The influence of dietary carbohydrate on accumulation of lipid in the liver varied with the type of fat in the diet. As in part 1, substitution of a fructose source for a direct glucose source resulted in the accumulation of less fat ($P < 0.01$) in livers of rats fed CO, but of more fat ($P < 0.02$) in livers of rats fed a hydrogenated oil (table 5). In most cases, differences were more pronounced at 4 than at 2 weeks.

Percentages of phospholipid in liver fat did not change progressively with time with any carbohydrate-fat combinations except rice starch or glucose with HPO, where a progressive increase was observed (table 5). It may be noteworthy that in the same rats the percentage of cholesterol in liver lipid decreased progressively with time.

When phospholipid and cholesterol content of livers were calculated on the basis of liver weight (mg/10 g of liver), no consistent pattern of changes with time was evident, and neither fraction contributed substantially to the changes in total lipid.

The concentration of cholesterol in the serum of rats fed HPO was lower than that in corresponding groups fed CO. At 4 weeks, differences were significant at the 1 or 2% level in all instances except that of HPO vs. CO with sucrose (table 5).

TABLE 5

Changes with time in liver lipids and serum cholesterol in rats fed different combinations of carbohydrates and fats (part 2)

Weeks fed diet	Direct glucose source with CO [1]	Fructose source with CO	Direct glucose source, with HPO	Fructose source with HPO
	Total liver lipid (% of liver)			
	RS and CO	S and CO	RS and HPO	S and HPO
2	3.44 ± 0.13 [2]	3.32 ± 0.18	2.96 ± 0.29	4.08 ± 0.37
3	4.36 ± 0.46	2.92 ± 0.08	2.72 ± 0.27	3.13 ± 0.14
4	5.15 ± 0.26	3.44 ± 0.20	3.14 ± 0.14	3.96 ± 0.33
	G and CO	F and CO	G and HPO	F and HPO
2	3.74 ± 0.27	3.06 ± 0.14	3.10 ± 0.44	3.51 ± 0.11
3	3.85 ± 0.34	2.71 ± 0.18	3.04 ± 0.16	3.87 ± 0.37
4	4.42 ± 0.31	3.34 ± 0.22	3.60 ± 0.19	5.24 ± 0.52
	Liver phospholipid (% of total lipid)			
	RS and CO	S and CO	RS and HPO	S and HPO
2	23.7 ± 3.6	29.7 ± 3.9	23.8 ± 5.2	23.3 ± 2.5
3	15.4 ± 1.1	15.2 ± 1.5	24.1 ± 4.8	23.3 ± 6.0
4	23.0 ± 2.2	30.0 ± 1.2	39.3 ± 1.5	32.1 ± 0.9
	G and CO	F and CO	G and HPO	F and HPO
2	23.9 ± 5.9	27.6 ± 5.4	27.6 ± 6.2	33.5 ± 1.8
3	24.2 ± 5.0	22.2 ± 7.6	36.5 ± 5.2	27.6 ± 4.2
4	26.0 ± 1.1	28.2 ± 0.9	41.9 ± 2.5	31.0 ± 3.4
	Liver cholesterol (% of total lipid)			
	RS and CO	S and CO	RS and HPO	S and HPO
2	12.6 ± 1.9	9.3 ± 1.2	12.0 ± 0.9	6.9 ± 0.8
3	11.0 ± 1.2	10.0 ± 0.2	9.1 ± 0.5	8.3 ± 0.9
4	9.0 ± 0.5	7.7 ± 0.4	8.7 ± 0.9	5.8 ± 0.7
	G and CO	F and CO	G and HPO	F and HPO
2	10.4 ± 0.4	9.9 ± 1.5	9.4 ± 1.1	8.7 ± 0.2
3	9.9 ± 1.1	9.6 ± 1.0	9.1 ± 0.7	6.6 ± 0.9
4	7.9 ± 0.6	7.5 ± 0.6	7.1 ± 0.8	6.0 ± 0.7
	Serum cholesterol (mg/100 ml serum)			
	RS and CO	S and CO	RS and HPO	S and HPO
2	230 ± 45	182 ± 13	130 ± 14	141 ± 22
3	254 ± 10	248 ± 12	158 ± 6	156 ± 7
4	213 ± 32	198 ± 14	108 ± 15	144 ± 26
	G and CO	F and CO	G and HPO	F and HPO
2	187 ± 25	130 ± 10	94 ± 9	104 ± 5
3	202 ± 14	226 ± 13	128 ± 4	149 ± 6
4	192 ± 17	186 ± 18	97 ± 14	117 ± 19

[1] Abbreviations: CO = corn oil, HPO = hydrogenated peanut oil, RS = rice starch, S = sucrose, G = glucose, F = fructose.
[2] SE of mean.

DISCUSSION

The above data support the thesis that differences in G-6-Pase and FDPase activities in livers from rats fed sucrose as compared with those fed rice starch are the result of the fructose component of sucrose. Diets containing fructose as the carbohydrate would provide approximately twice as much fructose as would those containing the same weight of sucrose, since sucrose is hydrolyzed into equal parts of fructose and glucose. Therefore, the greater activity of the 2 enzyme systems after feeding fructose diets as compared with sucrose diets is probably the result of the greater amount of fructose being metabolized. The action of dietary glucose in increasing G-6-Pase activity above that attained with rice starch may be related to a more rapid absorption of the monosaccharide, releasing a greater glucose load on the liver at one time.

Differences in response of the enzyme systems to dietary HPO as compared with HCO are possibly associated with chain length of their component fatty acids, since HPO yields chiefly stearic and palmitic acids, whereas HCO yields a large proportion of shorter chain acids. Since the metabolic fate of absorbed fatty acids depends partly on their chain length (13), this could be a factor in determining influences of dietary fat on glucose metabolism.

Labile phosphorus values represent the total amount of phosphorus derived from hydrolysis of the high energy bonds of ADP and ATP. As ATP yields twice as much labile phosphorus per mole as does ADP, decreases in labile phosphorus have been assumed to indicate decreases in available ATP or ADP + ATP (7). It is conceivable that the depression in labile phosphorus values observed in groups fed HPO is related to the lack of essential fatty acids in the HPO diets, since most of the ATP of the cell is generated by oxidative phosphorylation (14), which is uncoupled in livers of rats fed diets deficient in essential fatty acids (15). No explanation can be offered at this time for the consistently higher labile phosphorus values obtained from all groups fed a source of fructose as compared with those fed a direct source of glucose (table 3).

Changes in activities of specific enzyme systems in response to changes in dietary constituents are believed by some investigators to be a reflection of alterations in throughput of the pathway(s) in which the enzymes participate (2, 4). Furthermore, Freedland and Harper (2) proposed that adaptations in the activities of the G-6-Pase and FDPase enzyme systems in response to dietary changes would provide a useful means of investigating metabolic pathways. On the basis of the above theory, the suggestion was made in a previous report from this laboratory (5) that a lack of parallelism observed in the responses of the G-6-Pase and FDPase enzyme systems to various carbohydrate-fat combinations might indicate that some fructose is converted to either glucose or glycogen, or both, by means other than the classical route via fructose diphosphate. In the current study, differences in patterns of response of the 2 enzyme systems to diets containing HPO provide further evidence for an alternate route from fructose to glucose-6-phosphate, bypassing fructose diphosphate. For example, feeding HPO with sucrose resulted in a 90% increase in G-6-Pase activity over the level in rats fed the control diet (rice starch with CO), but FDPase activity in the same rats was almost identical with the control level (table 3). Also, these same data strongly suggest that dietary HPO may influence the metabolism of fructose, because although no increase in FDPase activity occurred in rats fed HPO with fructose (above), a substantial increase did occur in rats fed CO with fructose.

The liver lipid data further illustrate the complexity of carbohydrate-fat interrelationships. Especially striking is the consistent observation that, with the type of diet used in these experiments, substitution of a fructose source for a direct glucose source results in accumulation of less fat in livers of rats fed CO, but of more fat in livers of rats fed a hydrogenated oil. Thus the type of carbohydrate in the diet can be an important factor to consider in studies concerning influences of dietary fat on lipid metabolism.

ACKNOWLEDGMENTS

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Hypertension and Cardiovascular Abnormalities in Starved-Refed Swine[1]

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ABSTRACT Heart rate and blood pressure were recorded daily in 2 groups of young adult swine during many months of experimentation involving repeated episodes of starvation (total feed deprivation) and refeeding. Electrocardiograms from the standard and augmented limb leads were recorded during various phases of the study. Severe stresses upon the cardiovascular system were produced as a result of unrestricted initial refeeding particularly, with glucose alone or with a diet high in glucose. Damage of apparently irreversible nature was produced in the myocardium, arteries, and arterioles as a result of the stresses in refeeding following starvation. Notable diastolic hypertension was evident after only 2 starvation-refeeding episodes, and persisted following the fourth episode until the animals were killed 4 to 6 months later.

Hypertensive cardiovascular disease has been found to be prevalent among certain human populations that have been realimented following starvation or prolonged semi-starvation (1–3). Studies with dogs (4–9) have suggested that repeated episodes of starvation and refeeding result in the development of persistent, benign diastolic hypertension, and that the nature and extent of blood pressure responses to refeeding may be modified by diet composition. Acute cardiovascular stress, culminating in heart failure, has been observed in man under conditions of unregulated realimentation following experimental starvation studies (10).

The purpose of the present communication is to report observations of chronic hypertension and other indications of cardiovascular damage in young swine subjected to repeated episodes of starvation and refeeding. Aside from the obvious pertinence of such observations to the currently popular recommendation by some physicians of repeated fasts for weight reduction and to renewed interest in the physiology and psychology of the "religious fast,"[4] the observations appear to confirm previous suggestions of a basic relationship between starvation, refeeding diet and cardiovascular disease.

EXPERIMENTAL

Young swine, 3 to 5 months of age, were kept in individual false-bottom cages (1.82 × 1.52 m) in a laboratory affording temperature control and forced ventilation. They were trained to lie down in a standard position (on the right side) and submit to procedures allowing determinations of heart rate, blood pressure, and electrocardiographic patterns. Blood pressure was measured indirectly (11) using an inflatable rubber cuff in non-yielding cloth cover placed around the upper foreleg. Electrocardiograms were recorded from the standard limb leads (I, II, III, aVl, aVr, and aVf) using a Sanborn Visocardiette instrument.[5]

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[4] Crahay, R. (Warocque-mons Institute, Belgium) 1963 Psychology of fasting. Abbottempo, *1* (1): 35.

[5] Visocardiette, Model 51, electrocardiographic instrument. Sanborn Company, Waltham, Massachusetts.

The animals were fed their daily ration at a single feeding in the early morning. Measurements of heart rate and blood pressures were made in the evening, 10 to 12 hours following consumption of the meal. Determinations were made only after assurance that the subject had been lying relaxed for at least 10 minutes. Measurements were recorded daily, with each recorded value representing the mean of at least 10 replicate measurements.

The animals were fed a natural, corn and soybean meal diet for swine (U of I diet 160)[6] which was modified or replaced by other diets at various stages in the study. Starvation periods were initiated by abrupt and total withdrawal of feed. Drinking water was available at all times.

The first series of experiments was an effort to determine whether starvation-refeeding stresses could be assessed in young swine, and to obtain preliminary evaluation of the stressful influences of various single nutrients or simplified diets when fed during initial re-alimentation following starvation. The second series consisted of repeated starvation and refeeding episodes in a planned series designed specifically to test the hypothesis that chronic hypertension is produced as a result of repeated episodes of starvation and refeeding. The first series of experiments yielded only "uncontrolled observations" in that no control animals were observed. In the second series, observations of hypertensive experimental animals were compared during the final phase of the study with observations similarly made upon nonfasted "control" animals of the same age and weight, and maintained under similar laboratory conditions. Upon termination of the experimentation, the animals were killed and examined postmortem for abnormalities and evidence of cardiovascular disease.

RESULTS

Series 1. In this initial series of experiments, 3 young swine of approximately 5 months of age (2 females and a castrate male) were subjected to 8 episodes of starvation-refeeding during approximately 40 months of observation. Three of the starvation periods were of long-term duration (29 to 33 days) and 5 were of short-term duration (5 to 8 days). These episodes were characterized by initial refeeding with single nutrients or with diets deliberately unbalanced for periods varying from 1 to 10 days. The series of episodes had not been prearranged, but rather reflect the effort to obtain preliminary information pertinent to the relative responses to specific nutrients during the initial phases of refeeding in any given episode. The sequence of dietary regimens to which the animals were subjected is summarized as follows:

Phase 1: *Initial control period.* Diet 160, intake equal to needs for weight maintenance, 30 days.

Phase 2: *Episode 1.* Starvation, 29 days; refined corn oil,[7] 5 days; diet 160 at approximately one-half maintenance needs, 9 days; diet 160 at approximately twice maintenance needs, 21 days.

Phase 3: *Episode 2.* Starvation, 33 days; glucose monohydrate,[8] 5 days; glucose monohydrate plus NaCl at 2% of mixture, 3 days; purified complete diet[9] containing 83% glucose monohydrate, 15 days; diet 160 at maintenance, 25 days.

Phase 4: *Subsequent control period.* Diet 160 at approximately 1.5 maintenance needs, 50 days.

Phase 5: *Short-term starvation episodes 3, 4, 5, 6 and 7.* Starvation, 7 days; glucose monohydrate, 1 day; diet 160, 6 days; starvation, 8 days; cornstarch, 2 days; diet 160, 41 days; starvation, 8 days; soybean protein,[10] 1 day; diet 160, 24 days; starvation, 8 days; corn oil, 2 days; diet 160, 18 days; starvation, 7 days; glucose monohydrate plus NaCl at 4% of mixture, 3 days; diet 160, 10 days.

Phase 6: *Episode 8.* Starvation, 30 days; single nutrient feeding (first glucose, next soybean protein, finally corn oil), 17 days; diet 160 at twice maintenance needs, 33 days; diet 160 at 3 to 4 times maintenance needs, 20 days.

Phase 7: *Sacrifice.* Postmortem examinations.

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[6] U of I diet 160: (in per cent) ground shell corn, 82.6; alfalfa meal, 2.4; soybean meal, 12.2; ground limestone, 1.0; bone meal, 1.0; trace mineral mixture, 0.5; and vitamin supplement, 0.3.

[7] Mazola, Corn Products Company, Argo, Illinois.

[8] Cerelose 2001 (refined corn glucose monohydrate), Corn Products Company, New York.

[9] Purified diet based on composition of diet 160 with ground corn and soybean meal replaced by appropriate amounts of glucose and purified soybean protein.

[10] ADM C-1 Assay Protein (purified soybean protein), Archer-Daniels-Midland, Cincinnati.

Detailed observations from each of the individual phases in this series of experiments are contained in progress report of the Arctic Aeromedical Space Laboratory (Project B-7952-12, contract AF 41(657)130), and are summarized in a technical report (12). In the initial control period (phase 1) heart rates remained uniformly in the range of 70 to 80 beats/minute, and blood pressures were stable at 133 ± 6/96 ± 7 mm Hg (means and sample standard deviations for systolic/diastolic pressures, respectively). Electrocardiographic patterns were consistent and reproducible from day to day in each of the animals.

Body weight losses during the first 2 starvation periods represented approximately 17 to 20% of the pre-starvation weights. During starvation, electrocardiograms revealed arrythmia and T-wave inversion as the most significant changes. Systolic pressures decreased and diastolic pressures increased, with consequent lowering of pulse pressures. The initial phases of refeeding in each of the first 2 episodes were characterized by considerable stress upon the animals as manifested by vomiting, diarrhea, weakness, tremors, and flushing of the skin. Electrocardiograms revealed striking changes in the QRST patterns, especially when the initial refeeding diet consisted of glucose or was high in glucose. Refeeding with glucose resulted in transitory tachycardia. The heart rate and blood pressure responses to starvation and refeeding in epiosodes 1 and 2 are summarized in figures 1 and 2. The points plotted represent means of the daily values for the 3 animals. Control data from phase 1 are imposed in the figures. During refeeding in episode 2 (fig. 2), blood pressures increased above pre-starvation levels. The first 10 to 20 days of refeeding with diet 160 were characterized by large daily fluctuations in both heart rates and blood pressures. These fluctuations are merely suggested, but not depicted clearly in figure 2 which shows only the means of daily values for the 3 animals. As a consequence of these observations the regimen was continued for an extended period (75 days total) in which the caloric intake exceeded slightly the requirements for weight maintenance. Although heart rates subsided to initial levels after 40 to 50 days, the blood pressures continued to rise and became somewhat stabilized at a level of 150 ± 6/104 ± 8 mm Hg during the latter 30 days of the feeding period. This elevated level was regarded as a new base from which to evaluate subsequent responses in the 5 short-term starvation-refeeding episodes (phase 5).

The heart rate and blood pressure responses to initial refeeding with single nutrients or simplified diets following short-term starvation are summarized in figure 3. Although the data reflect some interesting comparisons, the effects cannot appropriately be compared directly or assigned solely to individual diet treatments since it is known that "carry over" effects may have been expressed along with diet effects in subsequent episodes.

During the periods of diet 160, feeding between the 5 short-term episodes of phase 5, it was observed that the daily fluctuations in blood pressures were greatly exaggerated and that the blood pressures increased markedly, exceeding the "control" level as re-established in phase 4. Individual readings as high as 230 mm Hg (systolic) were recorded during the last 10 days of phase 5, and the means of values during this period were 183 ± 10/139 ± 10 mm Hg. Several electrocardiographic abnormalities (e.g., frequent occurrences of sinus tachycardia, pronounced arrythmia, and variable changes, such as notching of QRS, T-wave inversion, elevation or depression of the S-T junction, or both, etc.), were observed during phase 5, and one animal demonstrated during starvation periods frequent occurrence of partial and recurring complete heart block (Wenkebach phenomenon).

In the final starvation episode (phase 6), the animals lost 15% of their pre-starvation weights. In refeeding, the heart rates and blood pressures were subject to such extreme variation, even on an hour-to-hour basis, and electrocardiograms so abnormal as to preclude any serious effort to compare effects of the several regimens employed. It was simply concluded that the animals were, at that point, definitely abnormal, apparently as a consequence of the dietary treatments to which they had been subjected. The final 50 days in which

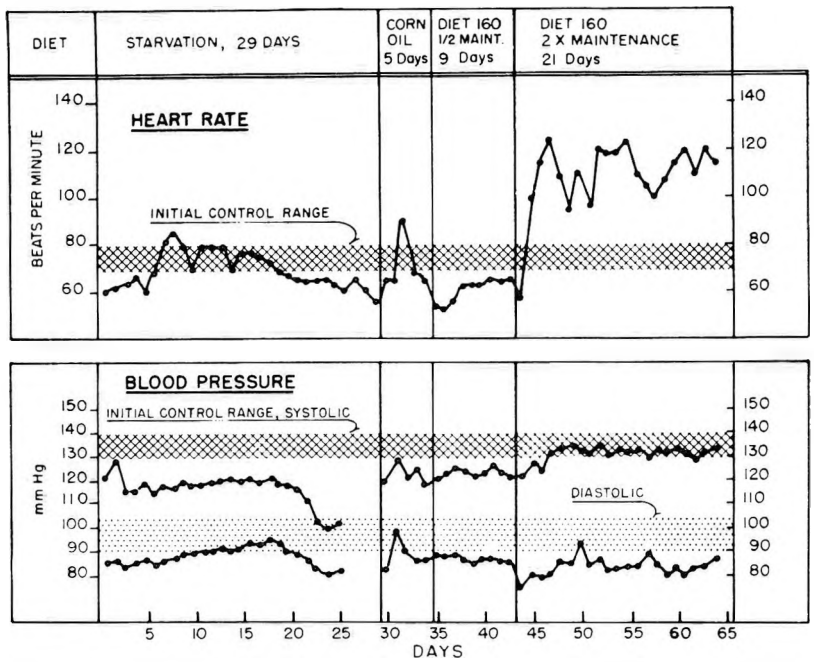

Fig. 1 Heart rate and blood pressure responses by young swine to starvation and refeeding with various diets (episode 1, series 1).

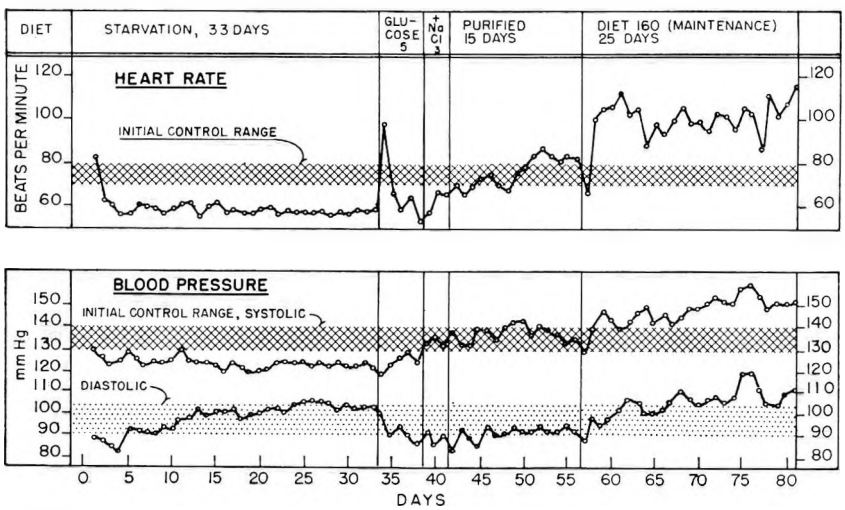

Fig. 2 Heart rate and blood pressure responses by young swine to starvation and refeeding with varied diets (episode 2, series 1).

diet 160 was fed were characterized by recurring periods of momentarily severe sinus tachycardia, with heart rates occasionally reaching 180 beats/minute. Blood pressures fluctuated widely, especially during the short periods of extreme tachycardia which were characterized by faltering pulse, but generally remained hypertensive, often with systolic pressures exceeding 200 mm Hg.

Series 2. Four castrate male swine were observed daily for heart rate and

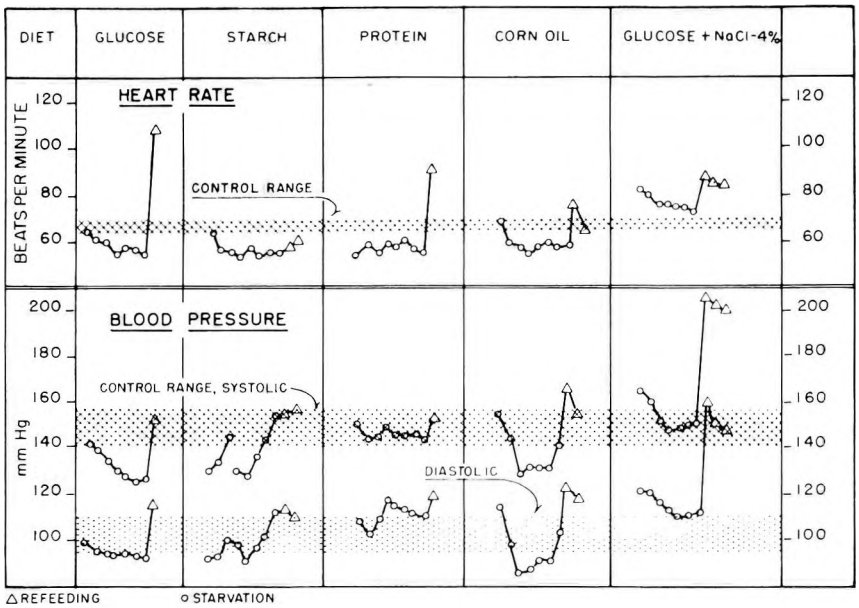

Fig. 3 Heart rates and blood pressures by young swine during refeeding with single-nutrient diets following short-term starvation periods (series 1). Points plotted are means of daily observations.

blood pressure changes during 16 months of experimentation, involving 4 long-term (30 to 50 days) starvation-refeeding episodes. The pigs were 3 months old at the onset of the experiment. Each starvation period was terminated by feeding, ad libitum, experimental diets of varying nature for varying lengths of time. Electrocardiograms (standard limb leads) were recorded periodically and frequently following periods of marked alteration in dietary regimen. The sequence of dietary regimens to which the animals were subjected is summarized as follows:

Phase 1: Period of preliminary adjustment and training (30 days).

Phase 2: Initial control period, diet 160 at level slightly in excess of maintenance needs (20 days).

Phase 3: Diet 160 at maintenance level (2 pigs) compared with the same diet, at 2 × maintenance (2 pigs), (10 days).

Phase 4: Diet 160 at 3 × maintenance, all pigs (5 days).

Phase 5: Starvation (28 days).

Phase 6: Refeed glucose monohydrate (3 kg) compared with starch (3 kg), 2 hogs each diet (1 day).

Phase 7: Refeed diet 160 plus glucose monohydrate (1:1) compared with the same diet plus cornstarch (1:1), at 2 kg/hog/day, 2 hogs each diet (28 days).

Phase 8: Diet 160 at 2.5 maintenance (2.3 kg), all hogs (40 days).

Phase 9: Starvation (40 days).

Phase 10: Refeed pure glucose monohydrate (3 kg) compared with corn oil (1.5 kg) mixed with cellulose (1.5 kg), 2 hogs each diet (1 day).

Phase 11: Refeed diet 160 (1.5 kg) plus glucose monohydrate (1 kg) or corn oil (0.75 kg), 2 hogs each diet (28 days).

Phase 12: Diet 160, maintenance level, all hogs (30 days).

Phase 13: Diet 160, 2 × maintenance, all hogs (10 days).

Phase 14: Starvation (40 days).

Phase 15: Refeed glucose monohydrate, all hogs (2.5 kg); 2 hogs received 150 mg supplemental thiamine (1 day, no feed following day).

Phase 16: Diet 160 (1.5 kg) plus glucose monohydrate (1.5 kg), with and without supplemental thiamine (150 mg daily), (8 days).

Phase 17: Diet 160 at 1.8 × maintenance, all hogs (30 days).

Phase 18: Diet 160 at maintenance, all hogs (20 days).

Phase 19: Diet 160 at 0.4 × maintenance, all hogs (20 days).

Phase 20: Diet 160 at 3.6 × maintenance, all hogs (20 days).

Phase 21: Starvation (50 days).

Phase 22: Diet 160, ad libitum, (approximately 3.5 kg), (30 days); kill hog 3c.

Phase 23: Diet 160 (approximately 2 kg), (140 days); kill hog 2c; observe "control" animals (180 to 330 days).

Phase 24: Diet 160, ad libitum, (4 to 5 kg), (20 to 30 days); kill hogs 1c and 4c and "control" animals.

Observations from each of the individual phases in this series of experiments are described in detail in progress reports to the Arctic Aeromedical Space Laboratory.[11] The responses in refeeding following starvation in this series were suggestive of severe distress, especially when the initial refeeding diet consisted of glucose or was of high glucose content. The general pattern of distress signs in refeeding was early lethargy after consumption of the initial meal followed by a marked increase in heart and respiration rates, then apparent nausea, vomiting, tremors, flushing of the skin, and general weakness sometimes leading to collapse. Diarrhea generally was evident within 2 to 3 hours after the first meal. The animals generally consumed the second, third and subsequent meals

[11] Progress reports 1–6, Contract AF 41(657)359, U.S. Air Force, Arctic Aeromedical Space Laboratory, Fort Wainwright, Alaska.

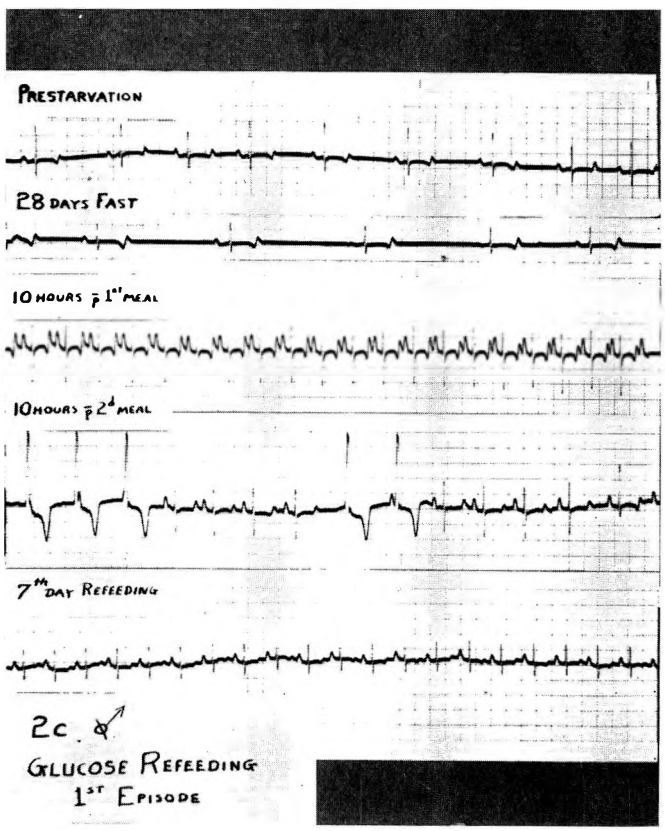

Fig. 4 Electrocardiograms from a young swine (lead II) during various stages of experimentation of starvation and re-alimentation (episode 1, series 2), (from top to bottom, respectively): prestarvation "control" period; twenty-eighth day of starvation; 5 hours following first meal of pure glucose; 10 hours following second feeding (high glucose diet); and 6 hours following feeding, seventh day fed high glucose diet.

heartily in spite of the distress signs initially and their repetition, although less severe, with subsequent feedings.

Upon refeeding in the first episode, one of the animals refed with glucose and exhibiting signs of extreme distress also demonstrated electrocardiographic evidence of myocardial anoxemia. The sequence of electrocardiographic patterns recorded is indicated in figure 4. This animal subsequently showed evidence of further myocardial anoxemia in later episodes. Another animal likewise showed similar evidence of acute anoxemia during refeeding after the fourth starvation period (phase 22).

Comparative responses during the various phases of experimentation were not sufficiently distinctive to support definite conclusions regarding the severity of refeeding stresses, but did suggest that the degree of stresses elicited by glucose was greater than by starch, fat or protein. The results showed no benefit to the animals from thiamine supplementation in early refeeding with high carbohydrate diets.

The data from series 2 demonstrate clearly the development of chronic hypertension in young adult swine as a consequence of repeated episodes of starvation and refeeding. Heart rate and blood pressure data from the entire series are summarized in figure 5. Each point plotted represents the mean of values from all 4 animals averaged for a given period, usually 10 days, with the range of observations in any given period indicated by sample standard deviation[12] plotted as the bars with each of the means. (Broken lines in the figure indicate the values for 1 or 2 individuals remaining on experiment near the end of the study.) A marked increase in heart rate at each refeeding phase is evident in the figure. More important, the figure shows a pronounced increase in systolic and diastolic pressures following the first episode which persisted throughout the second starvation. This observation resembles that reported by Wilhelmj et al. (8).

[12] $\sqrt{\Sigma x^2/n-1}$.

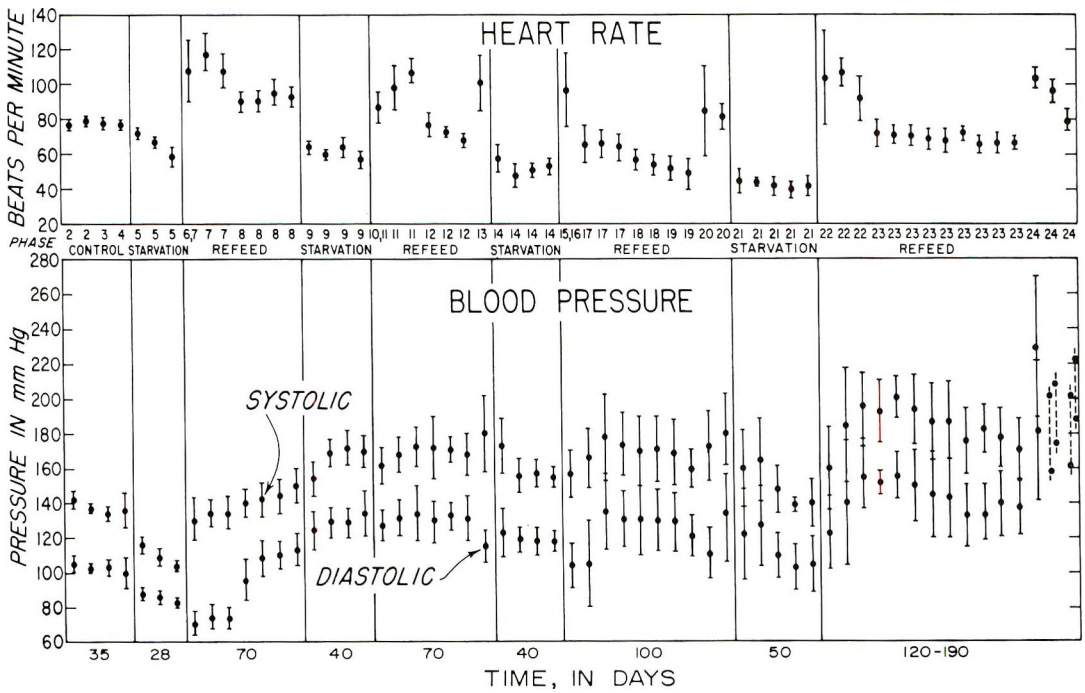

Fig. 5 Heart rates and blood pressures of young swine in repeated episodes of starvation and refeeding (series 2).

Blood pressure subsequently increased to, and persisted at, a hypertensive level. During the last 120 to 190 days of the study, the pressures remained hypertensive in the range 160 to 230 mm Hg, systolic, and 120 to 180 mm Hg, diastolic. Blood pressures for the "control" (normal, non-starved) animals of the same age and weight remained (except for females during periods of estrus) in the range 120 to 140 mm Hg, systolic, 90 to 110 mm Hg, diastolic. Changes in respiration rates tended to follow closely the changes in heart rates.

GROSS PATHOLOGY

Gross pathology findings revealed several abnormalities in the starved-refed animals which are of significance in relation particularly to cardiovascular disease. There was mild-to-moderate hypertrophy of the hearts, especially the left ventricles, and in one or two cases pronounced hypertrophy. The heart from one animal, which had exhibited electrocardiographic evidence of acute myocardial anoxemia (see fig. 4) during a refeeding episode contained a large area (approximately 3.5 × 5 cm) of fibrosis in the right ventricle along the mid-region of the right coronary artery. This lesion is depicted in figure 6. The musculature of the ventricular wall in this area had been replaced completely by scar tissue. The coronaries were probed, but no occlusion was found. Whether or not there had been occlusion of the branches, could not be discerned at the time of slaughter. In other starved-refed animals there was mottling of the heart tissue suggesting degenerative to necrotic changes. In 5 of the 7 starved-refed swine the aortas contained several roughened, fibrotic placques which varied in size from approximately 0.5 × 1.0 mm to 8 × 8 mm. These were generally most prominent in the aortic arch, but placques were found also in the pulmonary and iliac arteries. In only one of the 4 control animals were placques observed, and in that case the size of placques and the number observed were notably smaller than in the starved-refed animals.

Fig. 6 Heart from young adult swine showing evidence of extensive infarction several months earlier during refeeding after starvation.

HISTOPATHOLOGY

Histopathology in the starved-refed swine included degenerative changes in the myocardium with loss of striations, some fatty displacement of muscle tissue and occasional evidence of focal myocarditis. The lesion was accompanied by histological suggestions of infarction (fig. 6). In most of the experimental animals there were patchy, small areas of myocardial tissue degeneration to necrosis. In one animal, particularly, there was thickening of the arterioles in the heart, primarily in the media but including the adventitia, with decreased lumen size and some perivascular reaction including edema and fibrin. There was patchy, intimal edema in the aortas with variable thickening of the intima and foci of chronic inflammatory cells, both on and beneath the intima. Among the collagenous and elastin fibers of the intima were depositions of metachromatic staining material, essentially mucinous in nature. In some instances there was mild deposition of lipid in the intima of the aortas; however, the intimal changes were considered to be relatively mild in comparison with changes reported in cases of human

atherosclerosis. The pulmonary vessels manifested initimal thickening and, in one animal, there was a placque-like area with hemorrhage in the intima accompanied by edema of the media. There was a marked thickening of arterioles with decreased lumen size in many of the organs examined, especially the lungs and hearts. Thickening was primarily in the media with some perivascular reaction including fibrin, edema and an occasional inflammatory cell.

The control animals were relatively free of the abnormalities found in the starved-refed animals.

DISCUSSION

Swine were selected as subjects for this study because of their willingness to consume purified diets and even single nutrients in large quantities during the period of distress following initial refeeding after starvation. In our experience, the pigs were quickly and easily trained for procedures required in blood pressure measurement and electrocardiographic recording (contrary to popular opinion), and they appeared relatively free from psychic and emotional influences which often affect blood pressures in the dog (5). Swine are highly susceptible to heart failure (13), and they exhibit a relatively high incidence of atherosclerosis (14). These, and perhaps other physiological as well as nutritional similarities between swine and humans, may lend pertinence of the observations in this study to applications in the human. The results demonstrate clearly the usefulness of swine in studies requiring dietary extremes and measurement of cardiovascular responses.

These observations of severe stress upon the cardiovascular system in swine resemble similar findings in the human (10) and the dog (6, 8), and they extend the present knowledge regarding the severity and apparent irreversibility of damage to certain tissues which may result from refeeding responses. The results demonstrate beyond question that the immediate responses to unrestricted refeeding with a diet high in glucose may result in severe and irreparable damage to the tissues of the heart, and they suggest, moreover, that stresses associated with refeeding following starvation may damage or otherwise predispose the arterial tissues to development of fibrotic placques. Recently, Constantinides (15) has re-emphasized the view that many different agents may promote atherogenesis merely by damaging the arterial wall, and the present observations suggest that stresses of refeeding following starvation may result in such damage.

Wilhelmj et al. (9) observed moderate systolic hypertension with normal or subnormal diastolic pressures in dogs during initial re-alimentation, but produced persistent, diastolic hypertension in such animals only after repeated episodes of starvation and refeeding with diets unusually high in fat (8). The animals used were mature adults. In the present study involving swine of much younger relative age, a significant trend toward diastolic hypertension was evident during refeeding in the first starvation episode (see fig. 5), and hypertension was evidently persistent and unalleviated by reduction of dietary intake (phases 17, 18, and 19) after the animals were subjected to 3 starvation-refeeding episodes. Whether one such episode of severe refeeding stress would elicit persistent hypertension in young swine, and perhaps other animals, is a question, but of obvious importance. The pathogenesis of the induced hypertension is unknown. It is hypothesized that the arteriolar thickening observed in an occasional parenchymatous organ may be a factor. However, the fact that renal histopathologic changes were minimal does not preclude the possibility of primary or essential hypertension in these swine (16). Although the observations suggest that stresses of refeeding following starvation may result in changes of the myocardial arterioles leading to marked thickening and decreased lumen size, as well as possible predisposition to infarction from fibrotic response, further study is needed to determine whether such arteriolar changes are comparable to early stages of arteriosclerotic disease in the human.

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Energy Intake and Fertility of Male Chickens[1]

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ABSTRACT To determine the effect of restricting the caloric intake of male chickens, 3 groups of 8 adult White Leghorn males were fed 90 g of feed/bird/day containing 2553, 2068 and 1584 kcal of ME/kg for 13 weeks. Volume and fertilizing capacity of semen, as well as body and testis weights were markedly reduced in the 2 groups of males fed the diets with lower calorie levels. There was no difference in the hatchability of eggs fertilized by semen from the 3 groups of males. The data indicated that energy restriction resulting in body weight loss of from 11 to 16% was associated with reduced semen volumes and that the males became completely infertile when their weight loss was 30%. The diet containing 2553 kcal of ME/kg and providing 230 kcal/bird/day approached the minimum necessary for normal body-weight maintenance.

Published experimental results on the effect of nutrition on fertility in adult male chickens are limited as indicated by recent reviews (1, 2). In 1943, Parker and McSpadden (3) showed that restriction of feed intake reduced the volume, sperm density and fertilizing capacity of cock semen. Recently Arscott and Parker (4) showed that reducing the protein level of the ration to as low as 6.9% had no adverse effect on fertility of adult cocks. Since feed restriction had an adverse effect, and protein restriction had no effect, experiments designed to determine the effect of calorie restriction were indicated. The present paper reports the results of such experiments.

EXPERIMENTAL

Twenty-four 7-month-old dubbed White Leghorn cockerels were housed in individual wire-floor cages on December 12, 1962. All these males were fed control ration 1 (table 1) until January 8, 1963 at which time they were divided into 3 groups and fed the 3 experimental diets shown in table 1. The males were distributed into their respective groups in such a manner that differences in average body weights between groups were no greater than 27 g and differences in semen volumes based on 3 preliminary collections or ejaculations were no greater than 0.01 ml. In addition, the males on the 3 treatments were distributed throughout the 3 tiers of a battery in such a way as to avoid positional effects. The experiment was concluded on April 11, 1963 after a 13-week experimental period when all birds were killed and testis weights obtained.

The rations shown in table 1 were formulated so that all ingredients were supplied at a constant level except for glucose monohydrate[2] and cellulose[3] which were used to vary the energy levels. The calculated protein content for each of the 3 rations was 13.1%; however, based on Kjeldahl analyses of the individual ingredients used in these rations (N × 6.25), a protein content of 11.7% was calculated. The calculated metabolizable energy (ME) content for rations 1, 2 and 3 was 2553, 2068 and 1584 kcal/kg of the diet, respectively. Throughout the experiment all birds were individually fed 90 g of their respective diets/day. This amount of feed was shown in a previous experiment[4] to be 3 g/day less than the intake level for ration 1 when similar birds were fed free-choice. Water was provided ad libitum and

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[2] Cerelose 2001, Corn Products Company, New York.

[3] Solka Floc BW-100, Brown Company, Berlin, New Hampshire.

[4] Unpublished data, Arscott, G. H., and J. E. Parker, 1962, in which it was found, using the experimental rations reported here, that the males fed the lowest energy rations ad libitum over a 14-week period increased their food intake up to 1.73 times (with an average of 1.44 times) that noted for the control ration without any adverse effect on semen volume and fertility.

TABLE 1

Composition of experimental diets

	Ration		
	1	2	3
	%	%	%
Oats, grey, ground	43.8	43.8	43.8
Animal fat [1]	1	1	1
Glucose monohydrate [2]	30	15	—
Cellulose [3]	4	19	34
Soybean meal, solvent, (44% protein)	11.2	11.2	11.2
Fish meal, (70% protein)	3	3	3
Alfalfa meal, dehydrated, (20% protein)	3	3	3
Limestone flour	1.8	1.8	1.8
Dicalcium phosphate	1.5	1.5	1.5
Salt, iodized	0.5	0.5	0.5
Vitamin-trace mineral premix [4]	0.2	0.2	0.2
Total	100	100	100

[1] Calogen (Swift and Company, Inc., Portland, Oregon) stabilized with Tenox R which is composed of 20% of citric acid (anhydrous), 20% of butylated hydroxyanisole and 60% of propylene glycol.
[2] See footnote 2 of text.
[3] See footnote 3 of text.
[4] Nopcosol M-3 (Nopco Chemical Company, Richmond, California) supplies per 454 g of mixture: vitamin A, 600,000 USP units; vitamin D, 200,000 ICU; vitamin E, 200 IU; riboflavin, 0.4 g; D-pantothenic acid, 0.6 g; niacin, 3 g; choline, 17.4 g; vitamin B_{12}, 0.8 mg; Mn, 10.896 g; Fe, 3.632 g; Cu, 0.363 g; I, 0.218 g; Zn, 4.994 g; butylated hydroxytoluene, 22.68 g.

body weights were obtained at weekly intervals. Daily periods of 14 hours of incandescent light were provided throughout the experiment.

The procedures used in determining semen volume, fertility and hatchability of fertile eggs have been described (4). Two or 3 White Leghorn pullets were artificially inseminated from each ejaculate using a dose of 0.05 ml of undiluted semen. A total of 1280 eggs was used during this experiment.

The data were subjected to regression analysis with statistical significance determined by *t* test.

RESULTS AND DISCUSSION

The results of the experiment show that reducing the calorie level of diets fed adult White Leghorn males significantly reduced the average rates of change in semen volume ($P < 0.01$ for both lower energy levels) and fertilizing capacity of semen ($P < 0.05$ and < 0.01 for the 2068 and 1584 kcal diets, respectively) in comparison with those of the controls (fig. 1). The average semen volume for the control males receiving 2553 kcal of ME/kg of diet was 0.56 ml, ranging from 0.65 ml at the start to 0.49 ml at the end of the experiment in April (fig. 1A). Such a decline in semen production of the control males is probably not due to the diet since a seasonal decline in semen volume of male fowls commencing in March or April has been observed (5, 6). Semen volumes of the other 2 groups declined greatly throughout the experiment, and for the final collection the average volume was 0.25 ml for the males receiving 2068 kcal of ME/kg and 0.005 ml for those receiving 1584 kcal of ME/kg. Only 1 of the 8 males in the latter group was producing any semen at the end of the experiment, whereas all males in the other 2 groups continued to produce.

Although the fertilizing capacity of semen from the control males (2553 kcal of ME/kg) fluctuated somewhat between collections there was no downward trend (fig. 1B). There were, however, pronounced declines in the 2 groups with reduced caloric intake. Fertilizing capacity of semen from the low-calorie males decreased noticeably below that of semen from the other 2 groups commencing with the 9-week collections and declined to zero with the final or 13-week collections. Fertility of semen from the intermediate-calorie males decreased with relation to that from the controls commencing with the 11-week collections and declined to 39% the thirteenth week.

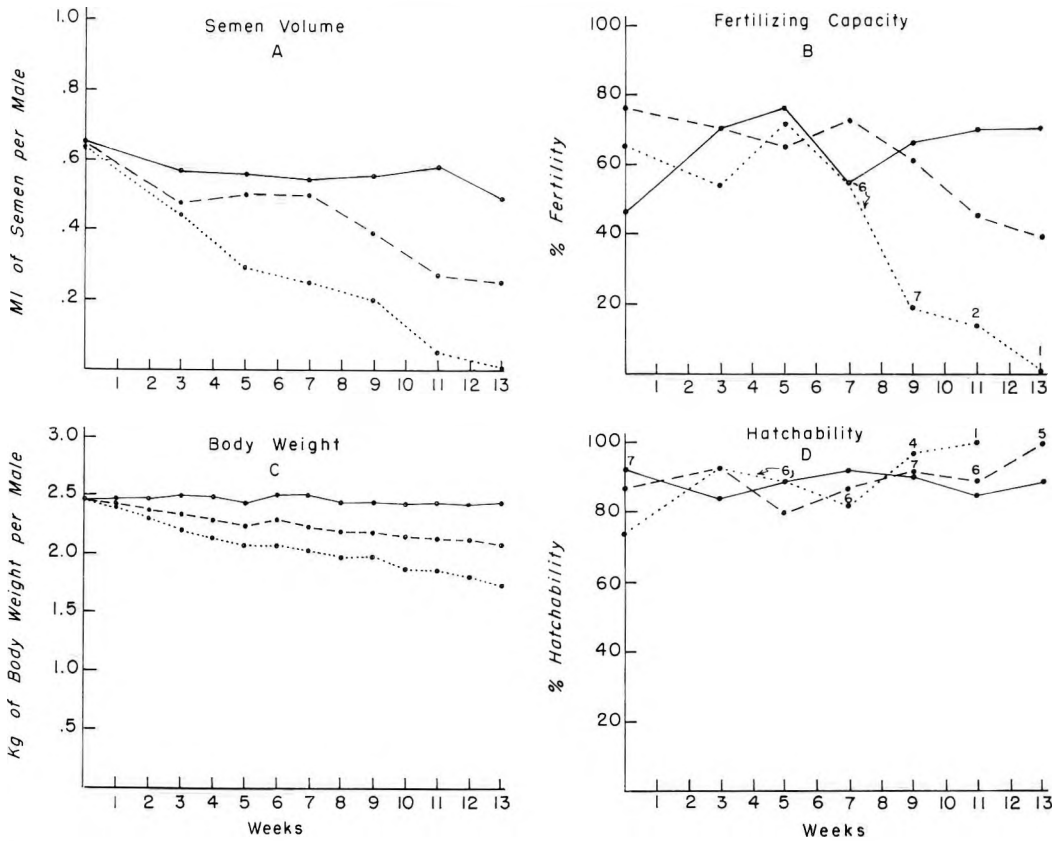

Fig. 1 Influence of caloric intake on volume and fertilizing capacity of semen and body weights of adult White Leghorn male roosters and on hatchability of fertile eggs. Legend: 2553 kcal of ME/kg of diet, ———; 2068 kcal of ME/kg of diet, – – – –; 1586 kcal of ME/kg of diet, · · · ·. Numerals indicate number of males from which data were derived when less than 8 males were involved.

The rate of change in body weights of the males was reduced significantly ($P < 0.01$) upon decreasing the calorie levels of the diet (fig. 1C). At the end of the experiment the average weights were 97.8, 84.7 and 69.7% of the original weights of the males receiving 2553, 2068 and 1584 kcal ME/kg of diet, respectively. The weight loss in both the low- and intermediate-calorie groups was gradual throughout the experiment. Re-examination of figure 1A shows that the first pronounced decrease in semen volumes due to treatment occurred from 3 to 5 weeks with the low-calorie diet and from 7 to 9 weeks with the intermediate-calorie diet. Since by the fifth week the low-calorie males had lost 16.1% of their original body weight and by the ninth week the intermediate-calorie males 10.9% of theirs, it may be concluded that whenever White Leghorn cockerels lose from 11 to 16% in body weight semen production decreases. As demonstrated by the performance of the low-calorie group, males become infertile when their body weight loss approaches 30%. With Rhode Island Red males, a heavier breed, fertility was greatly reduced (3), but not destroyed at body weight losses of similar magnitude induced by restriction of the total diet. As previously noted in this experiment, all males ate equal amounts of feed (90 g/bird day). There was no mortality.

Figure 1D shows that hatchability of fertile eggs from hens artificially insemi-

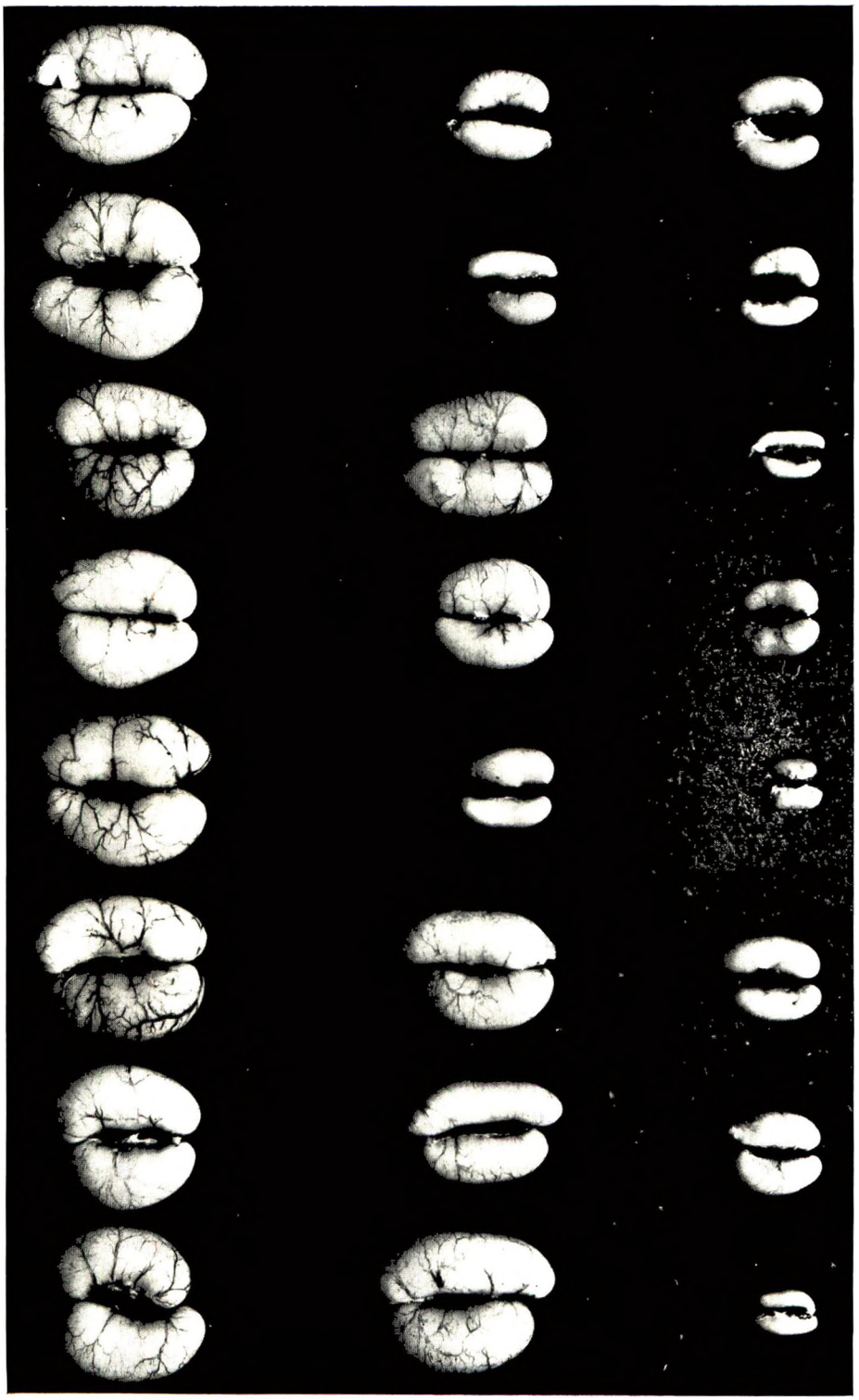

Fig. 2 Effect of energy intake on testis size.

Legend: 2553 kcal of ME/kg of diet, top row; 2068 kcal of ME/kg of diet, middle row; 1584 kcal of ME/kg of diet, bottom row ($\times$ 0.47).

nated with semen from males assigned to the 3 energy levels was not adversely affected.

Decreasing the calorie level of the diet resulted in reduced testis size (fig. 2). At the end of the experiment average weights of both testes per male were 18.45 ±2.88,[5] 9.44 ± 6.31 and 1.86 ± 1.25 g for groups fed 2553, 2068 and 1584 kcal of ME/kg of diet, respectively.

In view of these results and those recently reported by us (4) it is probable that the decreased fertility resulting from total feed restriction previously cited (3), at least in part, was due to a deficiency of calories in the diet.

In relating these energy levels to the level of 90 kcal of ME/kg of body weight/day reported necessary to maintain a positive nitrogen balance for the adult rooster by Leveille and Fisher (7) and adjusting their data to the average initial body weight (2.480 kg) for the 3 energy levels under investigation, a value of 223 kcal/bird/day results. The energy levels supplied by the rations used in this experiment were 230, 186 and 143 kcal/bird/day for rations 1, 2 and 3, respectively. Since 230 kcal/bird/day maintained normal reproductive function with only a 2% decrease in body weight, this level of energy intake apparently approaches the minimum necessary for normal body weight maintenance, whereas 186 and 143 kcal/bird/day proved inadequate. Although 230 kcal are in close agreement with the value cited (7) no experimental evidence was presented by these investigators that this was a minimal value for protein-containing diets nor was any reference made to male reproductive performance. A level of 230 kcal/bird/day is considerably below the level normally fed to chicken breeder males.

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[5] SD of the observations within a group.

Effect of Stress from High Protein Diets on Vitamin A Metabolism in Chicks

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ABSTRACT High protein diets fed to young chicks for 4 weeks produced a stress as evidenced by hypertrophy, hyperactivity and depletion of phospholipid content of the adrenal cortex and at the same time, caused an increased demand on vitamin A nutrition. No adrenal hypertrophy nor hyperactivity occurred in 4-week-old chicks fed high protein diets and simultaneously injected daily with corticosterone. However, daily injections of corticosterone increased the level of vitamin A in the blood plasma and generally caused a decrease in liver vitamin A levels. Although vitamin A deficiency was not found to affect adrenal size or corticoid production the in vitro addition of vitamin A to incubated adrenals from chicks fed high protein diets caused a marked increase in corticosterone production. Liver glycogen formation during the first 4 hours of refeeding high protein-fed chicks after a one-day fast was not impaired by vitamin A deficiency. However, liver glycogen per gram of total feed consumption was significantly decreased in vitamin A-deficient chicks 24 hours after refeeding the high protein diet following the fasting period. These results indicate an important association between vitamin A nutrition and stress produced by high protein feeding.

Evidence has been presented (1,2) showing that the consumption of high protein diets by young chicks is accompanied by marked reduction in liver vitamin A reserves and increases in adrenal size. According to Selye (3) a hypertrophied and hyper-producing adrenal cortex is associated with the first step (the alarm reaction) of an organism's reaction to stress. Vitamin A nutrition has been implicated as an adjunct in the protection of animals against a variety of stresses such as cold exposure (4, 5) X-irradiation (4), and infectious diseases (6–8). Interpretation of the evidence for this, however, has been somewhat controversial (9, 10).

Wolf et al. (11) and Van Dyke et al. (12) presented evidence that vitamin A directly influences hormone production of the adrenal cortex to such a marked extent that severe vitamin A deficiency was considered tantamount to a "chemical adrenalectomy" in the rat. Previous observations by Wolf et al. (13) showed that formation of liver glycogen from labeled acetate, lactate or glycerol was markedly reduced in vitamin A-deficient rats. Glick (14) observed a reduced heterophil count in blood of vitamin A deficient chicks which may indicate a reduced functioning of the adrenal cortex (15).

Evidence also has appeared indicating that the adrenal cortical hormones may influence vitamin A metabolism. Clark and Colburn (16) reported that large doses of cortisone acetate injected into normal or adrenalectomized rats caused a depletion of liver vitamin A. McGillivray (17) also observed that 4 mg of cortisone injected daily into rats caused a release of vitamin A from liver stores with elevated levels of vitamin A alcohol in the blood. Injected ACTH produced the same results, to a lesser extent.

The present report presents experimental results with chicks indicating (a) that the dietary level of isolated soybean protein influences vitamin A metabolism and adrenal cortical production; (b) that injected corticosterone influences the size and production of the adrenals; (c) that injected corticosterone influences vitamin A metabolism; and (d) that vitamin A influences adrenal cortical production.

MATERIALS AND METHODS

White Plymouth Rock X Vantress male chicks hatched from eggs of a controlled flock of hens were used in all experiments. These chicks, when fed vitamin A-free diets, consistently showed vitamin A defi-

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ciency symptoms at 2 to 3 weeks of age. The chicks at one day of age were randomly allotted into pens of a thermostatically controlled, wire-floor brooder. Duplicate treatments were randomly allotted to the individual pens. The high and moderate isolated soybean protein[1] diets used in these experiments were similar to those reported previously (2). The low protein diet had the same nutrient composition, except that it contained 14.5% isolated soybean protein and 71.465% glucose monohydrate. The moisture-free protein (N × 6.25) content of the diets, by analysis, was: high, 79.8%; moderate, 24.2%; and low, 14.4%.

Vitamin A determinations were conducted by the method of Ames et al. (18) on livers which, following dissection, were immediately immersed in liquid nitrogen and stored at −10°C. Blood plasma vitamin A was determined by the method described by Garbers et al. (19). Liver glycogen was determined by the anthrone reagent method of Carroll et al. (20). In vitro corticosterone production was determined in quartered adrenal glands incubated in Krebs-Ringer bicarbonate buffer with ACTH[2] in the medium according to the procedures described by de Roos (21, 22). The medium was then extracted with iso-octane (AR), methylene chloride (AR), and 30 N sulfuric acid, according to the method of Silber et al. (23) as modified by Moncloa et al. (24), except that the medium was not extracted with 0.1 N NaOH because it appeared that an alkaline extraction destroyed corticosterone. Corticosterone was then determined in the fluorescence attachment of a Beckman Model DU Spectrophotometer,[3] using a blue-green filter[4] for the exciting light, and reading the emitted light at 525 mμ. The histochemical determination of the adrenal phospholipids was accomplished by acid hematein stain according to the procedures outlined in Lillie (25), after the entire organ was fixed in calcium chloride formalin, embedded in 25% gelatin, and frozen sectioned at 10 μ. Analysis of variance of the data was accomplished by methods outlined by Steel and Torrie (26).

Injected corticosterone was first suspended in a solution containing (in per cent): NaCl, 0.9; polyethylene sorbitan monooleate (Tween 80), 0.1; absolute ethanol, 10.0; distilled water, 89. After chicks reached 7 to 10 days of age, 350 μg of corticosterone/100 g body weight were injected daily intramuscularly. Control chicks were injected with the solution without added corticosterone. In experiments where vitamin A was mixed into the diet or administered via gelatin capsules, vitamin A palmitate[5] was used. A heat-sterilized vitamin A solution[6] was used when injection was the mode of vitamin A administration.

Experiment 1.[7] High and moderate isolated soybean protein diets were fed to chicks with and without daily corticosterone injection. At one week of age, 14 of the 20 chicks/treatment were selected according to the average weights to be continued on experiment for 3 additional weeks. Corticosterone injection was started when the chicks were 1.5 weeks of age. The chicks fed the moderate protein diet were fed the equivalent amount of feed that was consumed by the high protein fed chicks. The diets were vitamin A-free. At 10 days of age, 20,000 IU of vitamin A were injected into 7 of the chicks in each pen. Livers were taken from chicks at 12, 36, 120 hours, and 17 days after vitamin A injection for liver vitamin A determination. Adrenals of the 4-week-old chicks were dissected, trimmed, weighed and used either for corticosterone analysis or for histochemical phospholipid determination. Adrenals were taken at 4 weeks from the vitamin A-deficient chicks receiving the moderate protein diets ("negative controls"). All vitamin A-deficient chicks fed the high protein diets were dead before the end of the 4-week experimental period.

Experiment 2. The second experiment was conducted to determine whether daily corticosterone injections have any effect on vitamin A levels in the blood plasma and

[1] ADM C-1 Assay Protein, Archer-Daniels-Midland, Minneapolis.
[2] Armour Pharmaceutical Company, Kankakee, Illinois.
[3] Beckman Instruments Inc., Fullerton, California.
[4] Corning (C. S. 4-72) Filter, Corning Glass Works, Corning, New York.
[5] PGB 250 (250,000 IU/g) Distillation Products Industries, Rochester, New York.
[6] 200,000 IU/ml; Hoffmann-LaRoche, Inc., Nutley, New Jersey.
[7] Preliminary report of this experiment by Stoewsand, G. S., A. van Tienhoven and M. L. Scott 1963 Influence of high dietary protein levels on adrenals of the chick. Federation Proc., 22: 609 (abstract).

liver of chicks consuming diets containing 3 different levels of protein.

High, moderate and low protein, vitamin A-free diets were fed ad libitum to 12 chicks/duplicated treatment. At one week of age one-half of the chicks within each of the dietary treatments were injected with corticosterone. One capsule of vitamin A (5000 IU) was given to 7 chicks in each pen at 10 days. Vitamin A was determined in the blood plasma and liver from 2 chicks on each treatment at 8, 9, 10 and 11 days after the vitamin A dosage was given.

Experiment 3. High and moderate protein diets either with or without additional 5000 IU of vitamin A/kg of diet were fed ad libitum to 20 chicks/duplicated treatment. At 13 days of age some of the chicks receiving the high protein diets showed symptoms of vitamin A deficiency. All obviously deficient chicks were removed from the pens, the remaining chicks were fasted for 24 hours, then refed the original diets, and 4 livers/treatment were collected at zero, 1, 4, and 24 hours after refeeding. Total liver glycogen and feed consumption were determined at each time interval.

Experiment 4. High and moderate vitamin A-free diets were fed to duplicated lots of 13 chicks/lot. At 10 days of age, 10,000 IU of vitamin A were injected into 6 chicks/pen. When mild deficiency symptoms were observed in the vitamin A-free chicks at 2 to 3 weeks of age, the adrenals of both vitamin A-deficient and vitamin A-sufficient chicks were used for corticosterone analysis, and some glands were used for histochemical phospholipid determination.

RESULTS AND DISCUSSION

Experiment 1. The results of the first experiment are summarized in table 1. The high protein diet fed to chicks for 4 weeks increased the size of the adrenal glands in confirmation of previous observations (2). However, daily injections of corticosterone nullified the adrenal weight increase in the high protein-fed chicks. Histological examination of the adrenals after hematoxylin and eosin staining showed some hypertrophy of adrenal cor-

TABLE 1

Effect of high and moderate isolated soybean protein diets, with or without corticosterone injection, on body weight, adrenal weight, corticosterone production and vitamin A liver storage

	Treatment	Mean 4-wk body wt	Mean adrenal wt [1]	Mean corticosterone production [2]	Vitamin A liver storage [3]		
					Time after dose [4]		
					12 hr	36 hr	120 hr	17 days
		g	*mg*	*μg/100 mg tissue*	*IU/liver*			
1	High isolated soy protein	308 ± 5 [5]	59.8 ± 3.9	16.5 ± 3.0	2,620	3,220	9,440	10,140
2	High isolated soy protein + corticosterone	305 ± 15	46.6 ± 4.7	4.3 ± 2.5	2,500	3,430	8,630	8,480
3	Moderate isolated soy protein	325 ± 19	50.9 ± 3.5	10.9 ± 2.1	4,120	5,990	8,380	11,380
4	Moderate isolated soy protein + corticosterone	321 ± 10	45.3 ± 1.6	7.9 ± 1.6	4,500	7,710	11,580	9,820

[1] Adrenal weight adjusted by analysis of covariance; both corticosterone and protein treatments had a significant ($P < 0.025$) effect on the mean adjusted adrenal weight.
[2] Analysis of variance shows that only corticosterone injection treatment had a significant ($P \leq 0.01$) effect on the mean corticosterone production.
[3] Analysis of variance shows that protein treatment and time have a significant ($P < 0.01$) effect on vitamin A liver storage.
[4] Dose = 20,000 IU vitamin A injected into each chick.
[5] SE of mean.

tical tissue especially at the periphery of the heavier organs. Microscopic determination of the relative amounts of cortical and medullary tissue using a blood counting grid, showed that apparently in all of the adrenals, regardless of size, approximately 70% of the gland was cortical tissue. Zarrow et al. (27) observed previously that corticosterone injections decrease chick adrenal weights presumably by depression of ACTH output from the anterior pituitary gland.

The corticosterone production of the adrenals in chicks fed the high protein diet was significantly greater ($P < 0.05$) than that of the adrenals of the chicks from the other treatments. It appeared that corticosterone production was directly correlated with the size of the adrenals. Injection of corticosterone significantly decreased the production of corticosterone by the adrenal glands. These results are in contrast with those of Nagra and Meyer (28) who measured corticosterone in adrenal venous blood of cockerels after daily corticosterone injections and observed no differences in treated or untreated chicks. In the present study, the corticosterone production of the adrenals of the corticosterone-injected, high protein-fed chicks was depressed to a much greater extent than the production of the adrenals from chicks fed the moderate protein diets and injected with the same amount of corticosterone.

Histochemically, the adrenal sections (fig. 1) of 4-week-old chicks fed the high protein diet showed decreased amounts and a less dense distribution of phospholipids than the adrenals of the chicks fed the moderate protein diet (fig. 2). Tepperman et al. (29) interpreted a lipoid depletion of the adrenal cortex as indicative of increased activation of the gland.

After the injection of the high dosage of vitamin A, the liver vitamin A reserves were decreased in the chicks receiving the high protein diet, only at 12 and at 36

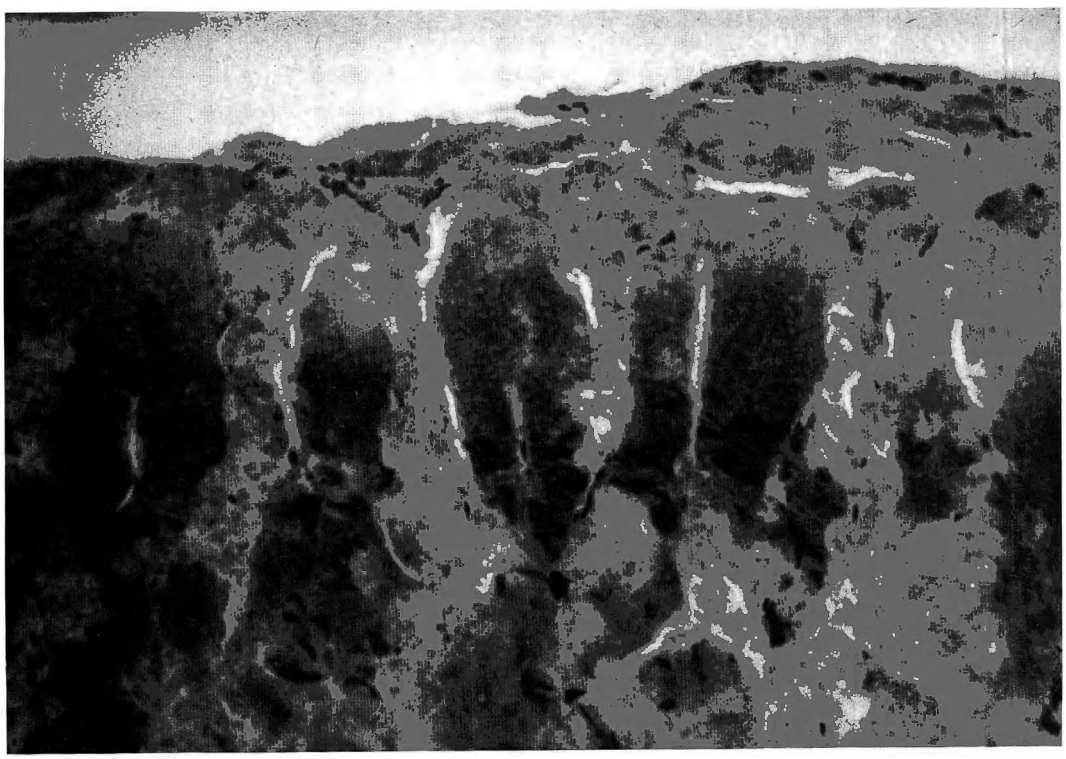

Fig. 1 Adrenal from 4-week-old chick fed the high protein, vitamin A-sufficient diet. Hypertrophy and hyperactivity present; phospholipid (dark area) shows marked depletion as compared with the normal. Acid hematein stain. × 100; 10 μ.

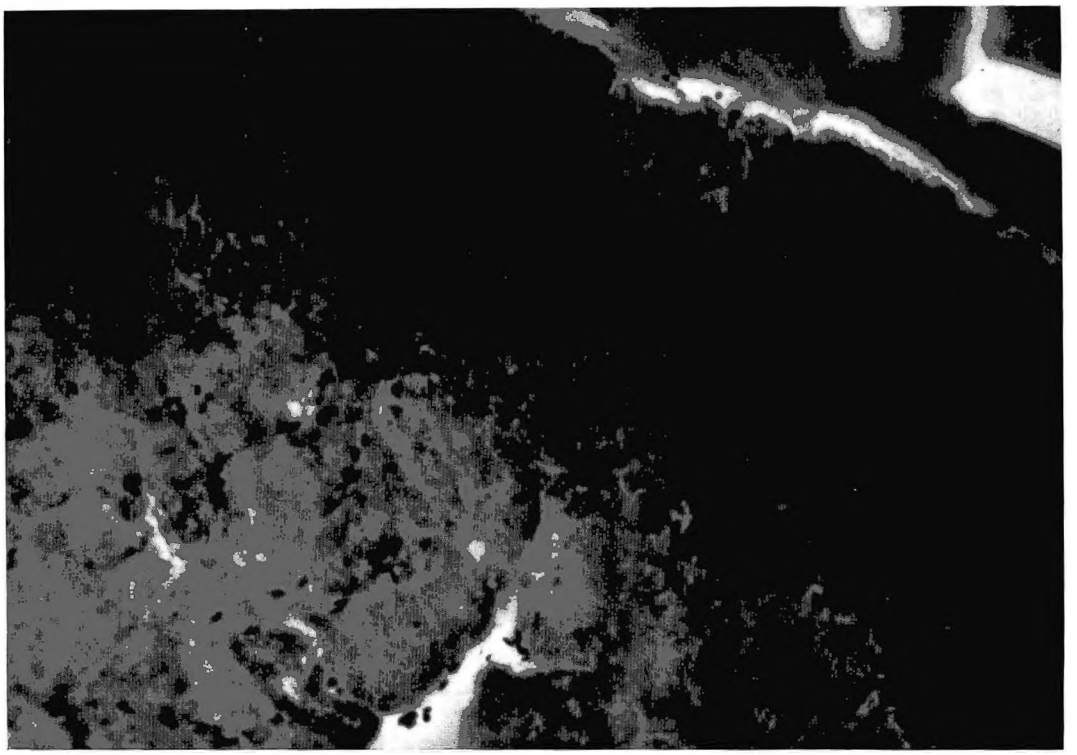

Fig. 2 Adrenal from 4-week-old chick fed moderate protein diet. Darkened area is phospholipid, which showed this normal distribution in all glands from moderate protein-fed chicks whether vitamin A-sufficient or vitamin A-deficient. Two-week-old chicks fed the high protein diet also showed this type of normal adrenal. Acid hematein stain. × 100; 10 μ.

hours after dosing. After 120 hours and after 17 days vitamin A liver storage of chicks on all treatments was very similar.

Experiment 2. The results of experiment 2 are summarized in table 2. The average vitamin A content of the blood plasma increased when corticosterone was injected daily into the chicks regardless of the dietary protein treatment. Concurrently the mean liver vitamin A values were, in general, lower in the corticosterone-injected chicks fed the high and low protein diets. The moderate protein-fed chicks had about the same liver vitamin A storage regardless of corticosterone treatment. In another experiment, with a very limited number of chicks fed the same moderate protein diet, it appeared that at 13 days after an oral dose of 5000 IU of vitamin A with daily corticosterone injections (350 μg/100 g body weight) the chicks had an average total vitamin A liver storage of 1100 IU. Chicks fed the same diet with no hormone injection had an average total vitamin A liver storage of 1366 IU. Thus, it appears that daily injections of relatively small amounts of corticosterone can to some extent mobilize liver vitamin A with concomitant increases of vitamin A in the circulation. Also, in contrast with experiment 1, corticosterone injections caused a decreased body weight, when the protein level of the diet remained constant. Others (27, 28) also have observed decreased growth when this steroid was injected daily into young chicks.

This experiment also shows that blood plasma vitamin A, as well as liver vitamin A, increased as the levels of isolated soybean protein in the diet were decreased. The blood plasma of the chicks fed the low protein diets had a "milky" white appearance which may indicate higher amounts of plasma lipid in the chicks receiving the low protein diet.

TABLE 2

Vitamin A status of chicks fed low, moderate and high isolated soy protein diets with or without daily corticosterone injections

	Treatment [1]		Mean blood plasma vitamin A [2,3]	Mean liver vitamin A [2,4]	Body wt
	Level of isolated soy protein	Daily injection of corticosterone			
			IU/ml	*IU/liver*	*g*
1	High	+	1.50 ± 0.15	451 ± 76	195
2	High	0	1.22 ± 0.16	544 ± 116	232
3	Moderate	+	1.65 ± 0.30	1557 ± 240	264
4	Moderate	0	1.29 ± 0.24	1508 ± 362	287
5	Low	+	2.58 ± 0.29	1454 ± 172	188
6	Low	0	1.84 ± 0.14	1690 ± 194	207

[1] One gelatin capsule containing 5,000 IU was given to chicks at 10 days of age.
[2] Analysis of variance showed that time effect is not significant ($P > 0.05$); therefore, the vitamin A data of 8, 9, 10, 11 days after vitamin A dose were averaged.
[3] Analysis of variance showed both dietary protein and corticosterone injection produced significant ($P < 0.01$) effects.
[4] Analysis of variance showed only the protein treatment had a significant ($P < 0.01$) effect.

Experiments 3 and 4. The results of experiments 3 and 4, which are presented in tables 3 and 4, are discussed together because the experimental designs were very similar. In these experiments special efforts were made to produce only a mild vitamin A deficiency in the two-week-old chicks, since it was desired to observe whether impairment of glycogen formation and corticosterone production is a primary lesion in vitamin A-deficient chicks before the severe ataxia and anorexia of acute vitamin A deficiency occur (30).

The data in table 3 show that the average amount of liver glycogen increased to a maximal level by 4 hours of refeeding after a 24-hour fast. Liver glycogen decreased after 24 hours of feeding. When the liver glycogen value is related to the amount of diet consumed, as indicated in the last column, it appears that liver glycogen formation after 4 hours of refeeding was not impaired by vitamin A deficiency. However, after 24 hours of refeeding, vitamin A deficiency in the high protein-fed chicks caused a significant reduction ($P < 0.05$) in the amount of liver glycogen formed per gram of feed intake as compared with chicks receiving vitamin A-sufficient high protein diets.

The adrenal corticoids are known to promote glycogenesis from protein and other nutrients (31). Wolf and co-workers (13) observed that glycogen formation from acetate, lactate, or glycerol was almost zero in vitamin A-deficient rats, possibly indicating adrenocortical insufficiency. However, Block and Cox (32) fed adrenalectomized mice pure casein after a 24-hour fast. These mice produced the same amount of liver glycogen, up to a maximal level at 16 hours after feeding, as the normal casein-fed controls. Their results showed, however, that liver glycogen was decreased in the adrenalectomized mice during the 16- to 64-hour period of refeeding pure casein as compared with the corresponding normal controls.

Corticosterone production of the adrenals of chicks fed high and moderate protein diets with or without vitamin A are presented in table 4. Adrenal glands in these two-week-old chicks fed high protein, vitamin A-sufficient diets were not enlarged, and did not produce more corticosterone than the adrenals of chicks fed the moderate protein diets. This perhaps was because chicks were consuming the high protein diet only for 2 weeks instead of 4 weeks as in experiment 1, since Leathem (33) has indicated that the age of the animal and the duration of the experiment are very important factors influencing adrenal hypertrophy from high levels of dietary protein.

The adrenal glands of the vitamin A-deficient two-week-old chicks fed either the high or moderate protein diets did not produce significantly lowered amounts of corticosterone, nor was there any gross change in the phospholipid distribution in their adrenals. Lowe et al. (34) observed in the

TABLE 3

Liver glycogen production of chicks fed vitamin A-deficient or vitamin A-sufficient, moderate and high protein diets

Time [1]	Treatment		Avg liver glycogen	Cumulative feed consumption	Liver glycogen/ g feed intake [2]
	Dietary protein	Vitamin A			
hours			*mg/100 g*	*g/chick*	*mg/100 g liver/g feed*
0	High	0	0	0	0
	High	+	0	0	0
	Moderate	0	2	0	0
	Moderate	+	2	0	0
1	High	0	94	1.75	54 ± 20
	High	+	118	2.5	48 ± 9
	Moderate	0	2186	5.2	418 ± 66
	Moderate	+	1757	6.4	274 ± 19
4	High	0	1733	3.8	460 ± 67
	High	+	1940	5.7	340 ± 53
	Moderate	0	7330	11.2	664 ± 34
	Moderate	+	7596	12.6	580 ± 168
24	High	0	658	13.0	48 ± 23 [3]
	High	+	2098	16.9	124 ± 23
	Moderate	0	4920	25.4	192 ± 15
	Moderate	+	3875	34.2	132 ± 20

[1] Time of refeeding after 24-hour fast.
[2] Mean of 4 chicks with SE of mean.
[3] Significantly ($P < 0.05$) lower value at 24 hours' refeeding.

TABLE 4

Corticosterone production of 2-week-old vitamin A-sufficient and vitamin A-deficient chicks consuming moderate and high protein diets

Treatment [1]			No. of chicks	Corticosterone production	2-week mean body wt	2-week mean adrenal wt
Isolated soy protein level	Vitamin A injected into chicks	Vitamin A incubated in media				
				μg/100 mg tissue	*g*	*mg*
High	+	0	6	12.58 ± 2.05 [2]	153	40.2
High	0	0	6	10.07 ± 2.40	134	34.5
High	+	+	2	26.94		
High	0	+	2	36.51		
Moderate	+	0	6	16.22 ± 0.96	203	45.7
Moderate	0	0	6	15.48 ± 1.84	208	44.2
Moderate	+	+	2	17.46		
Moderate	0	+	2	17.85		

[1] Analysis of variance of the data shows that vitamin A incubation and protein × vitamin A incubation are significant ($P < 0.01$) effects. The three-way interaction (i.e., protein × vitamin A injection × vitamin A incubation) is also significant ($P < 0.05$).
[2] SE of mean.

vitamin A-deficient rat's adrenal cortex that phospholipids migrate into the zona glomerulosa from the normal distribution in the zona fasciculata. In the chick there are no cortical zones. When hypertrophy or increased activity of the gland occurs, a decreased amount of phospholipid is noted throughout the cortex (fig. 1). Phospholipid distribution did not appear to be affected at 2 or 4 weeks of age by vitamin A deficiency (fig. 2). In vitro addition of 10,000 IU of vitamin A to incubated adrenal glands significantly increased the production of corticosterone. The increased corticosterone production was two- to threefold from the adrenals of chicks fed

the high protein diet, whereas only a slight increase was observed from the adrenals of the chicks consuming the moderate protein diets. Studies with adrenals from vitamin A-deficient rats have shown decreased amounts of corticosterone production (11, 12). Van Dyke et al. (12) and Wu Chang and Willis[8] observed that addition of vitamin A to rat adrenal homogenates increased the in vitro production of corticosterone.

The results of these experiments show that very high levels of isolated soybean protein fed to chicks for at least 4 weeks promote a hypertrophy and hyperactivity of the adrenal cortex. This "alarm stimulus" or "stressor agent" effect of high dietary protein appears to decrease vitamin A reserves. Hill (35) observed that chicks fed increased levels of protein had an increased susceptibility to an infectious disease and that an increased level of vitamins, including vitamin A, counteracted this dietary protein effect.

Corticosterone was used in the present studies because de Roos (22) has shown that it is the major glucocorticoid of the adrenal cortex of the chick. When corticosterone was injected daily into chicks fed high protein diets, the adrenals did not hypertrophy and also secreted much less corticosterone than uninjected chicks. Apparently, the production of corticotropin (ACTH) causes the adrenal cortex to hypertrophy, whereas injected corticosterone depresses this production by "feedback" mechanisms (31), although the chick's adrenal may be partially independent of the anterior pituitary gland (36, 37). Corticosterone injections increased the levels of vitamin A in the blood possibly by mobilization of vitamin A from the liver. This hormone effect, therefore, may be at least in part responsible for the depletion of vitamin A liver reserves of chicks fed high levels of protein. The effect of high dietary protein and vitamin A deficiency upon liver glycogen may indicate an abnormal turnover of liver glycogen in vitamin A-deficient chicks.

These results indicate that vitamin A nutrition bears an important relationship to adrenal function as an adjunct in the protection of the chick from the stress produced by feeding high protein diets.

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Studies on the Digestion of Soybeans

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ABSTRACT Protein digestibility of properly heated soybeans was significantly higher than of raw and overheated soybeans. Availability values of 5 essential amino acids, as determined by the fecal analysis method, were significantly higher for heated soybeans. Using rats trained for spaced-feeding, it was established that the rate of food passage through the stomach was considerably slower for raw soybeans. Fractionation of intestinal contents in insoluble residue, trichloroacetic acid (TCA)-soluble and TCA-precipitable nitrogen, revealed that insoluble residue and TCA-precipitable nitrogen were always higher in the case of raw soybean feeding. Using a diet containing a complete amino acid mixture to which a crude preparation of the water-soluble trypsin inhibitor factor (TIF) was added, the addition of raw TIF resulted in a slower rate of stomach emptying. The insoluble intestinal nitrogen and TCA-precipitable nitrogen fraction were significantly higher when the amino acid diet was supplemented with raw TIF. The addition of TIF also interfered with the amino acid absorption through the intestinal wall.

Since the early work of Osborne and Mendel (1) it has been known that properly heated soybeans are nutritionally superior to raw soybeans. The raw product contains heat labile factors possessing trypsin inhibiting (2), hemagglutinating (3) and possibly other activities (4) which result in reduction of food intake and poor growth.

Amino acid supplementation studies (5, 6) have shown that the nutritional quality of the raw product can be improved by addition of certain amino acids, but it appears that the resulting diet can never stimulate growth to the same extent as that obtained for rats fed heated soybeans (7).

Although earlier publications (8–13) suggest that nitrogen, sulfur and methionine absorption are similar in rats fed either raw or heated soybeans, results were reported recently (14, 15) indicating a decrease in nitrogen and methionine absorption in rats fed the raw product. Both threonine and valine also tend to be less available from the raw product although no significant difference was reached (15).

The assumption that amino acids are less available in the raw product can, however, not be made without reservation in view of results reported by Lepkovsky and his associates (16, 17). These workers observed that an excessive flow of digestive enzymes was caused by feeding a diet containing either raw soybeans or a crude preparation of trypsin inhibitor from soybeans. It was therefore suggested (6, 18) that the low nutritional value of raw soybeans is due to an excessive excretion of growth-limiting amino acids incorporated in the proteins of the overstimulated enzymatic secretion.

The problem is still a very complex one and warrants further investigation. The present communication reports on protein digestibility and availability to rats of 8 essential amino acids present in raw, heated and overheated soybeans, respectively. A study was also made of the gastrointestinal contents and plasma amino acids of rats fed the respective soybean products and diets containing a crude preparation of soybean trypsin inhibitor.

MATERIALS AND METHODS

Digestibility of protein and availability of amino acids. A local variety of soybeans (Swasiland) was milled in a hammer mill and the resulting meal extracted with petroleum ether (60 to 80 bp). One batch was used without further treatment (raw soybeans). A second batch was spread out in 1-cm thick layers in stainless steel trays and autoclaved for 30 minutes at 121°C. After autoclaving, the moist cake was dried in a draught-air oven at 50°C (heated soybeans). Another batch

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was autoclaved at 126°C for 2 hours and dried at 60°C (overheated soybeans).

The experimental diets contained: (in per cent) corn oil, 5; salts,[1] 5; cod liver oil,[2] 1; water-soluble vitamin mixture,[3] 0.25; choline chloride, 0.15; raw, heated or overheated soybean meal at such a level that the protein content was approximately 10; and cornstarch to make 100.

True digestibility of the soybean protein was determined by the "balance sheet" method of Mitchell (19) using adult male rats (Wistar strain) of approximately 250 g. Three sets of 3 rats each were allocated at random to a 3 × 3 Latin square design (20) so that at the end of the experimental period each set of rats had been given all 3 experimental diets but in a different sequence (21). Collection of feces was made for 5 days after a preliminary adaptation period of 3 days. A nitrogen-free diet, similar to that described above except for the addition of soybean meal, was fed before and after the experimental period in order to estimate the metabolic nitrogen. Each of these nitrogen-free periods had a duration of 8 days divided into a 3-day preliminary and a 5-day collection period. Rats were housed individually in stainless steel metabolism cages.

The collected feces were homogenized and strained through a sieve to separate out hairs. Aliquots of the fecal suspension were taken for Kjeldahl determination and acid hydrolysis. Acid hydrolysis was carried out with 3 N HCl acid in an autoclave for 8 hours at 121°C. Since it is a comparative study, no correction for possible destruction of amino acids under the conditions of hydrolysis was attempted. Amino acids were determined in the ration and the fecal acid hydrolysates by microbiological assay (22) using *Leuconostoc mesenteroides* for all amino acids except threonine, which was determined with *Streptococcus faecalis*. To estimate the metabolic amino acid excretion the feces of rats during the nitrogen-free period were also analyzed (21).

For digestibility the statistical analysis was based on individual animals. For the availability study the feces of the 3 rats in each set were pooled.

Study of gastrointestinal contents and free amino acids in plasma. Male rats of approximately 150 g were trained to consume large quantities of food in very short periods (23). After about 2 weeks' training they could consume about 3 g of diet in a 30-minute period. During this training a diet similar to that described for the digestibility determination, but containing 25% of casein as protein source, was used.

In the experiments with soybeans the experimental diet contained 55% of the raw or heated product. The amino acid diets contained a complete amino acid mixture (24) and were supplemented with 5% of a crude preparation of the soybean trypsin inhibitor. A control diet contained the same amount of trypsin inhibitor which had been autoclaved for 30 minutes at 121°C. The crude trypsin inhibitor (TIF) was prepared by the method of Lyman and Lepkovsky (16) involving an ammonium sulphate precipitation, dialysis and treatment with cold acetone.

Rats that had consumed approximately 3 g of the experimental diet within the 30-minute feeding period were killed at different time intervals.

Stomachs and small intestines were ligated and removed from the carcasses. The contents of the stomachs were washed out with water and dry weight determined. The intestinal contents were washed into a centrifuge tube. After centrifugation the supernatants were made up to 50 ml. The soluble protein was removed from the supernatant by addition of 20% trichloroacetic acid (TCA) to give a final TCA concentration of 5%. This mixture was heated in boiling water for 10 minutes and the TCA-precipitated material removed by centrifugation (TCA-precipitable fraction). This fractionation study is based on the assumptions made by Chen et al. (25).

The nitrogen of the insoluble residue, the TCA-precipitable fraction and that in the supernatant (TCA-soluble fraction) was determined by a semimicro-Kjeldahl method using mercuric oxide as catalyst.

[1] Richardson, L. R., and P. G. Hogan 1946 J. Nutrition, 32: 459.
[2] Cod liver oil was supplemented with α-tocopherol to provide 100 g of diet with 10 mg of vitamin E.
[3] Harper, A. E. 1959 J. Nutrition, 68: 405.

Blood samples were taken from the portal vein of rats used in the gastrointestinal content study. The rats were anesthetized with ether, an abdominal incision made, and about 4 to 5 ml of blood withdrawn from the portal vein into a heparinized syringe, the needle pointing away from the liver. The free amino acids were determined quantitatively by the 1-fluoro-2, 4-dinitrobenzene method (26).

RESULTS

Digestibility and amino acid availability. True digestibilities and amino acid availabilities are shown in table 1. The absorption of protein was significantly improved by heat treatment of the soybeans, whereas overheating decreased digestibility, although this was not significant.

Except for the lysine, threonine and methionine all amino acids were significantly more available in the heated soybean than in the raw product. Overheating as compared with the properly heated product had no statistically significant effect on the availability of isoleucine, leucine, phenylalanine and valine. Methionine availability was significantly higher in the overheated product, whereas no significant difference (although it approximates significance) could be found between the availability of methionine of heated and raw soybeans. The availability of all the amino acids under consideration is lower than the overall digestibility of the protein.

Stomach contents. The rate of a stomach emptying of rats fed a single meal of a diet containing 55% of either raw or heated meal is illustrated in figure 1A. Each point on the curve represents the mean value of 5 rats, each of which had consumed 3 g of the diets within the one-half hour time limit allowed for food consumption. Rats were killed just after the feeding period (zero hour), and after 1, 2, 3 and 5 hours. The amount of dry matter recovered is expressed as percentage of the total amount ingested. The rate of passage through the stomach was slower for the raw soybean than for the heated product.

Figure 1B shows the rate of stomach emptying at different time intervals of rats fed a complete amino acid diet supplemented with 5% of either the raw preparation of the trypsin inhibitor or the heated inhibitor. Stomach emptying was slower in rats fed the diet containing the raw factor. At zero hour more of the amino acid ration had passed through the stomach than in the case of the soybean diet. From then on, however, the soybean passed through the stomach at a faster rate than the amino acid diets.

Intestinal contents. The amount of insoluble nitrogen, TCA-soluble nitrogen and TCA-precipitable nitrogen of intestinal contents of rats fed raw or heated soybeans at different time intervals after ingestion are shown in table 2.

The insoluble nitrogen of the intestinal contents of rats fed raw soybeans was constantly higher than for the heated soybeans. The largest accumulation of insoluble residue occurred at the third hour after

TABLE 1

Availability of amino acids and protein digestibility in soybean samples

Amino acid	Soybean samples		
	Raw	Heated	Overheated
Histidine	62.6	85.6 [1]	76.4
Isoleucine	61.9	74.5 [1]	70.2 [1]
Leucine	65.9	76.3 [2]	76.3 [2]
Lysine	64.1	71.1	62.6 [3]
Methionine	49.4	56.9	65.2 [1]
Phenylalanine	83.0	91.6 [1]	91.7 [1]
Threonine	66.0	73.7	74.6
Valine	61.7	80.8 [2]	74.6
Protein digestibility	82.9	89.7 [2]	84.0

[1] Significant difference at $P \leq 0.05$ (compared with raw soybean).
[2] Significant difference at $P \leq 0.01$ (compared with raw soybean).
[3] Significant difference at $P \leq 0.05$ (compared with heated soybean).

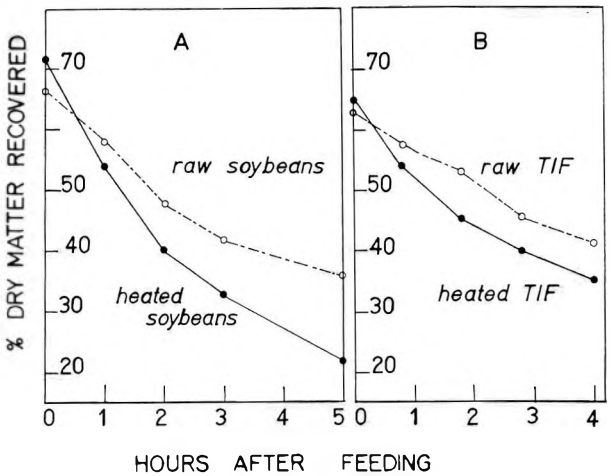

Fig. 1 Stomach emptying of rats fed raw and heated soybeans (A) and rats fed an amino acid-containing diet supplemented with raw and heated trypsin inhibitor factor (TIF). (B). Each point on the curve represents the mean value of 5 rats.

TABLE 2

Fractionation of intestinal contents of rats fed raw and heated soybeans

Sample	0 hr	1 hr	2 hr	3 hr	4 hr	5 hr
	mg	*mg*	*mg*	*mg*	*mg*	*mg*
			Insoluble nitrogen in intestinal contents			
Heated soybean	8.96 ± 0.72 [1]	12.71 ± 0.62	12.22 ± 0.55	14.66 ± 1.21	10.21 ± 0.94	8.71 ± 0.80
Raw soybean	11.57 ± 0.91	15.37 ± 1.00	14.39 ± 0.77	17.57 ± 0.73	13.02 ± 0.41	11.76 ± 0.52
			TCA-soluble nitrogen			
Heated soybean	8.16 ± 0.21	4.76 ± 0.50	8.07 ± 0.47	7.49 ± 0.25	5.60 ± 0.37	3.99 ± 0.61
Raw soybean	4.92 ± 0.54	5.18 ± 0.33	5.96 ± 0.61	16.02 ± 0.51	4.06 ± 0.44	4.55 ± 0.73
			TCA-precipitable nitrogen			
Heated soybean	2.85 ± 0.14	2.16 ± 0.42	3.10 ± 0.39	2.45 ± 0.18	3.99 ± 0.15	2.24 ± 0.25
Raw soybean	6.75 ± 0.36	7.84 ± 0.19	7.46 ± 0.21	6.02 ± 0.30	4.76 ± 0.18	6.09 ± 0.37

[1] Mean of 5 rats ± SE of mean.

food ingestion for both diets. The TCA-soluble nitrogen of rats fed the heated soybean diet is significantly higher than that for raw soybeans at zero hour. This tendency is, however, not as clear-cut at the other time intervals. The TCA-precipitable fraction of the intestinal contents of rats fed the raw soybean diet was significantly higher, at all time intervals, than that of rats fed the heated soybean diet. The amount of TCA-precipitable nitrogen was from 1.2 to 3.6 times higher for the rats fed the raw soybeans.

In table 3 results are shown on the fractionation of intestinal contents of rats fed a complete amino acid diet supplemented with 5% trypsin inhibitor. Rats fed a similar diet supplemented with heated trypsin inhibitor were used as a control group. The TCA-soluble nitrogen fraction of rats fed the diet containing raw TIF was significantly lower than for rats fed the heated trypsin inhibitor. The TCA-precipitable nitrogen fraction was significantly higher in the intestinal contents of rats consuming the raw inhibitor. The amount of TCA-precipitable nitrogen for rats fed raw trypsin inhibitor was 1.5 (at 2 hours after feeding) to 3 times (3 hours after feeding) the amount precipitated by TCA for the corresponding intestinal contents of rats ingesting the heated trypsin inhibitor. The insoluble intestinal nitrogen was significantly higher

TABLE 3

Fractionation of intestinal contents of rats fed amino acid diets supplemented with raw and heated trypsin inhibitor factor (TIF)

Diet	0 hr	1 hr	2 hr	3 hr	5 hr
	mg	*mg*	*mg*	*mg*	*mg*
			Insoluble nitrogen in intestinal contents		
Heated TIF	—	3.90 ± 0.50 [1]	4.21 ± 0.39	—	—
Raw TIF	—	6.77 ± 0.49	7.05 ± 0.71	—	—
			TCA-soluble nitrogen		
Heated TIF	7.09 ± 0.32	10.09 ± 0.33	9.12 ± 0.45	4.57 ± 0.80	7.74 ± 0.39
Raw TIF	5.88 ± 0.41	8.16 ± 0.18	—	2.73 ± 0.63	5.59 ± 0.28
			TCA-precipitable nitrogen		
Heated TIF	1.93 ± 0.43	2.26 ± 0.30	3.70 ± 0.50	1.95 ± 0.28	2.79 ± 0.51
Raw TIF	5.85 ± 0.31	6.71 ± 0.28	5.80 ± 0.22	5.95 ± 0.55	5.27 ± 0.32

[1] Mean of 5 rats ± SE of mean.

for rats fed the diet containing the raw TIF at the 2 time intervals tested.

Table 4 shows the TCA-soluble and TCA-precipitable nitrogen fractions of duodenal, jejunal and ileal contents of rats fed the amino acid diet supplemented with raw and heated inhibitor. The small intestines of 3 rats were each divided into 3 equal sections and duodenal, jejunal and ileal contents pooled. The data shown in the tables are the N values for the pooled samples. The TCA-precipitable nitrogen fraction was considerably higher in all sections of the intestines of rats consuming the raw trypsin inhibitor. There was, in general, good agreement between the pattern of TCA-precipitable nitrogen for the 3 different intestinal sections at the different time intervals for both diets. The duodenal and ileal section of the rats fed both diets contain the highest TCA-precipitable nitrogen at 3 hours after feeding, whereas the jejunum had the highest TCA-precipitable nitrogen at the first hour after food ingestion. The TCA-soluble nitrogen fraction in the different parts of the intestinal tract was, in general, lower for rats consuming the raw trypsin inhibitor. The duodenal TCA-soluble nitrogen was constantly lower than for either the jejunal or ileal contents of rats fed both diets. Of all values listed for the TCA-soluble fraction those for the jejunum at one hour after feeding were the highest.

The effect of prolonged feeding of diets containing the raw trypsin inhibitor on the different nitrogen fractions of the intes-

TABLE 4

TCA-precipitable and TCA-soluble fraction in duodenum, jejunum and ileum of rats fed a complete amino acid diet supplemented with either 5% heated or raw trypsin inhibitor factor (TIF)

Hours after feeding	Heated TIF			Raw TIF		
	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
	mg	*mg*	*mg*	*mg*	*mg*	*mg*
			TCA-precipitable nitrogen			
0	0.93 [1]	2.28	2.18	1.24	2.63	3.83
1	1.30	2.92	2.66	1.55	5.90	4.35
3	1.45	2.18	4.35	2.49	3.95	5.50
6	0.68	2.00	1.50	1.00	1.60	2.80
			TCA-soluble nitrogen			
0	5.19	—	7.63	4.98	6.95	7.35
1	3.94	11.45	6.80	4.50	11.02	8.05
3	4.52	6.35	5.80	3.22	4.68	5.20
6	1.70	5.00	3.50	2.00	2.80	2.85

[1] Each value obtained with 3 rats.

tinal contents is shown in table 5. All rats were allowed to feed for a period of 2 hours daily except on the day on which they were killed, when their feeding period was restricted to 30 minutes. Only those rats that had consumed 3 g within the set time limit were killed for analysis. The animals were killed 3 hours after food ingestion. The values for 2 and 8 days of feeding of the experimental diets represent the mean of only 2 rats per diet. The 4- and 6-day experiments were carried out with 4 animals per diet.

The insoluble residual nitrogen of the intestines is significantly higher for rats fed the raw TIF-containing amino acid diet. The TCA-precipitable nitrogen remained considerably higher for rats fed the raw TIF at all time intervals. Contrary to the short-time experiments reported above, the TCA-soluble nitrogen was not higher for rats fed the heated soybean inhibitor.

Values reported in table 6 illustrate the effect of different levels of the raw trypsin inhibitor on the total intestinal contents. All animals were killed 3 hours after food ingestion. A significant decrease in TCA-soluble nitrogen resulted with the addition of 5% of the raw trypsin inhibitor as compared with diets containing 0.5% ($P \leq 0.001$), 1.0%, 2% and 4% ($P \leq 0.05$). The TCA-precipitable nitrogen fraction of rats fed 5% trypsin inhibitor is significantly higher than that of rats fed 4% ($P \leq 0.05$) and 2, 1 and 0.5% and no inhibitor ($P \leq 0.01$). A tendency for increased insoluble intestinal nitrogen can also be detected with increasing supplementation.

Free amino acid in portal plasma. Figure 2 shows the concentration of free proline, serine, threonine and aspartic-glutamic in the portal plasma at different time intervals after food ingestion. Rats were given 3 g of the complete amino acid diet supplemented with 5% of raw trypsin inhibitor. As control, 3 g of the amino acid diet without TIF was fed. The fasting levels (24 hours) of these amino acids are also indicated. All points on the figure represent the mean value of at least 3 in-

TABLE 5

Analysis of intestinal contents of rats fed diets containing heated and raw trypsin inhibitor factor (TIF) for prolonged periods

Duration of feeding period	Heated TIF			Raw TIF		
	TCA-precipitable nitrogen	Total soluble nitrogen	Insoluble nitrogen, intestinal contents	TCA-precipitable nitrogen	Total soluble nitrogen	Insoluble nitrogen, intestinal contents
days	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
2	1.04	4.51	2.00	2.85	4.59	4.32
4	1.81 ± 0.21 [1]	4.10 ± 0.35	1.95 ± 0.19	3.02 ± 0.18	5.26 ± 0.22	5.16 ± 0.46
6	1.34 ± 0.18	3.49 ± 0.29	2.45 ± 0.38	2.94 ± 0.09	5.45 ± 0.19	5.26 ± 0.38
8	1.32	5.90	2.96	2.85	5.95	7.16

[1] Mean of 4 rats ± SE of mean.

TABLE 6

Effect of graded levels of crude soybean trypsin inhibitor factor (TIF) supplementation on the intestinal contents of the rat 3 hours after feeding

TIF	Intestinal contents		
	TCA-soluble nitrogen	TCA-precipitable nitrogen	Insoluble nitrogen
%	*mg*	*mg*	*mg*
0	4.94 ± 0.92 [1]	1.79 ± 0.28	6.57 ± 1.69
0.5	5.65 ± 0.20	2.00 ± 0.80	6.97 ± 1.25
1.0	6.88 ± 0.70	2.53 ± 0.20	8.84 ± 2.00
2.0	6.02 ± 0.34	2.64 ± 0.34	10.37 ± 1.29
4.0	5.44 ± 0.26	3.28 ± 0.47	10.1 ± 1.5
5.0	2.73 ± 0.63	5.95 ± 0.55	—

[1] Mean of 4 rats ± SE of mean.

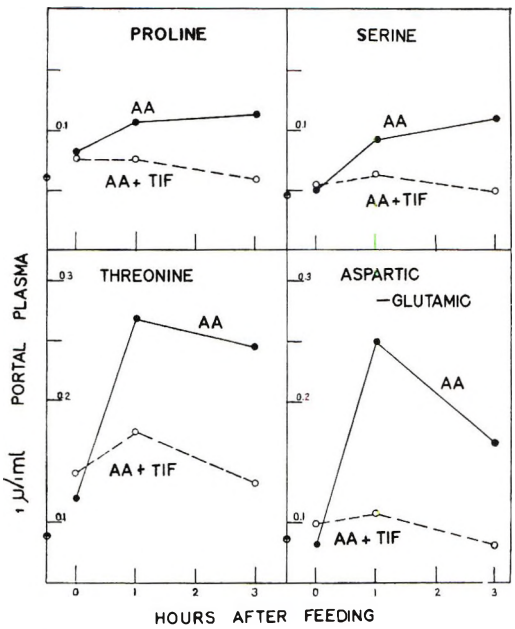

Fig. 2 Concentration of free portal plasma amino acids of rats fed an amino acid diet (●——●) and an amino acid diet supplemented with raw trypsin inhibitor factor (TIF). (○- - - -○) (◓ starvation level after 24 hours fasting). Each point on the curve represents the mean value of at least 3 individual rats.

dividual rats. Values for all 4 amino acids were less at 1 and 3 hours in the portal plasma of rats fed the trypsin inhibitor. At zero hour the portal amino acids were similar for both diets (proline and serine) or were slightly higher in rats fed the trypsin inhibitor (glutamic-aspartic and threonine).

DISCUSSION

In agreement with recently published results on the absorption of soybean protein (5, 14), in the present investigation significantly more nitrogen was excreted in feces (as shown by the digestibility data) of rats fed raw soybeans than in excreta of rats fed heated soybeans.

Autoclaving of soybeans generally improved the retention of individual amino acids although the difference was not always statistically significant. Four of the 8 amino acids assayed in the overheated soybeans showed reduced availability but only lysine availability was significantly lower. The amino acid content was determined in each of the 3 experimental diets so that if heat damage resulted in destruction of amino acids or in formation of amino acid-carbohydrate complexes, which might not be acid-hydrolyzable, no errors in availability determination would result. If this aspect is not taken into consideration and the amino acids determined only in the original raw product, it may result in a lower assay in feces relative to the diet of rats fed the heat-damaged protein and hence availability would be overestimated. The availability of methionine in all 3 soybean meal samples was considerably lower than any of the other amino acids and compares well with the values on methionine availability observed by Melnick and Oser (10), but are not as high as the values reported by Kwong et al. (14). In support of Borchers' results on threonine availability (5), in the present study this amino acid became slightly more available in heated soybeans. Borchers also reported a similar tendency for valine availability and the present results indicate that the availability of valine is significantly improved by proper heat treatment.

The study on rate of stomach emptying indicates that a crude preparation of raw TIF from soybeans is responsible for the slow passage of the experimental diet through the stomach of rats. Lyman and Wilcox reported an identical effect of soybean TIF on stomach emptying (27). The slow gastric emptying rate of rats fed amino acid diets as compared with a diet containing protein is in agreement with the observations of Chen et al. (25). The reduced food intake, always noted in rats fed raw soybeans, may possibly be partly attributed to slower gastric emptying. Further investigation, however, is required in this respect.

The consistently higher insoluble nitrogen fraction in intestines of animals fed raw soybeans indicates that either more mucosal debris is sloughed off during food passage or that the raw soybean is less soluble. The higher insoluble nitrogen content obtained on feeding the TIF-supplemented amino acid diet would support the thesis that slough-off is increased by the raw TIF. The addition of 5% of raw TIF to the amino acid diet resulted in a

supplementation of approximately 4% of protein to the diet since TIF contains about 80% protein. A study of the effect of heat treatment on the solubility of soybean protein (28) and crude TIF (29) showed, however, that raw TIF and soybean have greater solubility than the respective heat-treated products. Hence, with respect to protein solubility, the effect on insoluble intestinal nitrogen would have been in the opposite direction. These results therefore support the view that raw TIF promotes slough-off of mucosal debris. The high intestinal insoluble nitrogen persists when animals are fed the TIF-supplemented diet over extended periods (table 5) and show a tendency to increase as the experimental period is prolonged. That this intestinal nitrogen fraction is associated with raw TIF in the diet is further substantiated by the observation that insoluble intestinal nitrogen increased with higher levels of TIF supplementation.

The high TCA-precipitable nitrogen in intestines of rats fed raw soybeans is in agreement with results reported on TCA-precipitable nitrogen in intestines of chicks fed raw soybeans (30). Bielorai and Bondi concluded from the latter results, as well as from data from an in vitro pancreatic digestion study, that the high TCA-precipitable nitrogen could be attributed to either an inhibition by trypsin inhibitors of the digestion of certain plant proteins or to a suppression of digestion of intermediate products of proteolysis. Results obtained in the present study with rats fed TIF-supplemented amino acid diets do not support this view. Even when the main dietary nitrogen source was an amino acid mixture a high TCA-precipitable nitrogen fraction was obtained with the addition of 5% raw TIF. Furthermore, the increase in the TCA-precipitable fraction cannot be attributed to the introduction of 4% protein into the diet by the addition of TIF, since it was found that an in vitro enzymatic digestion of TIF reduced the TCA-precipitable nitrogen to a very low level (29). A similar small amount of TCA precipitate was reported by Bielorai and Bondi when "inhibiting extracts prepared from raw soybean meal" were digested with pancreatin (30). In view of these observations it can be accepted, therefore, that the high TCA-precipitable nitrogen in intestinal contents of rats fed the raw TIF is, in all probability, due to the nitrogen present in the trypsin-soybean TIF complex and proteins incorporated in the excessively stimulated digestive enzymes. This brings the whole concept into agreement with the reports of Lepkovsky and his coworkers (16, 17).

A comparison of the concentration of amino acids in the portal blood of rats fed amino acid diets with and without TIF supplementation indicates that raw TIF interferes with the absorption of both essential and non-essential amino acids. Low methionine and lysine concentrations in portal plasma of rats fed raw soybean meal (31) are therefore possibly the result of impaired absorption through the intestinal wall. The mechanism involved is difficult to visualize at present.

From the results presented in this study it can now be concluded that the difference in protein digestibility and availability of amino acids between raw and heated soybean products, as determined by conventional methods, can be partly and possibly completely attributed to increased mucosal slough-off, stimulated enzymatic secretion and impaired amino acid absorption. These effects are all brought about by factors present in a crude preparation of the so-called soybean TIF. The difference between the nutritive value of raw and autoclaved soybeans is therefore not due to a lower digestibility or poorer utilization of the raw product but to an extra demand for protein made on the animal in order to keep up with the faster epithelial cell regeneration and the sharply increased digestive enzyme secretions. Furthermore, this drain on the animal is probably aggravated by impaired food intake resulting from the slow stomach emptying observed in animals fed raw soybeans.

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Free Amino Acids in Plasma and Tissues of Rats Fed a Vitamin B_6-deficient Diet[1]

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ABSTRACT Amino acids were determined in plasma, muscle and liver tissue of rats receiving vitamin B_6-deficient or control diets. In the vitamin B_6-deficient animals, there was a pronounced increase in the glycine-to-serine ratio, an increase in cystathionine, and an increase in aspartic acid accompanied by a decrease in alanine. The changes in plasma amino acids were accentuated by a dietary supplement of glycine.

The role of the coenzyme forms of vitamin B_6 in the non-oxidative degradation and interconversion of amino acids is well documented (review, (1)). There is also evidence (2) that pyridoxal phosphate is involved in amino acid transport mechanisms. It therefore seemed appropriate to investigate whether alterations would occur in the amounts or proportions of amino acids present in the blood plasma and tissues of weanling rats fed diets lacking in vitamin B_6.

EXPERIMENTAL

Male weanling rats (Sprague-Dawley strain) weighing between 50 and 60 g were caged individually and divided into groups of 6 rats. In the first experiment one group was fed a control diet[2] and the other was fed an identical diet with the exception that pyridoxine·HCl was omitted from the vitamin mixture. The experiment was continued for 4 weeks and was repeated. In this experiment additional animals provided control and pyridoxine-deficient groups that were maintained for a 6-week period. Also, 7.5% glycine was added to the diet of a control group and one receiving the diet devoid of pyridoxine. All animals were fed ad libitum.

Amino acids were measured by the method of Tallan et al. (3) in picric acid extracts of pooled samples of blood plasma, of muscle, and of liver tissue taken from 6 non-fasting rats. The analyses were made by ion-exchange chromatography using a Beckman-Spinco instrument.

RESULTS

Rats fed the pyridoxine-free diet for 4 weeks gained an average of 56 ± 1.7[3] g and rats fed the identical diet with pyridoxine added gained 163 ± 2.4[3] g. The average values obtained for plasma amino acids in 2 separate experiments are shown in table 1. In general, the plasma amino acid concentrations tended to be lower in animals fed the pyridoxine-free diets and the total amount of the essential amino acids decreased to a greater extent than the total of the nonessential amino acids. Decreased food consumption, decreased absorption or increased tissue oxidation could all have been contributing factors to these observations. However, other changes in plasma amino acids appear to result from alterations in metabolic amino acid interconversions. The value for glycine increased, whereas that for serine decreased with a resultant pronounced increase in the ratio of glycine to serine. The level of cystathionine also increased. Aspartic acid increased with a concomitant decrease in alanine suggesting the possible reduction in trans-

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[2] Composition: (in per cent) vitamin-free casein, 18; cottonseed oil, 45; salt mixture (Hegsted, D. M. et al., J. Biol. Chem., *138*: 459, 1941), 4.0; choline chloride, 0.15; vitamin mixture, 0.02; dextrose to total 100. The vitamin mixture contained: (in mg/100 g diet) thiamine·HCl, 0.5; riboflavin, 0.5; nicotinic acid, 2.5; Ca pantothenate, 2.0; pyridoxine·HCl, 0.25; menadione, 0.05; biotin, 0.01; folic acid, 0.02; inositol, 10.0; vitamin A and D mixture (Pfizer Crystalites, Chas. Pfizer and Company, Inc., New York, units/g: vitamin A = 500,000 USP, vitamin D = 50,000 USP), 0.8; α-tocopherol, 0.01 and vitamin B_{12}, 0.002.

[3] Average ± SE of mean.

TABLE 1

Free amino acids in plasma, muscle and liver of weanling rats fed the control or the vitamin B_6-deficient diet

Amino acid	Plasma acid content		Muscle amino acid content		Liver amino acid content	
	Control group	Vitamin B_6-deficient group	Control group	Vitamin B_6-deficient group	Control group	Vitamin B_6-deficient group
	μmoles/100 ml		*μmoles/100 g wet wt*		*μmoles/100 g wet wt*	
Essential [1]						
Threonine	39.6	28.9	140	110	95	102
Valine	22.3	16.4	24	17	27	22
½ Cystine	3.3	1.0	—	—	—	—
Methionine	6.1	5.1	7	7	5	6
Isoleucine	9.4	7.0	9	7	14	12
Leucine	12.3	12.4	15	12	26	23
Tyrosine	11.3	7.5	20	11	11	9
Phenylalanine	5.3	3.9	6	4	9	7
Lysine	58.4	36.7	—	—	—	—
Tryptophan	3.4	1.5	—	—	—	—
Histidine	4.9	4.8	—	—	—	—
Arginine	7.8	4.2	—	—	—	—
Nonessential						
Aspartic acid	0.5	1.3	35	34	132	114
Serine	27.8	18.0	192	108	176	98
Glutamine and asparagine	62.0	58.0	596	534	574	590
Proline	43.7	31.0	123	81	43	39
Glutamic acid and citrulline	12.8	13.6	160	93	222	377
Glycine	11.7	34.4	216	653	120	256
Alanine	40.2	27.4	227	132	384	233
Ornithine	4.3	4.6	—	—	—	—
Cystathionine	0.2	1.8	< 0.5	3	2	274

[1] Includes cystine and tyrosine.

amination between these 2 compounds. There is evidence that all of the enzymes involved in the metabolism of these amino acids, serine adolase (4), cystathionase (5), and the transaminases (6), are vitamin B_6-dependent.

In the vitamin B_6-deficient rats the alterations in the amino acid content of plasma were, for the most part, reflected by alterations of free amino acids in muscle and liver tissue (table 1). However, with respect to cystathionine the increase in muscle was very slight as compared with the increase of this compound in liver tissue.

Some of the experimental animals were fed the diets for an additional 2 weeks. During this interval the animals fed the control diets gained an average of 67 ± 1.9 g and those fed the vitamin B_6-deficient diets, gained an average of 10 ± 1.5 g. The amounts and proportions of the amino acids in plasma and muscle tissue of both groups of animals remained approximately the same as the values shown in table 1 with the exception that the ½ cystine value was less than 0.5 μmoles and the glycine had increased to 39.4 μmoles/100 ml of plasma in the vitamin B_6-deficient animals.

When 7.5% glycine was added to a pyridoxine-free diet and fed to rats for a 4-week period, many of the changes in plasma amino acids previously noted in vitamin B_6-deficient rats were accentuated as shown in table 2. Although plasma values for glycine and serine also increased in animals fed the control diet supplemented with glycine (compare with values in table 1), the increase in glycine was much greater in the vitamin B_6-deficient animals and serine increased also. This appears to constitute additional evidence that the metabolism of glycine is altered by a vitamin B_6-deficiency.

Previous studies (7, 8) have indicated that the amino acid concentrations in plasma of experimental animals are affected by the amino acid composition of the diet. The present study has shown

TABLE 2

Plasma amino acids of weanling rats fed the control or the vitamin B_6-deficient diet supplemented with 7.5% glycine

Amino acid	Control + glycine group	Vitamin B_6-deficient + glycine group
	μmoles/100 ml	*μmoles/100 ml*
½ Cystine	1.2	< 0.5
Aspartic acid	< 0.5	2.0
Serine	43.3	91.9
Glycine	187.0	787.8
Alanine	36.5	30.5
Cystathionine	0.2	3.3

that the plasma amino acids are also responsive to alterations in amino acid metabolism incurred by a dietary lack of vitamin B_6. The determination of transaminase activity has been suggested as a method for studying pyridoxine nutriture by Brin et al. (9). It appears that the measurement of amino acid proportions in plasma should also receive consideration in this respect. A reversal of the ratio of glycine to serine accompanied by an increased level of cystathionine might prove to be characteristic of a pyridoxine deficiency state.

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Effects of Dietary Supplements on Young Rats Fed High Levels of Zinc[1]

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ABSTRACT The effects of several dietary supplements on growth and mineral metabolism of young rats fed high levels of zinc were observed in a series of experiments. Soybean meal offered some protection against subnormal growth, but with a level of 0.75% of zinc, this protection was not as good as that obtained with either liver or distiller's dried solubles. The addition of 20% of distiller's dried solubles to a diet containing 0.75% of zinc completely prevented the marked decreases in weight gain, hemoglobin level and liver copper associated with zinc toxicity. Results of several experiments suggest that dietary protein per se is not the primary factor involved in the subnormal growth associated with zinc toxicosis that is being supplied by the addition of liver or distiller's dried solubles. Results of this study indicate that a high level of protein may accentuate, rather than reduce, the severity of zinc toxicity in rats under certain conditions. This observation does not agree with a previous report by other investigators.

Although Sutton and Nelson (1) first demonstrated quantitatively in 1937 that high levels of dietary zinc were associated with decreased weight gains of rats, the exact nature of this interference of zinc remains an enigma. Grant-Frost and Underwood (2) contended that the depressing effect of zinc on growth was caused largely by reduced food consumption. Results showing that supplements of liver will partially alleviate the detrimental effects of high levels of zinc on growth (3, 4), however, suggest the possible involvement of some necessary growth factor. Results obtained by Magee and Matrone (4) indicated that the factor(s) of liver extract that alleviates the subnormal growth of zinc-fed rats is primarily organic in nature. Recently, McCall et al. (5) reported that the severity of zinc toxicity on the growth of rats depends upon the source and the level of dietary protein.

The present investigation was conducted to compare the effects of some purified proteins, of some growth factor sources and of various liver fractions on the weight gains of rats fed a high level of zinc. In addition the influence of these supplements on hemoglobin levels and on the copper, iron and zinc contents of the livers of zinc-fed rats was considered also.

EXPERIMENTAL

General procedures

Young male rats of the Sprague-Dawley strain (3 weeks of age) were used throughout all phases of this study. The animals were housed in individual wire-bottom cages, given free access to feed and water and were usually maintained on experiment for 5 weeks. Rats in each experiment were randomized into replications according to initial body weights. The test treatments were randomly assigned to individual cages within a replication.

The basal diet used in this study consisted of the following (in per cent): casein, 19;[2] cornstarch, 63;[3] vegetable fat, 10;[4] mineral mix, 4;[5] vitamin mix, 2;[6] cellulose, 2;[7] and oleum percomorphum.[8]

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[2] Vitamin-Test Casein, Nutritional Biochemicals Corporation, Cleveland.

[3] Globe Easy-flow Corn Starch 3366, Corn Products Sales Company, Greensboro, North Carolina.

[4] Crisco, Procter and Gamble Company, Cincinnati.

[5] Salt Mixture W, Nutritional Biochemicals Corporation, Cleveland. The composition of this salt mixture is listed as: (in per cent) $CaCO_3$, 21.000; $CuSO_4 \cdot 5H_2O$, 0.039; $FePO_4 \cdot 2H_2O$, 1.470; $MnSO_4$, 0.020; $MgSO_4$, 9.000; $KAl(SO_4)_2 \cdot 12H_2O$, 0.009; KCl, 12.000; KH_2PO_4, 31.000; KI, 0.005; NaCl, 10.500; NaF, 0.057; and $Ca_3(PO_4)_2$, 14.900.

[6] Each 100 g of vitamin mix contained: (in milligrams) 0.1% vitamin B_{12} (with mannitol), 0.1; biotin, 1; folic acid, 5; thiamine·HCl, 25; pyridoxine·HCl, 25; 2-methyl-naphthoquinone, 50; riboflavin, 50; nicotinic acid, 50; Ca pantothenate, 150; *p*-aminobenzoic acid, 500; (in grams) inositol, 5; choline chloride, 7.5; DL-methionine, 30; and cornstarch, 56.6. All vitamins and methionine were purchased from Nutritional Biochemicals Corporation, Cleveland.

[7] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[8] Each kilogram of diet contained 24 drops of oleum percomorphum, Mead Johnson and Company, Evansville, Indiana.

Zinc carbonate was used as the source of zinc. Supplements were incorporated into the basal diet at the expense of equal amounts of starch.

Oxyhemoglobin determinations, by the method of Shenk et al. (6), were made on blood samples taken from the tails of the animals at the termination of each experiment.

At the end of an experiment, rats from several randomly selected replications were killed, and the livers removed. The copper and iron content of the livers was determined by the methods of Parks et al. (7) and Kitzes et al. (8), respectively, as modified by Matrone et al. (9). The method outlined by McCall et al. (10) was used for the determination of liver zinc.

A randomized block design was used in the animal experiments, and all data were subjected to an analysis of variance. Statements of significance are based on odds of at least 19 to 1 ($P \leq 0.05$). Least significant difference (L.S.D.) values for each criterion studied were calculated for each experiment to give an indication of the difference between 2 treatment means that is required to show significance in a particular experiment.

Protein study (experiments 1, 2 and 3)

Since high levels of dietary zinc have been shown to cause an increase in the fecal and the urinary excretion of nitrogen (11, 12) and in view of the work of McCall et al. (5), a reasonable premise was that the factor(s) in a commercial liver preparation that alleviates the subnormal growth of zinc-fed rats is associated with the protein fraction of the liver material. To test this premise, 3 experiments were designed to explore the effects of several types and levels of high protein sources on zinc-fed rats. In experiment 1 the responses of zinc-fed rats to 5% supplemental levels of a commercial liver extract,[9] distiller's dried solubles,[10] soybean meal, casein, egg albumen, blood albumen, soy protein[11] and zein were compared. A 2^3 factorial design was used in experiment 2 with 14 and 19% levels of casein, 5% of commercial liver extract and 0.75% of zinc. The purpose of experiment 3 was to compare the effects of 0.5 and 0.75% of zinc on rats maintained with diets containing 10, 20 and 30% of casein and to compare the responses of rats to 0.5 and 0.75% of zinc when levels of 20 and 30% of casein or soybean meal were used as the source of dietary protein.

Source of factor study (experiments 4 and 5)

The purpose of experiment 4 was to determine whether the livers of young rats contained the "zinc toxicity alleviating factor" which has been detected in a commercial liver extract and to determine whether a high level of dietary zinc prevented the formation of the liver factor in young rats. Three sources of liver preparations used in this experiment were livers from rats fed a 0.75% level of zinc, livers from normal rats and a commercial liver preparation known to contain the factor under study. In preparation for this experiment, 128 rats were divided into 2 equal groups. One group received the basal ration; the other group received the basal ration containing 0.75% of zinc. Both groups were maintained with their respective dietary regimens for 5 weeks. At the end of this time, all animals were killed, and the livers removed. The livers were dried in an oven at 60°C, ground in a Waring Blendor and used as dietary supplements in experiment 4.

Experiment 5 was designed to investigate the "zinc toxicity alleviating" properties of varying levels of distiller's dried solubles.

RESULTS

Protein study

Experiment 1. None of the purified protein compounds tested alleviated the subnormal growth of zinc-fed rats significantly (table 1), indicating that dietary protein per se is not the primary factor involved in the subnormal growth associated with zinc toxicity. The results also indicate that the unidentified factor(s) that alleviates the subnormal growth of zinc-fed rats is not restricted to liver alone, since supplementing the high zinc diet with

[9] Liver Fraction L, Nutritional Biochemicals Corporation, Cleveland.

[10] We are indebted to the Distillers Feed Research Council and the John F. Young Company, Cincinnati, for the gift of distiller's dried solubles.

[11] Alpha Protein, Nutritional Biochemicals Corporation, Cleveland.

TABLE 1

Response of zinc-fed rats [1] to several high protein supplements

Supplement	Wt gain at 5 weeks	Hemoglobin	Liver constituent		
			Cu	Fe	Zn
	g	*g/100 ml blood*	*μg/g dry weight*		
None	224 ± 6(14) [2]	13.8 ± 0.2(14)	12.0 ± 0.7(8)	364 ± 19(8)	64 ± 4 (8)
0.75% Zn	107 ± 8(14)	6.2 ± 0.4(14)	4.9 ± 0.8(8)	162 ± 16(8)	572 ± 55(8)
0.75% Zn + 5% commercial liver [3]	182 ± 8(14)	9.1 ± 0.4(14)	6.3 ± 0.8(8)	148 ± 7 (8)	281 ± 29(8)
0.75% Zn + 5% distiller's dried solubles [4]	166 ± 13(14)	9.3 ± 0.4(14)	7.8 ± 0.7(8)	145 ± 14(8)	353 ± 40(8)
0.75% Zn + 5% soybean meal [5]	138 ± 11(6)	6.0 ± 0.4(6)	4.4 ± 0.7(4)	142 ± 9 (4)	354 ± 57(4)
0.75% Zn + 5% casein	111 ± 5(8)	7.7 ± 0.3(8)	4.4 ± 0.6(4)	161 ± 24(4)	552 ± 57(4)
0.75% Zn + 5% egg albumen	106 ± 5(8)	6.5 ± 0.3(8)	4.5 ± 0.3(4)	166 ± 6 (4)	565 ± 38(4)
0.75% Zn + 5% blood albumen	126 ± 13(8)	8.7 ± 0.3(8)	5.1 ± 0.5(4)	168 ± 14(4)	598 ± 45(4)
0.75% Zn + 5% soy protein [6]	122 ± 9(8)	7.2 ± 0.4(8)	7.0 ± 1.8(4)	161 ± 7 (4)	481 ± 45(4)
0.75% Zn + 5% zein	112 ± 13(8)	6.8 ± 0.4(8)	4.5 ± 0.3(4)	210 ± 19(4)	638 ± 95(4)
L.S.D.$_{0.05}$ [7]	26	0.8	2.1	48	142
L.S.D.$_{0.01}$	35	1.2	2.8	66	194

[1] Sprague-Dawley rats averaging 64 g in weight initially.
[2] Figures represent mean ± SE. Numbers in parentheses indicate number of animals in each group.
[3] Liver Fraction L, Nutritional Biochemicals Corporation, Cleveland.
[4] Product of the John F. Young Company, Cincinnati.
[5] 44% protein solvent-extracted soybean meal.
[6] Alpha Protein, Nutritional Biochemicals Corporation, Cleveland.
[7] Least significant difference at specified probability levels.

either liver, distiller's dried solubles or soybean meal resulted in highly significant increases ($P \leq 0.01$) in weight gain.

The addition of 5% levels of liver, distiller's dried solubles, casein or blood albumen resulted in highly significant increases ($P \leq 0.01$) in hemoglobin levels, whereas the addition of 5% of soy protein resulted in an increase which was significant at the 5% level of probability.

In this experiment the zinc-fed animals supplemented with distiller's dried solubles showed a highly significant increase ($P \leq 0.01$) in liver copper, whereas the addition of soy protein to the high zinc diet resulted in an increase in liver copper which was significant at the 5% level of probability. None of the supplements tested significantly increased the iron content of the livers of rats fed the high level of zinc. The results revealed a partial, but highly significant decrease ($P \leq 0.01$) in the accumulation of zinc in the liver when supplements of liver, distiller's dried solubles or soybean meal were added to the high zinc diet.

Experiment 2. The results of this experiment (table 2) were similar to those noted in the previous experiment; namely, the significant decreases in weight gain, hemoglobin level, liver copper and liver iron, and the marked increase in liver zinc associated with zinc toxicity. Analyses of the data also revealed a significant liver × casein interaction ($P \leq 0.05$) with respect to weight gain, hemoglobin level and liver zinc content. The data suggest that when a level of 0.75% of zinc is fed, the severity of this level on growth may be less when the protein level is low. The growth re-

TABLE 2

Response of zinc-fed rats [1] to various levels of casein in the presence and absence of liver [2]

Level of dietary casein and supplements	Wt gain at 5 weeks [3]	Hemoglobin [3]	Liver constituent [4]		
			Cu	Fe	Zn
	g	*g/100 ml blood*	*μg/g dry weight*		
19% Casein	227 ± 5	13.6 ± 0.2	12.1 ± 0.9	330 ± 20	58 ± 2
19% Casein + 0.75% Zn	98 ± 6	6.2 ± 0.4	3.9 ± 0.4	135 ± 15	477 ± 46
19% Casein + 0.75% Zn + 5% liver [5]	189 ± 8	8.7 ± 0.4	5.4 ± 0.6	145 ± 9	236 ± 36
19% Casein + 5% liver	236 ± 6	14.1 ± 0.3	12.7 ± 0.1	284 ± 14	60 ± 6
14% Casein	222 ± 4	14.0 ± 0.3	12.2 ± 0.2	320 ± 15	56 ± 6
14% Casein + 0.75% Zn	124 ± 10	6.9 ± 0.3	4.7 ± 0.2	142 ± 19	347 ± 30
14% Casein + 0.75% Zn + 5% liver	173 ± 12	8.7 ± 0.4	5.8 ± 0.6	136 ± 6	236 ± 18
14% Casein + 5% liver	226 ± 10	13.3 ± 0.2	12.6 ± 0.1	346 ± 39	64 ± 6
L.S.D.$_{0.05}$ [6]	20	0.9	1.6	60	67
L.S.D.$_{0.01}$	27	1.2	2.2	82	92

[1] Sprague-Dawley rats averaging 51 g in weight initially.
[2] Figures represent mean ± SE.
[3] Each figure is the mean of 6 animals.
[4] Each figure is the mean of 4 animals.
[5] Liver Fraction L, Nutritional Biochemicals Corporation, Cleveland.
[6] Least significant difference at specified probability levels.

sponse of zinc-fed rats to the liver supplement, however, was slightly higher when the level of dietary protein was 19% of casein. There is also an indication that a high level of protein may enhance the deposition of zinc in the liver under conditions of zinc toxicity.

Experiment 3. Response criteria of the rats to the various dietary regimens used in this experiment are shown in table 3. Analyses of the data revealed that increasing levels of dietary protein did not alleviate the highly significant decreases ($P \leq 0.01$) in weight gains associated with the feeding of high levels of zinc. This was true whether the source of protein was casein or soybean meal. The difference in weight gains between the controls and the animals fed either level of zinc increased as the level of protein increased. In general, rats maintained with the soybean meal diets containing either 0.5 or 0.75% of zinc had higher weight gains than did rats fed the corresponding casein diets. The growth response exhibited by animals receiving 30% of soybean meal and 0.75% of zinc was not as great as had been previously noted in this study when either 5% of liver or distiller's dried solubles was added to a diet containing 0.75% of zinc.

The data indicated that high levels of protein, casein or soybean meal, did not prevent the marked decrease in hemoglobin level associated with the feeding of 0.75% of zinc. Hemoglobin levels of rats fed 10% casein diets containing 0.5% of zinc were significantly higher ($P \leq 0.05$) than hemoglobin levels of rats fed 20 or 30% casein levels with 0.5% of zinc in the diet. The depression in hemoglobin level was not as great in rats fed 0.5% of zinc when the protein source was soybean meal.

Increasing the level of dietary protein did not prevent the significant decrease in liver copper level associated with zinc toxicity. The data indicated that as the protein level increased, the difference between hemoglobin levels of the controls and those of animals fed zinc increased.

When 0.75% of zinc was added to the diet, there was no significant difference between the liver iron levels of animals fed different levels of protein, although the iron levels of animals receiving casein were higher than those of animals receiving soy-

TABLE 3

Effects of high levels of dietary zinc on rats [1] fed various levels of casein and soybean meal [2]

Protein source	Protein level	Zinc	Wt gain at 5 weeks [3]	Hemoglobin [3]	Liver constituent [4]		
					Cu	Fe	Zn
	%	%	g	g/100 ml blood	μg/g dry weight		
Casein	10	none	172 ± 14	12.0 ± 0.3	9.4 ± 0.8	316 ± 36	48 ± 7
		0.50	150 ± 12	8.1 ± 0.4	5.4 ± 1.1	118 ± 11	326 ± 51
		0.75	90 ± 8	5.3 ± 0.6	4.6 ± 0.8	144 ± 15	554 ± 82
	20	none	225 ± 5	13.2 ± 0.2	11.6 ± 0.8	334 ± 22	64 ± 5
		0.50	181 ± 8	6.6 ± 0.2	4.3 ± 0.3	162 ± 12	365 ± 31
		0.75	78 ± 9	5.0 ± 0.5	3.5 ± 0.3	146 ± 13	615 ± 53
	30	none	218 ± 10	13.1 ± 0.2	10.0 ± 1.2	398 ± 40	70 ± 10
		0.50	128 ± 16	6.2 ± 0.7	2.7 ± 0.3	226 ± 16	484 ± 29
		0.75	59 ± 6	5.5 ± 0.7	2.7 ± 0.2	144 ± 12	727 ± 64
Soybean meal [5]	20	none	158 ± 6	12.2 ± 0.2	9.6 ± 0.3	310 ± 2	44 ± 8
		0.50	164 ± 7	9.2 ± 0.4	6.2 ± 1.1	120 ± 6	345 ± 31
		0.75	121 ± 16	6.5 ± 0.4	5.8 ± 0.5	119 ± 7	564 ± 50
	30	none	224 ± 5	12.9 ± 0.4	11.5 ± 0.9	282 ± 25	59 ± 9
		0.50	170 ± 9	7.9 ± 0.4	5.8 ± 0.8	120 ± 8	336 ± 42
		0.75	127 ± 16	5.9 ± 0.4	4.6 ± 1.2	107 ± 12	685 ± 78
$L.S.D._{0.05}$ [6]			29	1.2	1.6	50	124
$L.S.D._{0.01}$			39	1.6	2.2	67	166

[1] Sprague-Dawley rats averaging 51 g in weight initially.
[2] Figures represent mean ± SE.
[3] Each figure is the mean of 6 animals.
[4] Each figure is the mean of 4 animals.
[5] 44% protein solvent-extracted soybean meal.
[6] Least significant difference at specified probability levels.

bean meal. Increasing the casein level from 10 to 30%, however, resulted in a highly significant increase ($P \leq 0.01$) in liver iron levels of animals receiving 0.5% of zinc. This response did not occur when soybean meal was used as the source of protein.

In general, increases in dietary protein levels, either casein or soybean meal, resulted in progressive increases in liver zinc accumulation in animals fed diets containing high levels of zinc. Soybean meal offered some protection against the marked increases in liver zinc deposition, but the resulting values never returned to levels observed in the control animals.

Source of factor study

Experiment 4. The addition of both sources of rat liver and the commercial liver preparation to diets containing zinc resulted in increases in weight gains which were highly significant at the 1% level of probability (table 4). The mean weight gains of animals fed liver supplements, moreover, were not significantly different, indicating that 1) zinc apparently did not prevent the formation of the "zinc toxicity alleviating factor" in the livers of rats, and 2) the factor present in commercial liver is similar to that found in rat liver. The similarity in the trends observed with the other criteria among the liver supplemented and the zinc-control diets substantiated the foregoing interpretation. Hemoglobin values and copper concentrations of the liver were higher, and iron and zinc content of the livers was lower in the liver-supplemented rats than those of the zinc-control rats. Comparisons of the differences between individual means with those of the zinc-control rats, however, did not attain statistical significance in some instances (table 4). Presumably, these variations reflected differences in the copper and the iron contents of the 3 dietary liver preparations tested in this experiment.

Experiment 5. Analyses of the data (table 5) showed highly significant linear increases ($P \leq 0.01$) in weight gain, hemoglobin level and liver copper with increasing levels of dietary distiller's dried solubles. Complete alleviation of the adverse effect of zinc toxicity on weight gain, hemoglobin level and liver copper was obtained with a level of 20% of distiller's dried solubles.

TABLE 4

Response of zinc-fed rats [1] to some liver supplements [2]

Supplement	Wt gain at 4 weeks	Hemoglobin	Liver constituent		
			Cu	Fe	Zn
	g	*g/100 ml blood*	*μg/g dry weight*		
None	181 ± 6	14.0 ± 0.3	10.9 ± 0.6	335 ± 22	85 ± 4
0.75% Zn	101 ± 6	6.3 ± 0.3	4.8 ± 0.6 [3]	146 ± 9 [4]	680 ± 77
0.75% Zn + 5% commercial liver preparation [5]	142 ± 7	9.2 ± 0.3	5.8 ± 0.6 [3]	121 ± 7 [4]	351 ± 10
0.75% Zn + 4% liver from normal rats	142 ± 4	8.2 ± 0.3	6.8 ± 0.4	122 ± 8	450 ± 24
0.75% Zn + 4% liver from zinc-fed rats	131 ± 6	6.9 ± 0.4	5.2 ± 0.3	115 ± 6	440 ± 38
L.S.D.$_{0.05}$ [6]	17	1.0	1.5	35	103
L.S.D.$_{0.01}$	23	1.3	2.0	47	139

[1] Sprague-Dawley rats averaging 51 g in weight initially.
[2] Figures represent mean ± SE. Each figure is the mean of 8 animals unless otherwise indicated.
[3] Mean of 6 animals.
[4] Mean of 7 animals.
[5] Liver Fraction L, Nutritional Biochemicals Corporation, Cleveland.
[6] Least significant difference at specified probability levels.

TABLE 5

Response of zinc-fed rats [1] to various levels of distiller's dried solubles [2]

Supplement	Wt gain at 5 weeks [3]	Hemoglobin [3]	Liver constituent [4]		
			Cu	Fe	Zn
	g	*g/100 ml blood*	*μg/g dry weight*		
None	185 ± 6	13.0 ± 0.3	10.4 ± 0.1	359 ± 38	44 ± 2
0.75% Zn	123 ± 9	6.5 ± 0.4	4.2 ± 0.6	164 ± 15	300 ± 72
0.75% Zn + 5% DDS [5]	164 ± 9	10.5 ± 0.4	6.7 ± 1.0	140 ± 8	197 ± 17
0.75% Zn + 10% DDS	169 ± 7	11.9 ± 0.4	9.3 ± 1.0	135 ± 13	204 ± 15
0.75% Zn + 20% DDS	185 ± 6	12.8 ± 0.2	10.4 ± 0.4	131 ± 8	111 ± 15
L.S.D.$_{0.05}$ [6]	15	1.0	2.0	44	71
L.S.D.$_{0.01}$	20	1.3	2.6	60	96

[1] Sprague-Dawley rats averaging 43 g in weight initially.
[2] Figures represent mean ± SE.
[3] Each figure is the mean of 8 animals.
[4] Each figure is the mean of 4 animals.
[5] Distiller's dried solubles supplied by the John F. Young Company, Cincinnati.
[6] Least significant difference at specified probability levels.

None of the supplemental levels of distiller's dried solubles tested in this experiment prevented the marked decrease in liver iron associated with zinc toxicity. The addition of distiller's dried solubles to a high zinc diet was actually associated with some additional decrease in liver iron accumulation.

The addition of distiller's dried solubles to a diet containing 0.75% of zinc resulted in highly significant decreases ($P \leq 0.01$) in liver zinc levels, indicating that this material partially prevented the deposition of excess zinc in the livers of young rats.

DISCUSSION

Results presented in this paper tend to indicate that a high protein level can accentuate the effect of zinc toxicity under certain conditions, although McCall et al. (5) have reported that the severity of zinc toxicity in rats could be reduced when the diet contained a level of 30% of protein. This intensification of the severity of zinc toxicity was particularly noticeable when a level of 0.75% of zinc was added to the diet.

The results of this study indicate that low supplemental levels of either com-

mercial liver extract or distiller's dried solubles give better protection against growth depression caused by a high zinc diet than do supplements of soybean meal and other protein sources furnishing equal or greater amounts of protein to the basal diet. This could be interpreted to mean that the "zinc toxicity alleviating factor" supplied by the commercial liver preparation and distiller's dried solubles is not dietary protein per se. This possibility is further strengthened by the fact that 30% levels of dietary casein or soybean meal did not prevent the marked decrease in weight gain of rats fed a high zinc diet.

Throughout this study the addition of either commercial liver extract or distiller's dried solubles to a high zinc diet significantly decreased the amount of zinc that accumulated in the liver. A logical explanation for this is that some compound(s) in these materials chelates with some of the excess zinc and prevents it from being deposited in the liver. This could have been the result of either a decrease in the absorption of zinc or an increase in the excretion of the absorbed zinc. Results indicating that either the addition of soy protein to a high zinc diet or the use of soybean meal as the protein source resulted in a significant reduction in liver zinc, but did not prevent subnormal growth, tend to suggest, however, the existence of a "growth stimulating" factor(s) which is being supplied by liver or distiller's dried solubles. Another indication of the existence of the "growth stimulating" factor is that, although the growth depression of zinc-fed rats was largely prevented by the addition of commercial liver or distiller's dried solubles, the liver zinc levels of animals receiving these materials never returned to a level observed in the normal animal. In all experiments conducted in this study, the amount of zinc that accumulated in the livers of zinc-fed rats supplemented with either commercial liver extract or distiller's dried solubles was approximately 3 to 5 times that amount observed in the livers of normal rats.

The improvement in hemoglobin observed throughout this study when commercial liver extract or distiller's dried solubles was added to the high zinc diet may be the result of the copper and iron supplied by these supplements. Chemical analyses of the commercial liver extract used in this study showed that it contained approximately 38 μg and 369 μg/g of dry weight of copper and iron, respectively, whereas the distiller's dried solubles contained approximately 124 μg and 417 μg/g of dry weight of copper and iron, respectively. There is the possibility, however, that a deficiency of another hematopoietic dietary essential, in addition to copper and iron, occurs when levels of zinc are fed and that the liver or distiller's dried solubles supplements supply this dietary essential.

The addition of 5% of commercial liver extract or distiller's dried solubles to a diet containing 0.75% of zinc resulted in significant increases in liver copper levels, whereas a supplemental level of 10% of distiller's dried solubles completely prevented the depression in liver copper associated with zinc toxicity. This suggests that the extra copper supplied by these supplements was absorbed and utilized for liver storage.

Although the commercial liver extract and distiller's dried solubles supplements appeared to enhance the deposition of liver copper, no significant effect was observed on the liver iron deposition in zinc-fed rats. These results suggest an alternate possibility that the extra iron supplied by these dietary supplements was absorbed and utilized for hemoglobin but not for liver storage. A mechanism for explaining this alternate possibility, however, is not readily apparent although stabilization of ferritin sulfhydryl groups by zinc may be involved.

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Protein and Lysine as Factors in the Cariogenicity of a Cereal Diet[1,2]

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ABSTRACT This investigation concerns the effect of certain alterations of a raw whole wheat diet on its cariogenicity for white rats. The alterations were: increasing the protein level by supplementation with dried egg white, casein and gluten at approximately equivalent nitrogen levels and by supplementation with the amino acids, L-lysine·HCl, DL-methionine, and DL-threonine. Littermate Sprague-Dawley strain rats were fed at weaning each series of comparative diets and maintained with them for 60 days. The criteria of carious experience were incidence and severity. The caries produced were the smooth surface type. Increasing protein level decreased the cariogenicity of the diets; dried egg white and casein supplementation decreased the cariogenicity of diets significantly more than did an equivalent gluten supplement; the supplementation of the diets by L-lysine·HCl, DL-methionine and DL-threonine did not result in a decrease of carious experience for these animals.

The possible role of protein in the etiology of dental caries has been suggested in studies by Bavetta and McClure (1). Of special interest was their evidence that increasing the casein content of the diets reduced caries. McClure (2) has implicated heat processing of cereals, thereby decreased lysine, as a factor in the cariogenicity of high cereal diets. However, Dodds (3) has shown a raw whole wheat diet to promote smooth surface caries to a greater degree than equivalent diets of processed wheat products. The raw whole wheat diet was also more cariogenic than comparable diets of raw and processed corn, rice and oats.

This raw whole wheat diet has been consistently cariogenic. It is low in protein and the essential amino acids, lysine, methionine and threonine. The present investigation concerns the effect on the cariogenicity of this high cereal diet of increasing the protein level, varying the type of protein supplementation, and supplementation by the amino acids, L-lysine·HCl, DL-methionine and DL-threonine.

EXPERIMENTAL

The experimental rats were of the Sprague-Dawley strain, bred in this laboratory from a colony which originated from the National Institutes of Health. Thirty rats, weighing 40 to 55 g, were fed each diet at weaning. Littermates, without regard to sex, were assigned to the diets within a series. The animals were housed 2/cage and received distilled water and food ad libitum. The food intakes were recorded twice a week, and the rats weighed weekly.

The base diet contained 78.52% of raw whole wheat (ground to a flour); 18% glucose monohydrate;[3] 2.0% liver powder; 0.88% $CaHPO_4$; and 0.60% $CaCO_3$. A vitamin A, D, and E supplement was given orally to each rat weekly.

Comparisons were made of the cariogenicity of this diet with increasing protein levels. This was accomplished by supplementation with wheat gluten. Comparisons were made of the cariogenicity of the gluten-supplemented diets with approximate nitrogen equivalent supplementation of dried egg white[4] and casein.[5] A second set of comparisons was made of the effect on cariogenicity by amino acid supplementation of the raw whole wheat diet at three protein levels. The amino acids were L-lysine·HCl, DL-methionine and DL-threonine. Glycine supplementation was made

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[3] Cerelose 2001, Corn Products Company, New York.

[4] Egg white solids spray, Henningsen Foods, Inc., New York.

[5] Casein, enzymatic hydrolysate, General Biochemicals, Chagrin Falls, Ohio.

TABLE 1

Effect of varying level and type of protein on the cariogenicity of raw whole wheat diets; supplementation of diets, protein analysis, caries experience, growth and food intake

Diet series	A 100	A 101	A 102	A 110	B 100	B 103	B 111	B 121	C 100	C 104	C 105	C 106
Diet variables												
Whole wheat flour, %	78.52	59.26	40.00	40.00	78.52	67.02	68.02	69.52	78.52	73.72	66.02	58.82
Gluten, %	—	19.26	38.52	—	—	11.50	—	—	—	4.80	12.50	19.70
Egg white, % [1]	—	—	—	38.52	—	—	10.50	—	—	—	—	—
Casein, %	—	—	—	—	—	—	—	9.00	—	—	—	—
Analysis												
Protein (N × 6.25), %	10.1	24.7	38.4	37.6	8.4	17.6	15.4	15.4	10.9	14.6	20.5	25.9
Carious experience												
No. rats	26	25	26	26	27	29	28	30	28	30	30	28
Carious rats, %	96	76	50	23	96	97	46	67	96	100	93	54
Avg no. carious teeth/rat	3.0 ± 0.35 [2]	1.8 ± 0.28	1.0 ± 0.28	0.3 ± 0.16	3.7 ± 0.35	3.7 ± 0.38	0.9 ± 0.24	1.2 ± 0.20	4.9 ± 0.18	4.6 ± 0.32	2.9 ± 0.36	1.4 ± 0.33
Avg carious score/rat	6.2 ± 0.95 [2]	3.0 ± 0.64	1.7 ± 0.53	0.4 ± 0.16	9.1 ± 1.17	8.6 ± 1.18	1.5 ± 0.50	2.0 ± 0.40	11.4 ± 1.17	10.2 ± 1.01	5.4 ± 0.94	2.0 ± 0.60
Avg wt increment, g/rat/day	1.0	1.2	1.3	2.7	1.1	1.1	3.3	3.2	1.4	1.5	1.8	2.2
Avg food intake, g/rat/day	8.6	8.7	8.9	10.7	8.9	9.5	12.3	12.5	10.4	10.6	10.4	11.1

[1] 0.2 ml saturated solution biotin given 3 times a week.
[2] SE.

TABLE 2

Effect of varying protein and amino acid levels on the cariogenicity of raw whole wheat diets; supplementation of diets, protein and amino acid analysis, caries experience, growth and food intake

Diet series	D 100 [1]	D 130	D 131	E 132	E 133	E 134	E 135	F 132	F 133	F 134	F 135
Diet variables											
Whole wheat flour, %	78.52	78.52	78.52	72.57	72.44	72.24	72.17	66.07	65.94	65.74	65.67
Gluten, %	—	—	—	5.00	5.00	5.00	5.00	11.50	11.50	11.50	11.50
Glycine, %	—	1.6	—	0.95	0.33	0.13	—	0.95	0.33	0.13	—
L-Lysine·HCl, %	—	—	2.0	—	0.75	0.75	0.75	—	0.75	0.75	0.75
DL-Methionine, %	—	—	—	—	—	0.40	0.40	—	—	0.40	0.40
DL-Threonine, %	—	—	—	—	—	—	0.20	—	—	—	0.20
Analyses											
Protein, (N × 6.25), %	11.3	13.1	13.1	16.0	16.0	15.8	16.6	18.5	18.5	18.5	18.4
L-Lysine, %	0.33	0.32	1.95	0.69	1.25	1.41	1.38	0.65	1.26	1.46	1.42
L-Methionine, %	—	—	—	0.31	0.33	0.65	0.56	0.27	0.34	0.71	0.68
L-Threonine, %	—	—	—	0.38	0.39	0.38	0.45	0.40	0.42	0.38	0.52
Carious experience											
No. rats	39	39	39	27	27	25	30	29	28	29	28
Carious rats, %	97	90	97	89	96	92	97	86	86	76	79
Avg. no. carious teeth/rat	3.1 ± 0.28 [2]	2.7 ± 0.30	3.0 ± 0.27	3.7 ± 0.44	4.1 ± 0.38	4.3 ± 0.44	4.8 ± 0.33	2.4 ± 0.33	1.7 ± 0.39	2.5 ± 0.30	1.8 ± 0.27
Avg carious score/rat	6.2 ± 0.69 [2]	4.7 ± 0.60	5.8 ± 0.66	7.5 ± 1.11	8.9 ± 1.05	10.7 ± 1.42	11.7 ± 1.09	4.2 ± 0.73	2.7 ± 0.91	4.7 ± 0.47	2.6 ± 0.56
Avg wt increment, g/rat/day	0.8	0.9	1.1	1.0	1.0	0.9	1.0	1.1	1.1	1.3	1.3
Avg food intake, g/rat/day	8.0	8.6	9.0	9.1	9.2	8.7	9.4	8.8	9.4	9.6	9.9

[1] Supplementation was made at expense of glucose monohydrate.
[2] SE.

to keep these diets of the amino acid series equi-nitrogenous.

With the exception of the first series with lysine supplementation, D 130 and 131, all supplementation was made at the expense of the raw whole wheat. In this instance it was at the expense of the glucose. The diets were analyzed for nitrogen, ash, calcium, phosphorus and the added amino acids.[6] The nitrogen was determined by the Kjeldahl method, calcium by the method of the AOAC (4), and phosphorus by the method of Simmons and Robertson (5).

The addition of calcium and phosphorus salts was the same in all diets and was planned to give a calcium to phosphorus ratio of approximately one. The ash content of all diets lay between 2.31 and 2.75%; the calcium between 0.53 and 0.62%; the phosphorus between 0.44 and 0.65%. The low phosphorus values obtained when supplementation at the expense of the whole wheat was high. Because of the constancy of these data, individual values are not presented. The nitrogen and amino acid analyses are presented in tables 1 and 2. The protein levels of the diets as fed are presented as N $\times$ 6.25.

After 60 days on experiment, the rats were killed and the heads autoclaved to facilitate removal of the jaws. The lower molar teeth were examined for smooth surface caries as described by McClure (6). Fissure caries were not found.

Incidence of carious rats was consistently high with the basal raw whole wheat diet. Severity of caries, total score per rat, has been variable from period to period, but comparisons with concurrent experimental diets have been considered satisfactory. This variability of severity scores has appeared to be related to the sanitary conditions prevailing in the laboratory. To achieve a routine providing comparable conditions of oral flora as affected by fecal contamination the following procedure was adopted: 4 to 6 fecal pellets were added to 100 ml 5% sucrose solution, stirred, and allowed to stand at room temperature 1 to 2 hours. When diets were weighed, twice a week, 0.5 ml of this was added to the fresh diet and this in turn added to the sieved leftover food; containers were changed weekly. The effect of this on carious score per rat can be observed by a comparison of diets A-, B-, C-, D-100 (tables 1 and 2), diets A- and D-100 preceded this treatment; incidence, percentage carious rats, was similar but the innoculation of the diet resulted in higher severity scores.

RESULTS

Increasing the protein level of the basal raw wheat diet resulted in a decrease in the cariogenicity of the altered diets. Wheat gluten as a supplement for the whole wheat, providing protein levels of 24.7 and 38.4%, resulted in diets on which the percentage of carious rats decreased from 96 with the basal diet, successively to 76 and 50. Severity, average carious score per rat, decreased with increasing protein levels, 6.2, 3.0, 1.7. These differences are significant ($P < 0.01$) (tables 1 and 3, series A). Smaller increases in

[6] Microbiological assay, Rosner-Hixon Laboratories, 7737 S. Chicago Avenue, Chicago.

TABLE 3

Significance of differences (t test) between mean total carious score/rat fed diets A, B, and C [1]

Diet series	A 100	A 101	A 102	B 100	B 103	B 111	C 100	C 104	C 105
A 101	2.80								
A 102	4.16	1.47							
A 110	5.85	3.97	4.27						
B 103				0.31					
B 111				5.95	5.50				
B 121				5.71	5.26	0.78			
C 104							0.76		
C 105							3.97	3.43	
C 106							7.11	6.94	3.04

[1] *t* value needed for significance: 1%, 2.66; 5%, 2.00.

TABLE 4

Significance of differences (t test) between mean total carious score/rat fed diets D, E and F [1]

Diet series	D 100	D 130	E 132	E 133	E 134	F 132	F 133	F 134
D 130	0.42							
D 131	1.72	1.29						
E 133			1.33					
E 134			1.75	1.03				
E 135			2.72	1.91	1.02			
F 133						1.27		
F 134						0.59	1.94	
F 135						1.42	0.05	1.91

[1] t value needed for significance: 1%, 2.66; 5%, 2.00.

protein level, 10.9 to 25.9% by gluten supplementation (tables 1 and 3, series C) resulted in diets with which the rats experienced no decrease in caries intensity or severity between 10.9 and 14.6% protein levels, but did at the 20.5 and 25.9% protein levels. This change in severity is highly significant ($P < 0.01$).

This evidence of decreasing cariogenicity of this high cereal diet with 2 increments of protein above 15% is most marked between 20 and 26%, as indicated both by the data on carious rats and severity. The observation that dried egg white at an equivalent protein level, series A, is more cariostatic than gluten is borne out by the results in series B by extending the comparison to include casein at a lower, 15 to 17%, protein level. In this series the gluten supplementation at this protein level resulted in no protection against incidence and severity of caries, as in series C, whereas the egg white and casein were significantly and equally cariostatic (tables 1 and 3).

Quality and digestibility of the supplemental proteins may modify the degree of caries protection offered. The basal raw whole wheat diet is deficient in lysine, methionine and threonine. In the initial gluten trial, the highest supplementation, 38.52%, was made at a level which was calculated to bring the lysine of the diet to 0.8% and to provide an adequate level of methionine and more than adequate threonine. This diet was cariostatic to a degree.

The raw whole wheat diet was modified by the supplementation of L-lysine·HCl, DL-methionine and DL-threonine to provide adequate levels of intake for growing rats (7). The results of these trials are shown in tables 2 and 4. In series D, only L-lysine·HCl was added. The protein was at the level of the basal diet, 11 to 13%. In series E and F, the effect on cariogenicity of the diets was observed by the addition of the amino acids at 2 levels of protein, adjusted by gluten supplementation to 16 and 18.5%. In these series, the levels of these deficient amino acids were increased to adequacy in E 135, F 134 and F 135 as judged by the analysis of the L-forms. In none of these trials was supplementation by these amino acids successful in providing a cariostatic effect that was statistically significant. The only indication of a significant difference between group means of carious score per rat was a greater incidence of the fully amino acid supplemented diet E 135 over the unsupplemented diet E 132 ($P < 0.01$). There was indication that the higher protein level, 18.5%, itself provided a degree of protection against caries, although series E and F are separate trials and not directly comparable.

DISCUSSION

When supplementation of the raw whole wheat diet by gluten was high, 38.52% (table 1, series A), an increased volume of urine was produced by the rats, probably to insure urea clearance when this imbalanced protein diet was fed. Drinking water had to be increased and it was thought this heightened intake might alter the condition in the oral cavity and so affect the carious experience. Drinking water in series A was measured for a 28-day period. The measurements were considered accurate enough to establish differences. With diets A-100, -101, -102,

and -110, the respective water intakes, g/rat/day, were 18, 27, 30 and 21. Although the water intake varied inversely with the carious score per rat, with gluten supplemented diets, the dried egg white-supplemented diet that resulted in low caries incidence and severity did not cause an excessive water intake. It was concluded that water intake was not related to the carious experience. This increased urine volume was noticeable at 12.5 and 19.7% gluten supplementation in diets C-105 and -106.

It is tempting to impute the cariogenicity of these raw whole wheat diets to the incomplete protein of wheat, and its primary limiting amino acid lysine. In augmenting protein content of the basal diets with gluten supplementation, lysine content is diluted. Threonine and methionine levels are enriched and still other essential amino acids are disproportionally increased. With such supplementation, lysine may be more limiting than before and if it is a factor in cariogenicity as well as growth, such diets should result in increased caries.

A criterion suggested by Howard and associates (8), as being applicable to supplementation of bread flour by other products, is that a "complete protein," for growth, should contain 5.3 g lysine/16 g nitrogen. As judged by this, diets A-101, -102, C-105, -106 are all less "adequate" than their respective reference diets A- and C-100; 1.6 to 2.6 g lysine/16 g nitrogen in the supplemented and 2.9 and 3.1 g lysine/16 g nitrogen in the basal diets. The carious experience is, however, markedly decreased in these cases.

In series A and C increasing gluten supplementation was accompanied by decreasing cariogenicity and increasing the protein level was cariostatic in these cases. In series A and B, within which protein level is constant and provided by both an "inadequate" supplementation (gluten) and adequate supplementation (dried egg white and casein) a further cariostatic effect is shown which appears to be related to quality. In these cases the calculated quality of the egg white and casein supplemented diets is "adequate" as judged by the desired lysine to protein ratio, growth is improved and carious experience is decreased.

In series D, E, and F (table 2) with amino acid supplementation, the manipulation of protein level by gluten supplementation was kept at gluten levels that were not cariostatic in series A, B and C. Thus any imbalance by decreasing lysine by dilution as well as increasing other essential amino acids was kept at a minimum. The supplementation by L-lysine·HCl, DL-methionine and DL-threonine achieved recommended quantitative values as shown by analyses. These quantitative statements are minimal ones and are on the assumption of "dietary N $\times$ 6.25 with a true digestability and a biological value of 100 per cent" (7). Neither raw whole wheat nor gluten fulfills this assumption, but the supplementations made were in the direction of a "balanced" dietary protein. It is possible that the ratio of other essential amino acids may be a factor in the role of total protein in caries prevention.

It was thought that the higher protein levels in the diets used by Bavetta and McClure (1) had permitted the cariostatic action of lysine to be evident and that the low protein levels of these raw whole wheat diets may have been primarily limiting. An increase in the protein level to 18.5%, in our experience, did not provide a basis for demonstrating a cariostatic role for lysine. McClure (6), however, has shown lysine additions to be cariostatic in cereal diets at protein levels of 9 to 14%, when the cereal products had been altered by heat treatment. He was unable to show a cariostatic effect for lysine in high millet diets (9), although he demonstrated the deficiency of lysine in this cereal by a marked improvement in growth when it was added to a millet diet.

The inadequacy of these caries-producing diets is emphasized by the limited growth that accompanies the cariogenicity, but in some cases with gluten supplementation there is a cariostatic action in spite of little improvement in growth (table 1). The failure to improve growth with lysine supplementation suggests other limiting factors. The lack of fat in these diets may

decrease the efficiency of protein utilization but cariostatic action is demonstrable with them.

Although protein level was a factor in the production of smooth surface caries in these rats, the results of these trials do not permit the conclusion that lysine is the responsible entity in the protein which related it to the carious process.

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Protein-Energy Relationship in Adult Rats

I. EFFECTS OF VARYING AMOUNTS OF DIETARY PROTEIN AND FOOD ENERGY ON NITROGEN UTILIZATION[1]

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ABSTRACT During ad libitum feeding for 20 days, adult female rats were given isocaloric diets supplying either 5 or 15% of the total calories as protein from lactalbumin; average nitrogen intakes per 5 days were 464 mg for group L and 1159 mg for group A, respectively. Then food intake was restricted to two-thirds of ad libitum amounts for 30 days; groups maintained previously with 464 mg N received 266, 450, 835 or 1279 mg N, and groups maintained previously with 1159 mg N received 434, 762, 1144 or 1492 mg N/5 days. Although ad libitum food intake of group L was higher than that of group A, gains in body weight were similar. Nitrogen retention, hepatic nitrogen concentration and activities of hepatic succinic dehydrogenase and xanthine oxidase were greater and hepatic fat concentration lower in group A than in group L. Increments in nitrogen intake during restriction minimized losses in body weight and increased the unit activities of 2 hepatic enzymes studied. Liver weights were reduced markedly in all restricted groups, hepatic nitrogen concentrations increased and percentage of hepatic fat decreased. Concentrations of carcass nitrogen and body fat were similar for both ad libitum and restricted groups.

Nutritional status studies of adult women in the North Central region showed an increased number of subjects in negative nitrogen balance when self-selected diets supplied less than 1800 kcal/day (1). Women failed to maintain nitrogen equilibrium with average daily intakes of 50 to 62 g of protein when the energy value of the diet was 1500 kcal or less. Average intakes of both food energy and protein by these women over 30 years old decreased with age. Thus, the problem of a desirable proportion of dietary protein to food energy becomes critical as people age. In view of these observations, it seemed worthwhile to investigate the interrelationship of energy and protein in adult rats with controlled intakes of various proportions of food energy and protein.

The metabolic response of normal adult rats to diets supplying varying amounts of protein and food energy has been described by Munro and Naismith (2), Calloway and Spector (3, 4) and by Rosenthal and Allison (5). Data in these reports stressed the importance of the protein-to-calorie ratio for optimal utilization of protein when the intake of food energy was limited.

The present study was undertaken to obtain further information that may assist in answering the following basic questions in relation to nitrogen utilization in adult rats fed suboptimal amounts of food energy: (a) What influence does the previous state of protein nutrition exert upon the metabolic response to caloric restriction? (b) What are the effects of reduced intakes of protein? (c) Are there any merits to feeding increased amounts of protein in terms of utilization of nitrogen by the animal? (d) What is the nature of changes in nitrogen utilization?

Normal adult female rats were fed different amounts of dietary lactalbumin with unrestricted and restricted intakes of food energy. Utilization of nitrogen under the experimental conditions used was derived from the following measurements: food intake, change in body weight, nitrogen balance, carcass nitrogen and fat, hepatic nitrogen and fat, hepatic enzyme

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activity (succinic dehydrogenase and xanthine oxidase) and hemoglobin in the blood. Eight experiments were carried out on female rats. Data from all experiments were pooled and summarized for this paper.

EXPERIMENTAL

The composition of the diets used is shown in table 1. Similar caloric densities (approximately 4.5 kcal/g of diet) were maintained in all diets by adjusting the carbohydrate component when amounts of lactalbumin were varied. Vitamin supplements and the average nitrogen content of the diets are shown in table 1.

Adult female animals of Wistar stock were selected on the basis of uniform growth performance and maintenance of body weight for at least 3 weeks. The initial average weight was 195 g at ages from 4.5 to 5 months. Experimental groups had comparable weights at the beginning of each study. Rats were housed separately in galvanized wire-mesh cages.

Each experiment consisted of 2 consecutive periods: 1) food provided ad libitum for 20 days, and 2) food restricted to two-thirds of ad libitum intake for 30 days.

Two groups of rats were fed ad libitum, diets supplying either 15% (group A) or 5% (group L) of the total caloric value as protein from lactalbumin. At the end of 20 days, some experimental animals were killed along with animals of the same age fed laboratory stock ration.[3] The remaining animals in each group were subdivided into several groups. For the next 30 days, consumption of food energy was restricted by giving each animal two-thirds of its ad libitum intake. Variations in total nitrogen consumed during the second period were achieved by feeding diets in table 1. When these diets were fed in restricted quantities, the absolute amounts of nitrogen supplied varied as follows: for groups of rats maintained previously with 15% of the total calories as protein, the absolute amount of nitrogen was increased by about one-third of the ad libitum intake in group B, remained the same in group C, was decreased by one-third in group D and by two-thirds in group E; for groups of rats maintained previously with 5% of the total calories as protein, the absolute amount of nitrogen during restricted feeding was increased approximately by three in group M, was increased by two in group N, remained the same in group O and was decreased by one-third in group P. The amount of protein in the diet was varied at the expense

[3] The stock diet supplied approximately 25% of the total energy value as protein.

TABLE 1

Composition of the experimental diets

	Diets [1]				
	1	2	3	4	5
	g	*g*	*g*	*g*	*g*
Lactalbumin [2]	8.0	23.3	11.9	34.8	50.0
Dextrin [3]	67.6	53.4	63.3	42.7	24.0
Lard	9.3	9.3	9.3	9.3	9.3
Butterfat	9.3	9.3	9.3	9.3	9.3
Salts [4]	4.0	4.0	4.0	4.0	4.0
Processed rice hulls [5]	2.0	2.0	2.0	2.0	2.0
NaCl	1.0	1.0	1.0	1.0	1.0
Total	101.2	102.3	100.8	103.1	99.6
Avg nitrogen, mg/g of diet (analyzed)	10.0	27.6	14.7	40.9	58.9

[1] In addition to the above diets, each animal received daily a dry vitamin mixture which supplied the following vitamins: (mg/day) thiamine, 0.04; riboflavin, 0.06; niacin, 0.5; pyridoxine, 0.04; Ca pantothenate, 0.1; inositol, 10.0; *p*-aminobenzoic acid, 10.0; biotin, 0.001; folic acid, 0.008; choline chloride, 5.0; and ascorbic acid, 1.0 mg. A liquid vitamin mixture was given separately which supplied the following vitamins daily: vitamin A, 90 IU and vitamin D, 9 IU contained in 50 mg of cod liver oil; *a*-tocopherol, 0.75 mg; and vitamin B_{12}, 0.0015 mg.

[2] Nutritional Biochemicals Corporation, Cleveland; estimated to contain 75% protein.

[3] Dextrin, corn, white; Fisher Scientific Company, Chicago.

[4] Prepared in the laboratory; for composition, refer to Osborne, T. B., and L. B. Mendel. J. Biol. Chem., 37: 557, 1919.

[5] Ruffex, for roughage; Fisher Scientific Company, Chicago.

of carbohydrate so that the total food energy from both protein and carbohydrate sources represented a constant proportion of the diet. Fat, mineral salts and roughage were reduced by the same quantity as the total food intake. Vitamin supplements, however, were constant throughout the entire period of 50 days. The specific diets fed to groups of animals and corresponding intake of nitrogen are presented in table 2.

Continuous nitrogen balances were obtained in some experiments. In other experiments, data were collected only during the last 5 days of ad libitum feeding and during the first 5 days of restricted food intake. Samples of urine and feces were composited for 5 days and analyzed for nitrogen by the macro-Kjeldahl procedure.

Specific gravity of clipped, eviscerated rats was measured by the water displacement method of Rathbun and Pace (6) and the percentage of body fat was calculated as follows:

Per cent of body fat = 5.362/specific gravity − 4.880. Liver and carcass were analyzed for nitrogen by the macro-Kjeldahl method.

Hepatic fat was determined by a modification of the method of Soderhjelm and Soderhjelm (7). Ten-milliliter samples of homogenized liver were extracted in Mojonnier flasks using a solvent mixture of ethyl alcohol, ethyl ether and petroleum ether. Extractions were evaporated from weighed containers on a water bath. Samples were dried for 15 hours in an air oven at 80°C (this procedure approached minimal values in preliminary experiments).

For measuring the activity of hepatic enzymes, the animal was stunned and decapitated; the liver was removed and chilled immediately in cracked ice. Representative portions of each lobe were weighed and homogenized in appropriate buffer solutions for the assays. Activity of succinic dehydrogenase was measured according to the manometric method of Schneider and Potter (8). Manometric determinations of cytochrome oxidase activity by the procedure of Schneider and Potter (8) were not successful until the recommended concentration of sodium ascorbate was doubled and the amount of liver increased by five. Since limited data were obtained on hepatic cytochrome oxidase activity, they are not included in this report. Xanthine oxidase activity in hepatic tissues was measured by the method of Van Pilsum (9).

For hemoglobin analyses, blood was withdrawn by a hypodermic syringe from the portal vein of anesthetized rats and stored in heparinized test tubes. Measured

TABLE 2

Average intakes of nitrogen, food and food energy per 5 days for groups of rats during ad libitum and restricted feeding

Ad libitum,[1] 20 days					Restricted,[2] 30 days				
Groups	No. of rats	N intake	Food intake	Energy value of intake	Groups	No. of rats	N intake	Food intake	Energy value of intake
		mg/5 days	*g/5 days*	*kcal/5 days*			*mg/5 days*	*g/5 days*	*kcal/5 days*
					B	4	1492	25.2	112
					C	17	1144	27.7	125
A	74	1159	40.9 [3]	186	D	17	762	27.3	124
					E	17	434	29.1	134
					M	15	1279	31.2	141
					N	16	835	29.9	136
L	73	464	44.5 [3]	205	O	17	450	30.8	142
					P	4	266	26.1	120

[1] During ad libitum feeding, groups A and L received diets that supplied 15% (diet 2) and 5% (diet 1) of the total calories as protein, respectively.

[2] During restricted feeding, food energy intake was restricted to two-thirds of the amount consumed ad libitum by each animal. Group A was subdivided into groups B, C, D and E; group L, into groups M, N, O and P. Nitrogen intakes varied as follows: increased by one-third of ad libitum amount for group B fed diet 5; remained the same as ad libitum intake for groups C and O fed diet 4 and diet 3, respectively; decreased by one-third of ad libitum intake for groups D and P fed diet 2 and diet 1, respectively; decreased by two-thirds of ad libitum intake for group E fed diet 3; increased 2 times and 3 times the ad libitum amounts for groups N and M fed diet 2 and diet 4, respectively.

[3] Significantly different at 1% level.

amounts of blood were pipetted into dilute ammonium hydroxide (0.5%). Hemoglobin concentration was determined by measuring absorption at 540 mμ with a Beckman DU spectrophotometer.

The data collected were evaluated for statistical significance by the *t* test according to Snedecor (10).

RESULTS

Data on nitrogen balance were collected for periods of 5 days each; hence average intakes of food and nitrogen during ad libitum and restricted feeding were expressed in terms of 5 days. For brevity in presentation of results, average nitrogen intake per 5 days will be referred to as "mg N".

During ad libitum feeding, rats that consumed 464 mg N (group L) had significantly higher intake of food than rats fed 1159 mg N (group A) as shown in table 2. Animals in group L may have eaten additional amounts of food to satisfy the demands for certain essential amino acids present in minimal quantities in the diet fed. On the other hand, increased food intake of rats in group L may be interpreted to suggest a need for additional amounts of energy to compensate for an increase in heat production associated with diets low in protein, an observation reported by Black and co-workers (11) for growing rats.

Although food energy intakes of ad libitum-fed rats differed significantly, similar gains in body weight were observed (table 3) whether the nitrogen intake was 464 mg (group L) or 1159 mg (group A). Furthermore, there were no significant differences between groups A and L in carcass weights and concentrations of fat and nitrogen in the carcass (fig. 1). Hepatic weights were similar (fig. 2) and total hepatic nitrogen was directly related to weights of the liver.

Continuous nitrogen balances based on 5-day periods showed greater nitrogen retention ($P < 0.01$) when the average nitrogen intake per 5 days was 1159 mg (group A) than when the amount consumed was 464 mg (group L). Rats in group A also had a significantly higher percentage of hepatic nitrogen and lower hepatic fat ($P < 0.01$) compared with rats in group L (fig. 2).

The above data suggested that differences in food energy intake of ad libitum-fed rats while consuming either 1159 or 464 mg of nitrogen might have resulted in gains or losses or both in body components such as fat, protein, water and possibly minerals. Studies of body composition would help clarify the effects of the observed difference in food intake on nitrogen utilization under the same experimental conditions.

TABLE 3

Average nitrogen balance and body weight changes on varying nitrogen intakes during ad libitum and restricted feeding

Groups	Avg N intake	Avg N balance [1]	Avg wt change [2]	Groups	Avg N intake	Avg N balance [1]	Avg wt change [2]
	mg/5 days	*mg/5 days*	*g*		*mg/5 days*	*mg/5 days*	*g*
			Ad libitum, 20 days				
A	1159	86 (39) [3]	4.6 (74)	L	464	42 (38)	2.3 (73)
			Restricted, 30 days				
B	1492	−131 (4)	−17.0 (4)	M	1279	41 (6)	−11.1 (15)
C	1144	−108 (8)	−21.3 (17)	N	835	14 (10)	−16.6 (16)
D	762	−104 (11)	−22.4 (17)	O	450	−38 (8)	−19.0 (17)
E	434	−120 (6)	−26.4 (15)	P	266	−114 (4)	−24.0 (4)

[1] Ad libitum: nitrogen balance for the last 5 days only with ad libitum feeding; restricted: nitrogen balance for the first 5 days only with restricted feeding.
[2] Difference between initial and final body weights for each period of feeding.
[3] Figures in parentheses indicate number of animals observed.

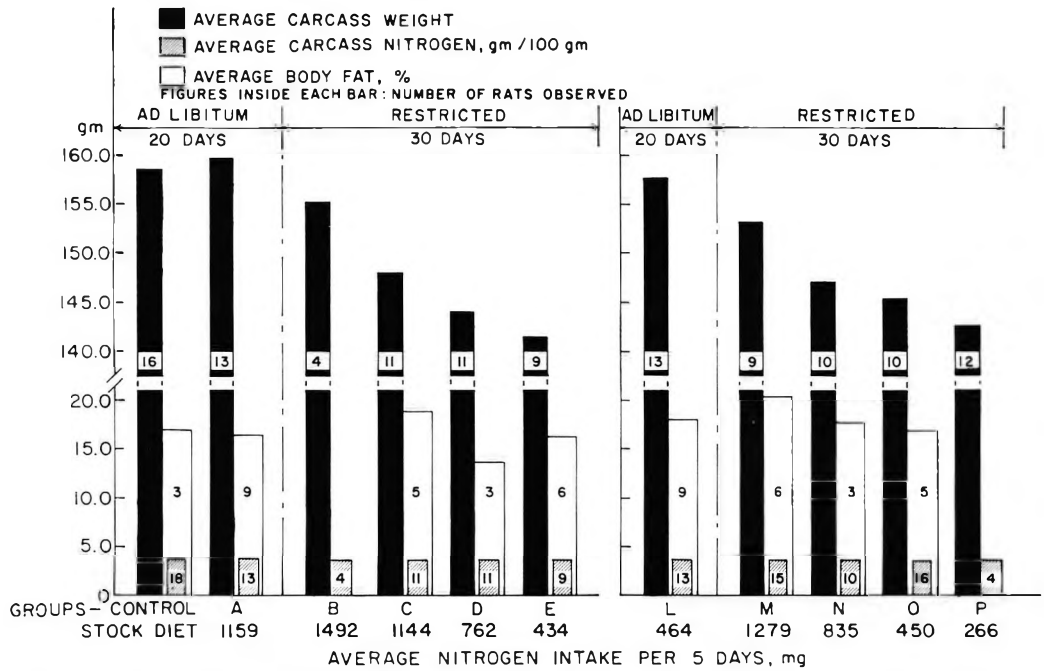

Fig. 1 Average carcass weights were similar for ad libitum (A and L) and stock control rats. Following restriction, carcass weights decreased significantly ($P < 0.01$) except for groups B and M. There were no significant differences in concentrations of body fat and carcass nitrogen in both ad libitum and restricted rats. Percentage of body fat was calculated from specific gravity data; no data were collected for groups B and P.

At the end of ad libitum feeding, both unit and total activities of succinic dehydrogenase and xanthine oxidase in hepatic tissues were significantly higher ($P< 0.01$) when rats were fed 1159 mg N (group A) than when given 464 mg N (group L). Stock control rats had significantly higher values ($P < 0.01$) than either groups A or L. Average unit activities for succinic dehydrogenase and xanthine oxidase are represented in figure 2. Unit activities of these 2 hepatic enzymes were also related directly to percentage of hepatic nitrogen. Observations by Miller (12, 13) indicated that changes in certain liver enzyme activities reflected variations in the concentration of hepatic protein.

Compared with corresponding ad libitum values, restricted animals showed appreciable losses in body weights (table 3) and in carcass weights (fig. 1) following 30 days of restricted food consumption. Weights of the liver were reduced markedly; concentrations of hepatic nitrogen increased and fat concentrations in hepatic tissues decreased (fig. 2). Unit activities of hepatic succinic dehydrogenase and xanthine oxidase increased as the percentage of hepatic nitrogen increased (fig. 2). When restricted feeding was imposed, losses of urinary nitrogen were transitory. All groups of restricted rats either approached or re-attained nitrogen equilibrium at the end of restriction.

Effects of protein intake during ad libitum feeding were evident in the nitrogen balance picture during restriction. When groups of rats consumed 1159 mg N prior to restriction (groups B, C, D, E), urinary excretion was greater than when rats received 464 mg N before restriction (groups M, N, O, P). This difference in nitrogen loss was particularly apparent in the first 5 days of restricted feeding (table 3). The magnitude of nitrogen loss during restriction may reflect differences in initial body stores of nitrogen in restricted rats. Rosenthal and Allison (14) observed

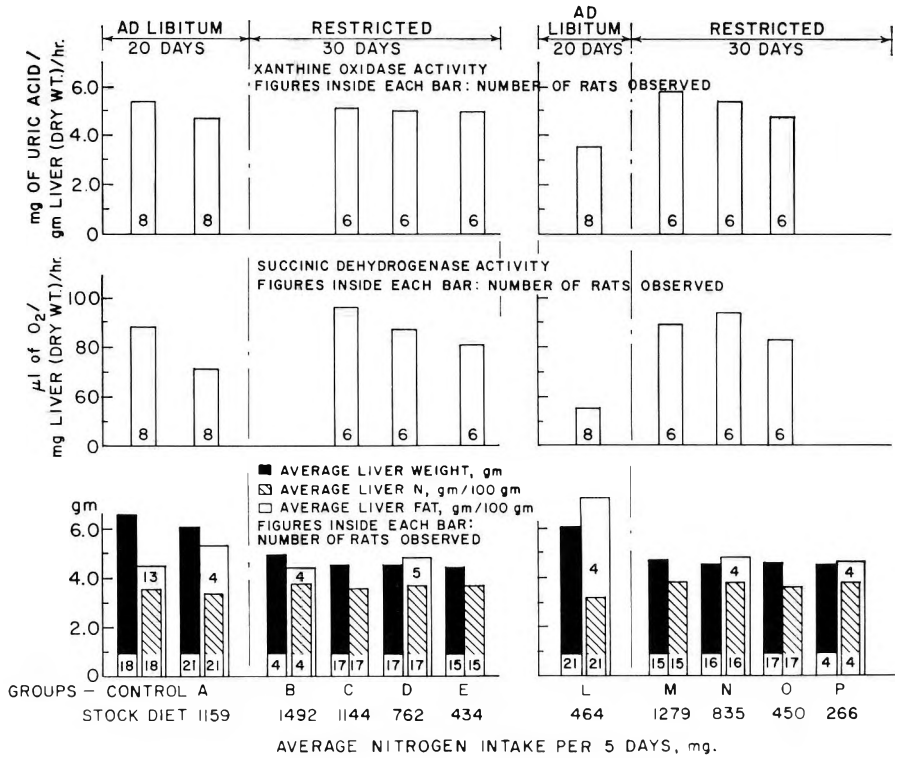

Fig. 2 At the end of ad libitum feeding, liver weights were significantly lower ($P < 0.01$) in groups A and L than in control group. Concentrations of hepatic nitrogen and unit activities of 2 hepatic enzymes were higher and hepatic fat concentration lower for control rats and group A compared with group L. Following restriction, liver weights were markedly reduced; unit activities of 2 enzymes increased as hepatic nitrogen concentration increased; fat concentration decreased. No data were collected for the following: Hepatic fat for groups C, E, M and O; hepatic enzyme activities for groups B and P.

that normal adult dogs with sufficient body stores showed greater initial losses of nitrogen when subjected to caloric restriction than dogs with limited reserves of body nitrogen. It is possible also that restricted rats previously fed 1159 mg N were less economical in their use of nitrogen than rats previously fed 464 mg of nitrogen.

When intakes of nitrogen were increased over ad libitum amounts consumed (group B, from 1159 mg to 1492 mg; groups M and N, from 464 mg to 1279 and 835 mg, respectively), losses in body weights (table 3) and carcass weights (fig. 1) were significantly less ($P < 0.01$) than when nitrogen intakes were reduced during caloric restriction (groups D and E, from 1159 mg to 762 and 434 mg, respectively; group P, from 464 mg to 266 mg).

During restriction, increments in nitrogen consumed from ad libitum intake of 464 mg N to 835 and 1279 mg (groups N and M, respectively) increased significantly the unit activities of hepatic succinic dehydrogenase ($P < 0.01$) and xanthine oxidase ($P < 0.05$) as shown in figure 2. Decreased intakes of nitrogen from ad libitum amounts of 1159 mg to 762 and 434 mg (groups D and E, respectively) resulted in a progressive decline in unit activities of hepatic succinic dehydrogenase but not in xanthine oxidase activity. When nitrogen intakes per 5 days were maintained at about 1150 mg during both ad libitum and restricted period (group C), the unit activity of succinic dehydrogenase was significantly higher ($P < 0.01$) than when the intake of nitrogen was maintained at

about 455 mg/5 days (group O). Xanthine oxidase activities for groups C and O were not significantly different. Hence, succinic dehydrogenase in hepatic tissues appeared to be more sensitive than xanthine oxidase to variations in nitrogen intake when food energy was restricted. Total activities of these 2 hepatic enzymes varied directly with total hepatic nitrogen which in turn depended upon the weight of the liver.

Groups of restricted animals fed different amounts of nitrogen had similar average values for nitrogen and fat per unit weight of liver (fig. 2). Concentrations of body fat and carcass nitrogen were not significantly different at the end of restriction (fig. 1).

At the end of ad libitum feeding, average hemoglobin concentrations in grams per 100 ml were 12.48 for 10 stock control rats, 12.90 for 4 rats fed 1159 mg N (group A) and 11.72 for 4 rats fed 464 mg N (group L). The average concentration of hemoglobin for group L was significantly lower than values obtained for both groups A ($P < 0.01$) and control rats ($P < 0.05$). Following caloric restriction, rats fed 1492 mg N with reduced food intake (group B) had a significantly higher average hemoglobin concentration ($P < 0.01$) of 14.11 g/100 ml (4 rats) compared with corresponding ad libitum value of 12.90 g (group A). For other restricted groups, no clear-cut relationships were evident between hemoglobin concentrations and nitrogen intake during the time food consumption was limited. Average hemoglobin values for these groups ranged from 12.11 to 12.90 g/100 ml at the end of restriction.

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Protein-Energy Relationship in Adult Rats

II. QUALITATIVE AND QUANTITATIVE VARIATIONS IN DIETARY NITROGEN AND EFFECTS ON DEPOSITION OF HEPATIC FAT AND ON NITROGEN UTILIZATION[1]

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ABSTRACT Groups of female adult rats were fed ad libitum a diet providing 5% of the food energy as lactalbumin or the same diet supplemented with amino acids calculated to be most limiting after comparison of the ratio of amino acids to tryptophan with that of the daily amino acid requirements of the adult rat. After 20 days of unrestricted feeding, no significant differences in food intake were attributable to added arginine, methionine, phenylalanine and valine. Both groups were comparable in body weight, nitrogen retention and in weights and nitrogen concentrations of liver and carcass. However, hepatic fat was significantly lower with supplementation than without. Restriction of food energy intake for 30 days to two-thirds of amounts consumed voluntarily was associated with decreased hepatic weight and nitrogen; concentration of hepatic nitrogen increased. Total hepatic fat, carcass weight and total carcass nitrogen were reduced markedly. These effects of caloric restriction were not modified by variations in quality or quantity of nitrogen fed. Restricted groups had similar concentrations of hepatic fat. Increased nitrogen intake during restriction minimized losses in body weight and urinary nitrogen. Concentrations of carcass nitrogen were similar for supplemented and unsupplemented groups whether restricted or not.

As part of experiments examining the relationship of dietary proteins and food energy to utilization of nitrogen, it had been observed that concentration of hepatic fat was significantly higher when adult female rats were fed a ration supplying 5% of the total energy value as protein, compared with feeding a diet providing 15% of the food energy as protein. Hepatic fat was reduced markedly after 30 days of caloric restriction (1).

Amino acid balance and its significance in deposition of hepatic fat have been reviewed by Harper (2). The importance of food energy intake in the deposition of fat in livers of rats fed suboptimal amounts of protein was emphasized in early studies by several investigators (3–6) because restricting intakes of food in young rats fed rations low in protein prevented fat accumulation in the liver. Recent observations by Yoshida and Harper (7) stressed the relationship of amino acid balance and caloric intake to fatty infiltration in the liver of weanling rats fed diets low in protein and deficient in threonine.

The present study was planned to examine the effects on deposition of fat in the liver and utilization of nitrogen when a diet low in protein was supplemented with its most limiting amino acids and fed ad libitum or in restricted quantities. Since investigators have reported a decrease in food consumption when weanling and adult rats were fed diets low in protein with imbalanced amounts of amino acids (8–10), data on food intakes were included.

EXPERIMENTAL

Investigations were carried out on adult female rats according to experimental procedures described in the preceding paper (1).

The composition of diets used is shown in table 1. Lactalbumin was the source of

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TABLE 1

Composition of the experimental diets

	Diets [1]		
	5	5A	15
	g/100 g	*g/100 g*	*g/100 g*
Lactalbumin [2]	6.7	5.5	20.0
Amino acids			
L-Arginine	—	0.24	—
L-Methionine	—	0.14	—
L-Phenylalanine	—	0.36	—
DL-Valine	—	0.16	—
Dextrin [3]	67.7	68.0	54.4
Lard	9.3	9.3	9.3
Butterfat	9.3	9.3	9.3
Salts [4]	4.0	4.0	4.0
Processed rice hulls [5]	2.0	2.0	2.0
NaCl	1.0	1.0	1.0
Avg nitrogen, mg/g of diet (analyzed)	8.2	8.2	23.8

[1] In addition to the above diets, each animal received daily vitamin supplements described in table 1, footnote 1, of the preceding paper (1).
[2,3,4,5] Sources and composition of these ingredients are shown in table 1 of the preceding paper (1).

protein and amounts were varied by adjusting the carbohydrate component of the diet. The diets were isocaloric (4.5 kcal/g) and nitrogen content was determined by the macro-Kjeldahl method. Each animal received daily vitamin supplements as indicated in the footnote to table 1.

Diets 5 and 5A each supplied 5% of the total caloric value as protein or as protein plus amino acids. Diet 15 provided 15% of the food energy as protein.

Diet 5A was isonitrogenous with diet 5 and contained 4 amino acids, arginine, methionine, phenylalanine and valine. These amino acids were added in quantities calculated to provide them to the rat in the same ratio to tryptophan as given in the daily amino acid requirements of the male adult rat by Mitchell (11). The amino acid composition of protein in lactalbumin was estimated from data by Block and Bolling (12).

Rats in group A were fed ad libitum diet 5 (lactalbumin only as source of protein) and rats in group E were fed diet 5A (lactalbumin plus arginine, methionine, phenylalanine and valine). The control group was fed laboratory stock ration. At the end of 20 days, control animals and some experimental rats were killed. The remaining animals in group A were subdivided into groups B, C and D and rats in group E into groups F, G and H. For the suceeding 30 days, the intake of food energy of each rat was restricted by feeding diet 5, diet 5A or diet 15 in amounts equivalent to two-thirds of its ad libitum intake. When reduced amounts of diet 5 were fed to groups C and G, total nitrogen intakes were decreased to two-thirds of that consumed ad libitum. Similarly, when diet 5A was fed to groups D and H, absolute intakes of nitrogen were reduced to two-thirds of that consumed ad libitum. With restricted amounts of diet 15, total nitrogen intakes of groups B and F were twice the amounts previously consumed.

Table 2 shows the variations in nitrogen intake with and without amino acid supplements during ad libitum and restricted feeding. The food energy value derived from the sum of protein and carbohydrate sources in each diet was a constant proportion of the total caloric value. During restriction, not only intakes of fat but also mineral salts and roughage were reduced by the same quantity as the total intake of food. Intakes of vitamin supplements remained constant throughout the entire experiment.

TABLE 2

Effects of amino acids supplementation on food intake, nitrogen balance and on body weight changes during ad libitum and restricted period

Periods	Ad libitum [1]		Restricted [2]		Ad libitum		Restricted	
Diets fed	5	15	5	5A	5A	15	5	5A
Groups	A	B	C	D	E	F	G	H
No. of rats	31	4	8	8	33	8	9	8
Avg nitrogen intake/5 days, mg	379	676	266	260	383	731	252	260
Avg food intake/5 days, g	43.6	28.0	31.4	30.3	45.3	30.2	29.8	30.5
Avg energy value of intake/5 days, kcal	208	128	145	140	210	138	136	142
Avg nitrogen balance, mg [3]	+43(17) [4]	−7(4)	−114(7)	−100(8)	+28(18)	−30(8)	−125(8)	−123(7)
Avg weight change, g [5]	0.0	−13.0	−21.5	−23.0	+0.4	−16.8	−23.2	−23.6

[1] Ad libitum, 20 days; groups A and E were fed a low-protein diet without and with amino acid supplement, respectively.
[2] Restricted, 30 days; intake of food energy was restricted to two-thirds of ad libitum amount. Groups B and F: nitrogen intakes were twice the ad libitum amounts consumed. Groups C and G received the diet without amino acid supplement, D and H were fed the diet with amino acid supplement; nitrogen intakes were reduced to two-thirds of ad libitum amounts.
[3] Ad libitum: nitrogen balance for the last 5-day period only; figures in parentheses indicate number of animals observed. Restricted: nitrogen balance for the first 5-day period only.
[4] Figures in parentheses indicate number of animals observed.
[5] Weight change represented by the difference between initial and final body weights during each period of feeding.

Experimental animals were weighed each day and records of daily food intake were kept. Nitrogen balance, nitrogen and fat in the liver, carcass nitrogen and blood hemoglobin were determined according to analytical procedures described in the preceding paper (1).

Data from experiments were pooled and evaluated statistically by the *t* test of Snedecor (13).

RESULTS

For clarity, average intakes of nitrogen per 5 days as shown in table 2 will be referred to simply as "mg N" for each experimental diet fed during ad libitum and restricted periods. Amino acid supplements will be designated by "S"; hence, nitrogen intake per 5 days of rats fed diet 5A ad libitum is S-383 mg N.

Supplementation of lactalbumin with amino acids had no significant effects on ad libitum intakes of food. Rats in group E consumed an average of 45.3 g of food/5 days with nitrogen intake of S-383 mg N compared with 43.6 g of food for group A fed 379 mg N (table 2). These observations indicate that limiting amounts of arginine, methionine, phenylalanine and valine in lactalbumin protein were not responsible for the increased food intake reported earlier (1) when rats were fed ad libitum a diet supplying 5% of the total calories as lactalbumin compared with feeding a diet providing 15% of the total calories as lactalbumin. Diet 5, used in the present study, was similar although not identical to the diet that supplied 5% of the total calories as protein in previous experiments. Results also suggest that the addition of amino acids did not produce an imbalanced mixture of amino acids with lactalbumin that could have affected food intake. For instance, Kumta and coworkers (10) reported a decrease in intake of food by adult rats when fed a diet made imbalanced by the addition of 0.4% of DL-methionine and 0.6% of DL-phenylalanine to 6% of fibrin.

When groups of rats fed ad libitum consumed either 379 mg N or S-383 mg N, there were no significant differences in body weight changes, nitrogen retention (table 2) and in weights and nitrogen concentrations of liver and carcass (fig. 1) fol-

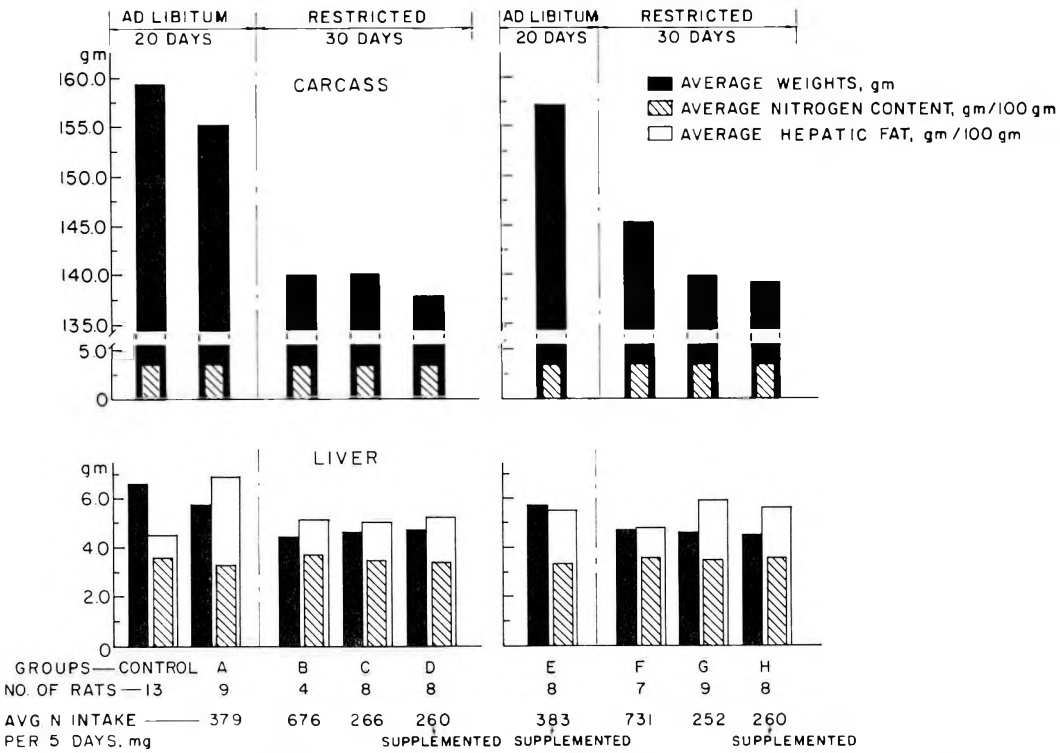

Fig. 1 Average weights and nitrogen concentrations of liver and carcass were similar for control rats and ad libitum groups A and E. Average hepatic fat concentration was significantly higher in group A than in group E. Following caloric restriction, carcass weights decreased significantly except for group F; carcass nitrogen concentrations were not significantly different from ad libitum values. Hepatic weights were reduced and nitrogen concentrations increased. Hepatic fat concentrations were similar for all restricted groups.

lowing unrestricted feeding. However, stock control animals maintained with laboratory ration exhibited significantly higher hepatic weights and concentrations of hepatic nitrogen ($P < 0.01$) in contrast with rats fed the experimental diets. The stock diet supplied approximately 25% of the total calories as protein. Carcass weights and carcass nitrogen concentrations of both experimental groups fed either 379 mg N or S-383 mg N were similar to those observed in stock control rats.

Deposition of hepatic fat during ad libitum feeding was affected markedly by amino acid supplementation. A significant reduction was noted in the total fat content ($P < 0.05$) and in fat concentration of livers ($P < 0.01$) when rats were fed S-383 mg N compared with rats fed 379 mg N. Stock control rats showed the least amount of hepatic fat (fig. 1).

The results obtained on mature rats during ad libitum period, indicate that alterations in amino acid composition of diets low in protein may modify certain metabolic responses without exerting any significant influence on others. From observations on relationships of amino acid imbalance to hepatic fat deposition in young rats, Despande and co-workers (8) have suggested that amino acid balance required for various physiological processes such as growth may be different from that needed for normal liver fat deposition.

Nitrogen losses for the first 5 days of restriction and weight losses following 30 days of restricted feeding were minimized when intakes of nitrogen increased from ad libitum amounts of 379 mg N to 676 mg N (group B) and from S-383 mg N to 731 mg N (group F) as shown in table 2. Losses in weight and initial nitrogen ex-

cretion increased when nitrogen intakes were reduced to two-thirds of amounts fed ad libitum (from 379 mg to 266 mg and S-260 mg for groups C and D, respectively; from S-383 mg to 252 mg and S-260 mg for groups G and H, respectively). Therefore, nitrogen intakes during restriction influenced the magnitude of weight loss and nitrogen balance; supplementation with amino acids did not modify these effects. After 30 days of restriction, the animals had adjusted to the regimen and approached nitrogen equilibrium with all experimental diets.

Compared with ad libitum values, marked reduction in liver weights observed in restricted animals (fig. 1) was accompanied by significant decreases in total hepatic nitrogen ($P < 0.01$) and corresponding increases in concentrations of hepatic nitrogen ($P < 0.01$ for groups B, F, H; $P < 0.05$ for groups C, D and G). The average concentration of hepatic nitrogen for group B was 3.7% which was significantly higher ($P < 0.01$) than 3.5 and 3.4% for groups C and D, respectively. However, there were only 4 rats in group B compared with 8 rats each for groups C and D. With the exception of group G, total hepatic fat decreased significantly for restricted rats ($P < 0.01$ for groups B, C, D, F; $P < 0.05$ for H).

The data on liver weights, hepatic nitrogen and total hepatic fat following restricted feeding, indicate that caloric restriction reduced liver size. In turn, total hepatic nitrogen and fat decreased and concentrations of hepatic nitrogen increased. These effects of caloric restriction were not modified by increasing or decreasing the intakes of nitrogen when food consumption was limited. Supplementation with amino acids before or during restriction had no significant effects on hepatic nitrogen and total hepatic fat following restricted feeding.

Average concentrations of hepatic fat were decreased significantly ($P < 0.01$) at the end of restriction for rats maintained previously with unsupplemented diet 5; values were 5.3% for groups B and C, 5.2% for group D compared with corresponding ad libitum amounts of 6.9%. On the other hand, when supplemented diet 5A was fed before restriction, average concentrations of hepatic fat did not differ significantly from the ad libitum value of 5.5% compared with 4.8, 5.9, and 5.6% for groups F, G and H, respectively. Average hepatic concentrations of fat were similar for all restricted groups. Therefore, variations in amounts of nitrogen consumed or supplementation with amino acids when food intake was limited had no significant effects on concentrations of hepatic fat.

Carcass weights were significantly lower than ad libitum values for groups B, ($P < 0.05$), C, D, G and H ($P < 0.01$) but not for group F as shown in figure 1. Total carcass nitrogen was reduced markedly by caloric restriction. Concentrations of carcass nitrogen were similar for all restricted groups (fig. 1).

Limited data were obtained on concentrations of blood hemoglobin. At the end of ad libitum feeding, average values in g/100 ml of blood were 12.21 for 5 rats fed 379 mg N, 12.49 for 3 rats given S-383 mg N and 12.48 g for 10 control rats. Following caloric restriction, results suggested a trend towards increased concentrations of hemoglobin with average values ranging from 12.97 g to 13.90 g/100 ml of blood; number of rats varied from 3 to 5/group. No significant effects of different intakes of nitrogen or supplementation during ad libitum and restricted feeding were evident.

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Antagonism of Poorly Invertible D-Amino Acids Toward Growth Promotion by Readily Invertible D-Amino Acids[1]

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ABSTRACT Replacement of L-histidine by D-histidine in purified L-amino acid diets promotes appreciably less rapid growth, particularly when the histidine is fed at one-half the normal level. At this level, the response to the D-histidine is almost completely inhibited by including the poorly invertible D-forms of lysine, threonine, isoleucine, leucine, and valine in the diet. The antagonism is not attributable uniquely to any single D-amino acid, but appears rather to be cumulative. Qualitatively the same *en masse* effect of the D-forms of this poorly invertible group of the essential amino acids could be demonstrated toward the growth promotion by one-half normal levels of the other readily invertible D-amino acids tested (D-methionine, D-phenylalanine, and D-tryptophan). The quantitative variations in response noted probably reflect differences in dietary concentration of the readily invertible D-amino acids tested and differences in their normal susceptibilities to attack by D-amino acid oxidase.

The D-isomers of the essential amino acids have previously been shown to vary widely in their capacities to promote growth in the rat when fed singly in lieu of their L-enantiomorphs. D-Lysine, D-isoleucine, and D-threonine are apparently quite unable to replace their L- counterparts for this purpose, and D-leucine and D-valine show only minor capacities to do so. On the other hand, D-methionine is as effective as L-methionine and D-tryptophan only moderately less so than L-tryptophan. The D-forms of phenylalanine, arginine, and histidine apparently occupy intermediate positions (1).

However, Phillips and Berg (2) noted that the simultaneous replacement by their D-isomers of several of the essential L-amino acids whose D-forms are readily utilizable when fed singly (methionine, tryptophan, phenylalanine, histidine, and arginine) produced a growth response which appeared to be disproportionately low. Wretlind (3) had observed somewhat earlier that in essential amino acid mixtures devoid of arginine, suboptimal quantities of D-methionine promoted essentially the same growth as L-methionine when 5 of the 8 other indispensable amino acids were fed in the L-form (histidine, leucine, lysine, phenylalanine, and tryptophan), but that it induced much slower growth when all of the other eight were fed in the racemic form. He suggested that stereonaturalization of the D-methionine may have been inhibited by the other D-amino acid components. Wachter and Berg (4) studied the collective effect of the D-forms of the poorly invertible group of the essential amino acids (D-alloisoleucine,[2] lysine, threonine, leucine, and valine) upon the growth induced by D-histidine in diets that contained the nonessential as well as the essential amino acids. At normal (0.4%) and suboptimal (0.2%) levels of L-histidine only a slight depression in growth was obvious (6 and 8%, respectively) when the extraneous D-amino acids were included, but in analogous tests in which the histidine was fed in the D-form at these levels, the growth suppression was marked (71 and 86%).

The present communication records an attempt to determine whether the effect of the extraneous D-amino acids might be attributed more particularly to one amino

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[1] This investigation was supported by U.S. Public Health Service Research Grant AM-03141, from the National Institute of Arthritis and Metabolic Diseases. A preliminary report was presented at the April 15, 1962, program of the American Institute of Nutrition in Atlantic City. Kamath, S. H., and C. P. Berg 1962 Antagonism among the D-amino acids toward growth. Federation Proc., *21*: 7 (abstract).

[2] DL-Isoleucine is not available on the market, hence D-alloisoleucine + L-isoleucine was employed instead. The D-alloisoleucine is not available for growth.

acid species than to another, and whether the degree of antagonism observed may differ markedly among the more readily invertible D-forms of the essential amino acids.

EXPERIMENTAL

The DL-lysine monohydrochloride, DL-leucine, DL-valine, and the D-alloisoleucine + L-isoleucine[2] employed in these tests were prepared by recrystallization of lots made available to us through a commercial source,[3] as was also the L-lysine monohydrochloride. The DL-threonine, DL-histidine, D-methionine, D-phenylalanine and most of the L-amino acids (both essential and nonessential) were also obtained commercially.[4] Part of the D-histidine had previously been prepared in the laboratory (5) and the remainder was resolved by minor modification of the procedure (6) employed earlier. The D-tryptophan was also prepared by resolution (7). Tests with snake venom (8) indicated that the D-forms of the histidine, tryptophan, methionine, and phenylalanine were essentially free of the L-isomers. The specific rotations of the L-amino acids and their chemical purity and the purities of the D- and DL-amino acids conformed well with the specifications of the National Academy of Sciences (9).

The composition of the L-amino acid mixture used in the control reference diet is shown in table 1. For convenience, the amino acids are grouped under sub-headings as essential (D-isomers poorly invertible and D-isomers readily invertible) and as nonessential. The subdivision of the essential amino acids is somewhat arbitrary (for details, see the introduction and (1)). In this study 2 types of changes were made in the reference mixture. One of the variations consisted of feeding one or all of the amino acids of the poorly invertible group in the DL-form at twice the L-amino acid level indicated in table 1. The other involved decreasing to one-half the amount of the readily invertible D-amino acid tested, with the same decrease in the L-isomer in the control diet.

In addition to the particular amino acid mixture employed, each diet also contained: (per 100 g) sucrose, 15.0; cellu-

[3] We are happy to acknowledge our indebtedness to the Dow Chemical Company for these amino acids and to Drs. H. C. White and R. P. Perkins through whose courtesy they were obtained.

[4] From the California Corporation for Biochemical Research.

TABLE 1

Composition of the L-amino acid mixture in the reference diet

Essential amino acids	g/100 g	Nonessential amino acids	g/100 g
D-isomers poorly invertible [1]		Alanine	0.6
Isoleucine	0.5	Aspartic acid	1.0
Lysine [3]	1.0	Cystine	0.2
Threonine	0.5	Glutamic acid	2.0
Leucine	0.8	Glycine	0.6
Valine	0.7	Hydroxyproline	0.1
		Proline	0.5
Total	3.5	Serine	0.2
		Tyrosine	1.0
D-isomers readily invertible [2]			
Arginine [3]	0.2	Total	6.2
Histidine [3]	0.4		
Methionine	0.6		
Phenylalanine	1.0		
Tryptophan	0.2		
Total	2.4		

[1] In testing the possible antagonism of the D-isomers of this group toward growth induction by a readily invertible D-amino acid, the poorly invertible amino acid was fed in the DL-form at twice the L-level indicated. Since DL-isoleucine was not commercially available, D-alloisoleucine + L-isoleucine was substituted. Of the 4 isomers of isoleucine, only L-isoleucine is available for growth.

[2] When the respective D- and L-forms of histidine, methionine, phenylalanine, or tryptophan were tested comparatively in this study each was incorporated in the diet at one-half of the level indicated; in all other tests the L-forms were fed at the levels shown.

[3] These figures represent the free lysine, arginine, and histidine present. The lysine and arginine were fed as the monohydrochloride and the histidine was fed as the monohydrochloride monohydrate. The HCl was neutralized by adding an equivalent amount of $NaHCO_3$ to the diet.

lose,[5] 2.0; salt mixture (10), 4.0; corn oil, 2.0; vitamin A and D concentrate,[6] 0.08; inositol, 0.1; choline chloride, 0.2; liver concentrate,[7] 0.4; and dextrin to make 100 g. To each kilogram of diet were added thiamine·HCl, 5.0; riboflavin, 10.0; pyridoxine·HCl, 5.0; nicotinic acid, 5.0; calcium D-pantothenate, 25.0; *p*-aminobenzoic acid, 300.0; α-tocopheryl acetate, 25.0; and 2-methyl-1,4-naphthoquinone, 2.0 mg; and biotin, 100; folic acid, 100; and vitamin B_{12} 15 μg.

The weanling male albino rats employed were of the Sprague-Dawley strain, weighing 40 to 50 g each. They were housed in individual cages in an air conditioned room maintained at 25 to 26°C. They were given food and water ad libitum. Body weights and food consumption were recorded every 4 days. To permit comparison with previous studies, the experimental period was set at 28 days, although many of the animals were maintained with the diets longer than this.

RESULTS AND DISCUSSION

Figure 1 presents the data obtained in the comparative tests with 0.2% of D- and 0.4% and 0.2% of L-histidine. In the first 5 experiments, responses to diets that contained all of the other essential amino acids in the L-form were compared with responses to diets that contained twice as much of each of the amino acids of the poorly invertible essential group, but in the DL-form. This amounted essentially to adding the D-forms of these amino acids in quantities equal to those of the L-forms already present. The data for each of the last 5 experiments show the response observed when a single L-amino acid of the poorly invertible group was replaced by twice as much of the DL-form. The designation DL-Ileu actually refers to D-alloisoleucine + L-isoleucine, the only D- and L-mixture of isoleucine isomers available on the market.

The data for experiments 1–5 agree reasonably well with the analogous tests made by Wachter and Berg (4). The depression of the response to 0.2% of L-histidine noted in experiment 4 was somewhat greater than had been noted previously, but the comparisons of the responses to L- and D-histidine were similar and equally striking (cf. exps. 4 and 5). Under the conditions employed in experiment 5, growth becomes rather erratic. Duplication of these conditions in experiment 16 promoted a small gain in weight (fig. 2) instead of the slight loss observed here.

Figure 1 shows further that when only one of the poorly invertible amino acids was fed in the DL-form, the effect upon the response to 0.2% of D-histidine was not nearly as striking as when the DL-forms of all were fed. Arranged in descending order of retardation, lysine > threonine > leucine > valine > isoleucine. The evidence does not appear to warrant the assumption that any one of the poorly invertible D-amino acids could be regarded as a unique antagonist toward the utilization of D-histidine. It appears rather that the poorly invertible D-amino acids collectively retarded the growth induced by the 0.2% D-histidine diet (cf. exp. 5). They presumably did this by competing with D-histidine for the D-amino acid oxidase or for some other enzyme involved in the inversion process.

In a series of tests based on oxygen uptake with a crude exract of acetone-dried sheep kidney, Bender and Krebs (11) observed that valine, isoleucine, and leucine reacted more rapidly than histidine, and histidine more rapidly than threonine and lysine. It is possible that both differences in rates of reaction and differences in dietary concentration may be involved. It is also possible that antagonism among the extraneous D-isomers of the poorly invertible group could affect their collective effect toward the utilization of D-histidine.

Figure 2 presents comparisons of the growth data obtained by feeding singly half-optimal levels of each of the L- and D-forms of the readily invertible group of essential amino acids, except arginine, in diets which contained the DL-forms of the less readily invertible group at twice the L-level. Under these conditions, retardation in varying degrees was noted with all

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

[6] Oleum percomorphum, Mead Johnson and Company, Evansville, Indiana. It contained 60,000 USP units of vitamin A and 8,500 USP units of vitamin D/g.

[7] Liver concentrate, N.F., was kindly provided by the Wilson Laboratories through the courtesy of Dr. S. W. Hier.

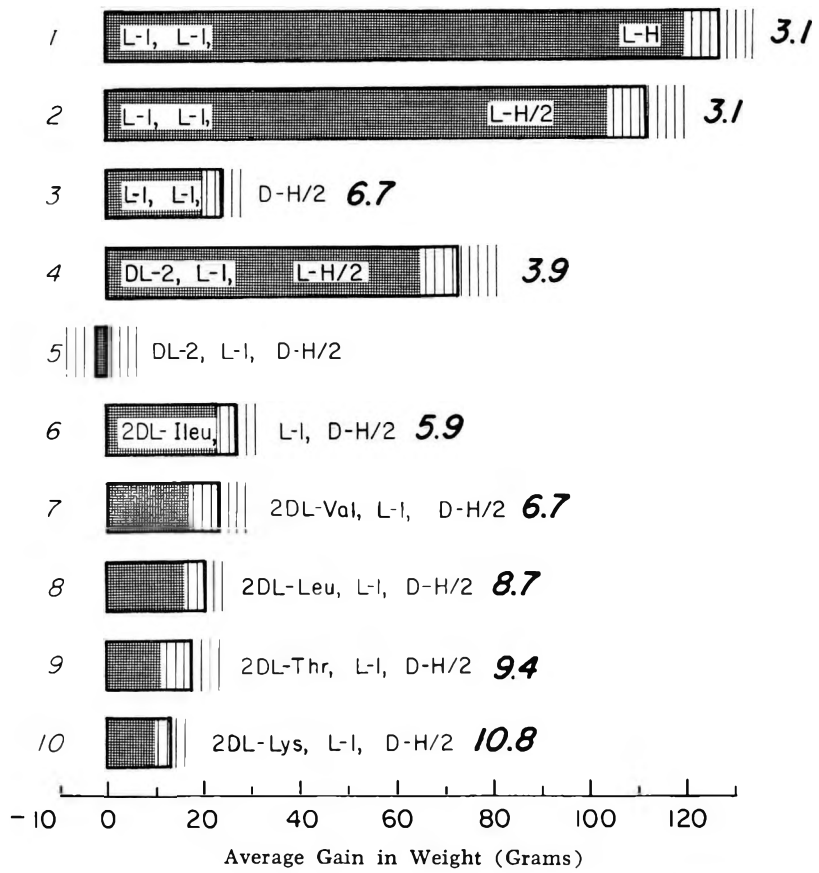

Fig. 1 The effect of poorly invertible D-amino acids upon the utilization of 0.2% of D-histidine.

Each bar represents the average growth of 4 male rats in 28 days. The standard error of the mean is indicated by the vertical shadings. The experiment number appears at the left of the bar and the grams of diet consumed per gram of gain in weight at the far right.

The composition of the basic amino acid mixture used in these studies is shown in table 1. Variations in this mixture are indicated by the symbols on or beside the bars.

The first letter and figure on the bar refer to the configuration and quantity of the group (or single member) of the essential amino acids whose D-forms are poorly invertible (see table 1). Thus, L-1 indicates only the L-forms in the quantities given in table 1; DL-2 indicates twice these levels in the DL-form. 2 DL-Ileu, 2 DL-Val, 2 DL-Leu, 2 DL-Thr, and 2 DL-Lys that only the specific amino acid designated was fed in the DL-form at twice the normal L-level, with all other members of the group in the L-form at normal levels.

The second letter and figure on the bar (L-1) refer to the group, less histidine, whose D-forms are readily invertible, in the quantities given in table 1. The histidine was fed in the L- or D-form, at the normal 0.4% or the one-half normal 0.2% level, designated as L-H, L-H/2 or D-H/2.

All of the diets also contained nonessential L-amino acids and glycine at the levels indicated in table 1.

The animals in experiment 5 showed an average loss of 2 g in weight and an average food consumption of 124 g in the 28 days.

of the readily invertible D-isomers. The growth depressions in the experiments with D-methionine and D-phenylalanine were essentially the same, the depression with D-tryptophan was much more marked, and the depression with D-histidine was the most srtiking. At half-optimal levels, the percentages of each of these amino acids in the diet differed (phenylalanine, 0.5; methionine, 0.3; histidine, 0.2; and tryptophan,

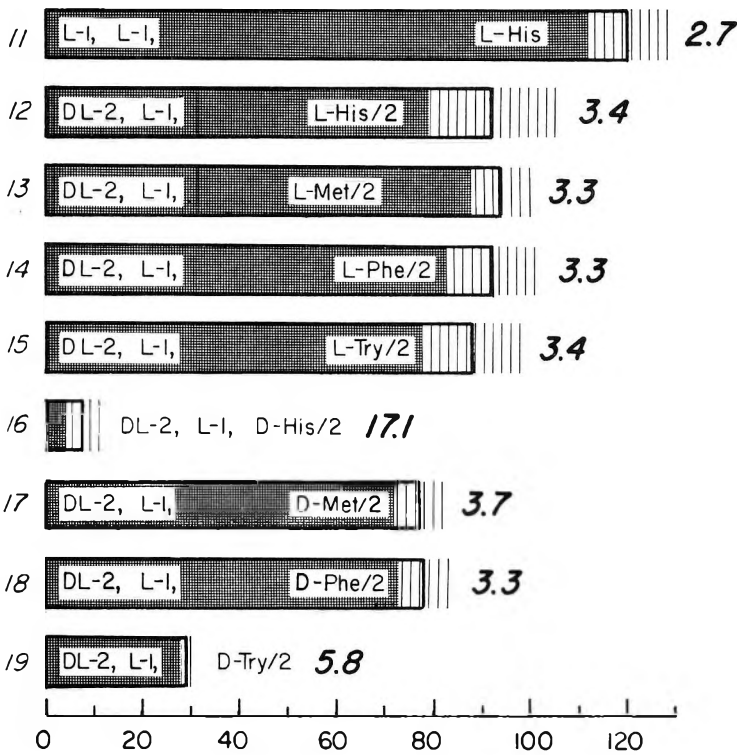

Fig. 2 The effect of poorly invertible D-amino acids upon the utilization of half-normal levels of methionine, phenylalanine, and tryptophan.

For aid in interpreting this figure, see the footnotes to figure 1.

In experiment 11, no variations were made in the composition of the diet given in table 1. In all of the other experiments, the group of essential amino acids whose D-forms are poorly invertible were fed in the DL-form at twice the L-level. This is indicated by the DL-2.

In experiments 12–19, the L-1 indicates that all of the group of essential amino acids whose D-forms are readily invertible, except the one specifically indicated, were fed in the L-form at the level shown in table 1. In experiments 12–15, histidine, methionine, phenylalanine, or tryptophan was fed in the L-form at half the normal L-level noted in table 1; in experiments 16–19, analogous tests were made of the D-forms of each of these amino acids.

All of the diets also contained nonessential L-amino acids and glycine at the levels indicated in table 1.

0.1). It seems fair to assume that, in the presence of the 3.5% of poorly invertible D-amino acids, a readily invertible D-amino acid present in a smaller concentration (e.g., 0.1% of D-tryptophan) could not compete as effectively for the enzymes involved in inversion as could another present in a larger concentration (e.g., 0.5% of D-phenylalanine). Susceptibility to enzymatic attack is undoubtedly also involved. The data of Bender and Krebs (11) make it clear that D-amino acid oxidase attacks some D-amino acids much more readily than others. Thus, D-amino acid oxidase from sheep kidney oxidized D-methionine twice as rapidly as D-tryptophan, 3 times as rapidly as D-phenylalanine, and about 13 times as rapidly as D-histidine.

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Antagonism of the D-Forms of the Essential Amino Acids Toward the Promotion of Growth by D-Histidine[1]

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ABSTRACT Growth in rats fed diets containing normal levels of the L-amino acids plus glycine, is only moderately retarded by replacing the L-histidine with its D-isomer. Tests were made of the simultaneous replacement with double quantities of the DL-isomers of either the group of essential amino acids whose D-isomers are poorly invertible (lysine, threonine, isoleucine, leucine, and valine) or of the other members of the group whose D-isomers are readily invertible (methionine, tryptophan, phenylalanine, and arginine). Such replacement in either group markedly impaired the response to the D-histidine diet, but only slightly that to the L-histidine diet. Doubling the L-amino acids in either group induced less retardation in the D-histidine diets. D-Lysine and D-threonine were largely, but not completely, responsible for the markedly retarded growth produced in the D-histidine diets which contained the poorly invertible group of amino acids in the DL-form. Replacement by its D-isomer of L-methionine, L-phenylalanine, L-tryptophan, or L-arginine also retarded the response to the D-histidine diet. Differences in percentage of the D-amino acid fed in the diet and in its susceptibility to attack by D-amino acid oxidase were probably responsible for the differences in degree of retardation noted.

Early work from this laboratory showed that D-histidine was utilized by the young rat for growth, although at a rate which was inferior to that noted with L-histidine (1). The L-histidine needed for the synthesis of new tissue is produced from the D-histidine by inversion (2). The process probably involves the initial production of the corresponding α-keto acid by oxidative deamination, followed by transamination to yield L-histidine (3).

In the presence of other D-amino acids, the growth response to D-histidine is markedly impaired. This has been shown primarily by tests in which the D-forms of the poorly invertible group of amino acids (lysine, threonine, isoleucine, leucine, and valine) have been employed (4). The impairment presumably occurs because this group of extraneous D-amino acids interferes with the process involved in the inversion of D-histidine.

The preceding paper (5) has shown that the interference by the poorly invertible group of D-amino acids could not be ascribed specifically to any single member of the group, some of which were, however, more effective than others, but was attributable, rather, to the collective effect of the several extraneous D-forms. The preceding paper also indicated that impairment of inversion by the poorly invertible D-amino acids could be demonstrated, not only with D-histidine, but in varying degree also with others of the readily invertible group of D-amino acids (D-methionine, D-phenylalanine, and D-tryptophan).

The present communication records the results of 3 series of tests, in all of which D-histidine was employed as the indicator at a level optimal for L-histidine. The first of these was designed to determine whether the retardation, particularly by D-lysine and D-threonine, could have been the result of imbalance, rather than of competition of the D-isomers for the enzymes involved in the inversion process. The data appear to indicate that imbalance could possibly have been a factor, but that competitive inhibition by the D-isomers was also involved.

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[1] This study was supported by U.S. Public Health Service Research Grant AM-03141, from the National Institute of Arthritis and Metabolic Diseases. A preliminary report was presented April 15, 1962 before the American Institute of Nutrition in Atlantic City. Kamath, S. H., and C. P. Berg 1962 Antagonism among the D-amino acids toward growth. Federation Proc., 21: 7 (abstract).

In the second study, the effect of the *en masse* addition to the diet of the other readily invertible D-amino acids (methionine, phenylalanine, tryptophan, and arginine) was tried. The inhibition of the growth induced when the diet contained D-histidine was at least as striking as the retardation effected by the poorly invertible group of D-amino acids.

The third series of tests involved comparisons of the growth inhibition produced when the other D-amino acids of the readily invertible group were substituted individually and collectively for their corresponding L-isomers in the amino acid mixture which contained D-histidine. The retardation which occurred with each in varying degree, was exceeded by their employment *en masse*.

EXPERIMENTAL

The conditions and procedures employed in these 3 series of studies were the same as those presented in the experimental section of the preceding paper (5). The composition of the reference L-amino acid mixture was also the same. However, the histidine was fed throughout at the 0.4% level. The dietary modifications are described in detail in the footnotes to the figures presented in the succeeding section. The sources and criteria of purity of most of the DL- and L-amino acids fed are indicated elsewhere (5). The DL-phenylalanine and DL-tryptophan, neither previously employed, were recrystallized from lots provided by a commercial source.[2] The DL-arginine was prepared by racemizing L-arginine and the D-arginine by resolving the racemized product.[3] Both were fed as the monohydrochlorides. Their N content and the specific rotation of the D-arginine monohydrochloride compared well with the theoretical values. The purity of all of the amino acids employed conformed closely with the specifications of the National Academy of Science (6).

RESULTS AND DISCUSSION

D-Histidine was chosen as the only unvaried readily invertible D-amino acid in these series of tests because it had repeatedly been shown to promote growth less well than its L-isomer, hence might be expected to be a relatively sensitive indicator of any antagonistic effects of dietary variations toward inversion and growth. In the preceding paper, histidine and the other readily invertible D-amino acids tested were fed in quantities equalling one-half of the normal L-levels. This was done primarily to increase their sensitivities to the antagonism of other D-amino acids. Wretlind had previously noted that when 1% of D-methionine was fed in a diet which contained all other essential amino acids in the DL-form, it produced essentially as rapid growth as 1% of L-methionine. When the methionine content was dropped to 0.25%, however, the response with D-methionine was considerably less than with L-methionine (6).

The results obtained in the first series of tests are presented in figure 1. They provide an extension of the data previously reported with 0.2% of D-histidine, in which none of the poorly invertible D-amino acids tested appeared to be primarily responsible for the antagonistic effect of the group (5). Experiment 4 shows that DL-lysine and DL-threonine together retarded the growth production by D-histidine (exp. 2) to a considerably greater extent than did DL-isoleucine,[4] DL-leucine, and DL-valine (exp. 10). However, the retardation was in neither instance as great as that produced when all 5 of these amino acids were fed in the DL-form (exp. 6). In these tests the L-form of each of the amino acids tested was replaced by twice its weight of the DL-mixture. This amounts essentially to adding an equal amount of the extraneous D-amino acid to the diet. When the L-forms of lysine and threonine were doubled, as in experiments 7 and 8, the growth retardation observed was not limited primarily to the diet which contained D-histidine, but was noted also in the tests with the diet which contained L-histidine. Hence, this retardation may have been induced primarily by creating an imbalance among the L-components of

[2] We are happy to acknowledge our indebtedness to the Dow Chemical Company for these and the several other lots of amino acids provided through the courtesy of Drs. H. C. White and R. P. Perkins.

[3] We are indebted to Miss Chien-chyou Wang and Mr. Don Shi-jseng Lin who prepared these products by procedures not yet published.

[4] The DL-isoleucine, so-called only for convenience in designation on the figures, is not currently available commercially. The product used was the D-alloisoleucine + L-isoleucine mixture. L-Isoleucine is the only one of the 4 isomers utilizable for growth.

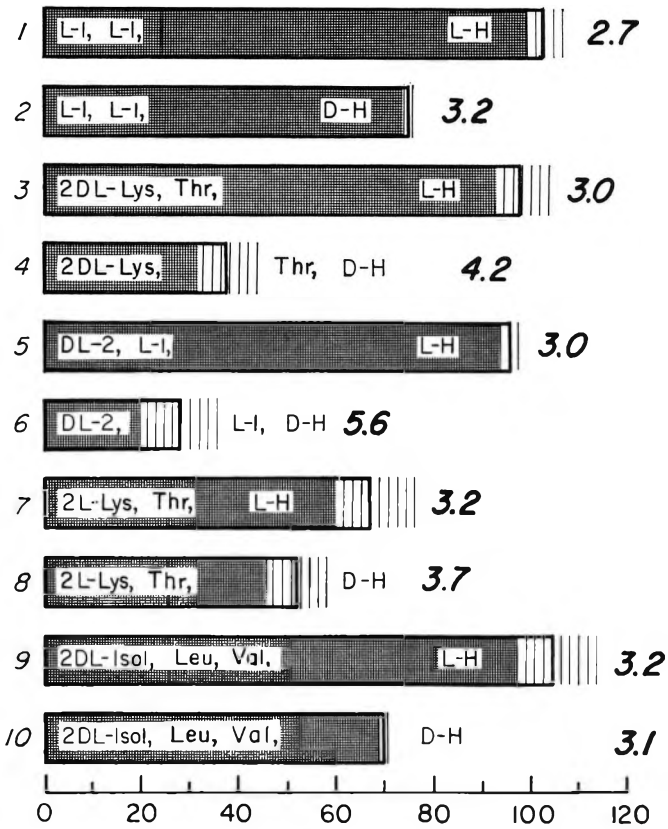

Fig. 1 Effect of D-lysine and D-threonine and extra L-lysine and L-threonine on the utilization of 0.4% of D-histidine.

Each bar represents the average growth of 4 male rats in 28 days. The vertical shadings indicate the standard error of the mean. The number at the left of the bar is the experiment number and the number to the right gives the grams of diet consumed per gram of weight gained.

The amino acid mixture used in experiment 1 contained normal levels of the essential and nonessential L-amino acids and glycine. The initial letter on each bar refers to the poorly invertible group of the essential amino acids. L-1 indicates normal levels of the L-forms; DL-2, twice as much in the DL-form; 2 DL-Lys, Thr, only lysine and threonine in the DL-form at twice the L-levels, the isoleucine, leucine and valine in the L-form at normal levels; 2 L-Lys, Thr, twice the L level of lysine and threonine, with normal levels of the L-forms of the other members of this group, and 2 DL-Isol, Leu, Val, twice as much D-alloisoleucine + L-isoleucine, DL-leucine, and DL-valine, with normal levels of L-lysine and L-threonine.

In experiments 1–10 all of the readily invertible amino acids, except histidine, were fed in the L-form at normal levels. This is indicated by the second letter (L-1) only on bars 1, 2, 5, and 6. L-H indicates 0.4% of L-histidine and D-H, 0.4% of D-histidine.

All of the diets contained, in addition, normal levels of the nonessential L-amino acids plus glycine.

the diet, as opposed to an antagonism toward inversion of the D-histidine.

Figure 2 shows that extraneous D-amino acids of the readily invertible group, like those of the poorly invertible group, impair the utilization of D-histidine for growth (compare experiments 17 and 18 with experiments 13 and 14, and also with experiments 2 and 6 in fig. 1). The retardation induced in the L-histidine series was minor (compare experiment 11 with experiments 13 and 17), but in the D-histidine series it

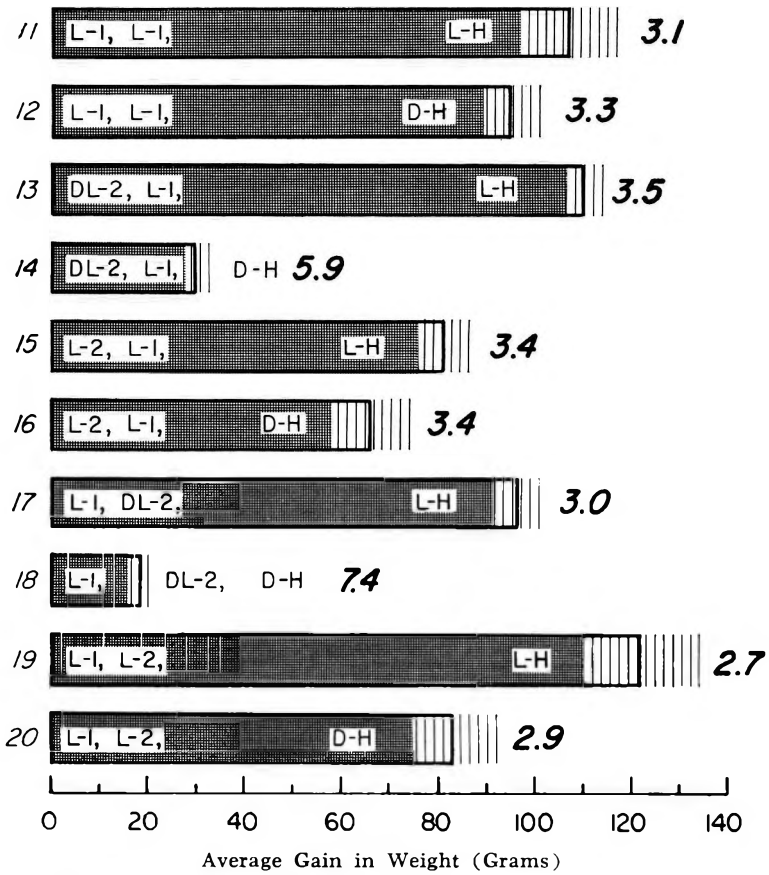

Fig. 2 Effect of extraneous D- or extra L-amino acids of the poorly invertible and readily invertible groups of essential amino acids upon the growth promotion of 0.4% of D-histidine. See footnote to figure 1 for general information.

The first letter on bars 11–20 refers to the poorly invertible group of essential amino acids, the second letter to the readily invertible group minus histidine, and L-H or D-H to 0.4% of L- or D-histidine. L-1 indicates normal L-amino acid levels, L-2 twice normal levels, and DL-2 the use of DL-amino acids at twice the normal L-level.

In addition, all of the diets also contained normal levels of the nonessential L-amino acids plus glycine.

was marked (compare experiment 12 with experiments 14 and 18). Doubling the L-amino acids of the poorly invertible group definitely retarded the response, both to L-histidine and to D-histidine (compare experiments 11 and 12 with 15 and 16). Doubling the L-amino acids of the readily invertible group actually accelerated somewhat the response to the L-histidine diet (compare experiments 11 and 19), but not to the diet which contained D-histidine (compare experiments 12 and 20). It therefore seems fair to assume that the inhibitory effect of the extraneous D-amino acids results from their competition for the enzymes needed to effect the inversion of the D-histidine. Such competition for D-amino acid oxidase could be expected to retard the oxidative deamination of D-histidine to the point where too little L-histidine could be produced from it to support even moderate growth.

Figure 3 presents the results obtained in tests in which one or all of the D-forms of the readily invertible group of essential amino acids was substituted for the L-forms fed in the control diets that contained L- or D-histidine. Experiments 23, 25, 27, and

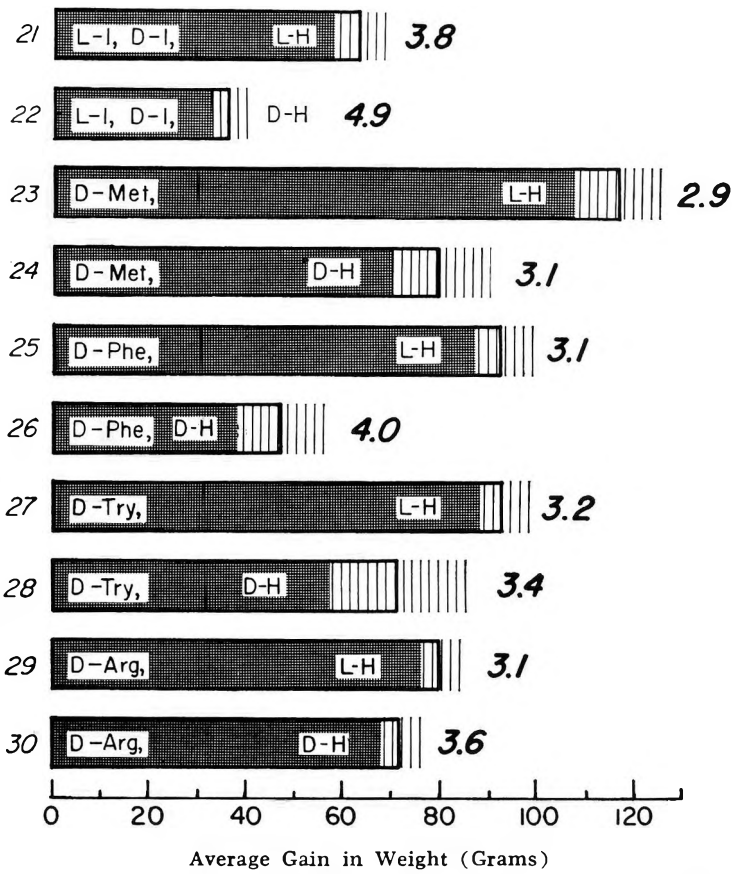

Fig. 3 Effect of the readily invertible D-amino acids on the utilization of 0.4% of D-histidine.

See footnote to figure 1 for general information.

The first letter (L-I) on bars 21 and 22 refers to use of the poorly invertible group of amino acids in the L-form at normal levels; the second letter (D-I) to the use of only the readily invertible group of D-amino acids at the normal level employed for the L-forms; and L-H and D-H to 0.4% of L- or D-histidine. These and the diets which follow all contained also normal levels of the nonessential L-amino acids, plus glycine.

In bars 23–30 only the varied components of the diet are indicated. The readily invertible D-amino acid indicated was substiuted quantitatively for the L-form otherwise fed. The histidine fed was 0.4% of L- or 0.4% of D-histidine (L-H or D-H). All of the other essential amino acids were fed in the L-form at normal levels. The diets also contained normal levels of the nonessential L-amino acids plus glycine.

29 show quite clearly that any one of the D-forms alone in a diet containing all of the other amino acids in the L-form promotes a greater growth response than when the diet also contains D-histidine instead of L-histidine (exps. 24, 26, 28, and 30). As might be expected, the antagonistic effects of these several D-amino acids together (exp. 22) exceed the effect of any one alone. The summation of the mutally antagonistic effects exerted by the 4 D-amino acids (methionine, phenylalanine, tryptophan, and arginine) in an L-histidine diet is shown in experiment 21. Again, it exceeds the effect of any one alone.

It is difficult to draw close comparisons between results obtained in studies of this type. The responses observed in experiments 12 and 11 show a more favorable comparison than usual between D- and L-histidine. The comparison noted between experiments 2 and 1 is more nearly typical.

The data in figure 3 (exps. 23, 25, 27, and 29) show the production of little, if any decrease in growth with D-methionine, only a moderate decrease with D-phenylalanine or D-tryptophan, but a sizable decrease with D-arginine. The position of D-arginine is complicated by the dual role which its L-isomer must play, both as a protein unit and as a component of the urea cycle. These and other factors involved in the relative degrees of utilization of the D-forms of the various essential amino acids have been discussed elsewhere at some length (3). Suffice it to say that the data in this study appear to indicate that the extent to which the various readily invertible D-amino acids of the essential group interfere with the utilization of D-histidine (exps. 24, 26, 28, and 30) depends both upon the concentration of the D-amino acid and upon the ease with which inversion makes it useful for growth. Thus, D-methionine was most readily invertible alone, but the presence of 0.6% of it in the diet which also contained D-histidine produced nearly the same degree of retardation as was produced by 0.2% of dietary D-tryptophan and 0.2% of dietary D-arginine, both of which are less readily invertible. D-Phenylalanine was apparently as readily invertible as D-tryptophan but probably retarded the response to D-histidine more strongly because it was present in a concentration of 1.0% of the diet.

It should not be assumed that antagonism among the D-amino acids is in every instance necessarily nonspecific. There is good evidence that the very slow growth which can be obtained when D-valine is incorporated in a diet that contains all other amino acids in the L-form (7, 8) can be completely inhibited by introducing D-leucine (8,9) into the diet. D-Isoleucine is relatively ineffective (9). The data in the present studies show plainly that the extraneous D-amino acids of the poorly invertible group vary markedly in their capacities to retard the growth response induced by D-histidine. The same is true of the provision singly of the readily invertible D-amino acids in lieu of their L-counterparts. In no instance was any single D-amino acid (nor were two or three) completely responsible for the growth inhibition of the group.

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Influence of Various Chelating Agents on the Availability of Zinc[1]

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ABSTRACT Chelates with stability constants for zinc ranging from 5.3 to 18.8 were tested for growth-promoting activity with turkey poults in a zinc-deficient diet containing isolated soybean protein. The chelates were fed at a level of 0.684 mmole/kg diet for about 20 days. EDDADP, HEDTA and EDTA were most active in improving growth under these conditions. NTA and EDDA were moderately effective in promoting growth while DHEG, IDA, HIEDA, EDDHA, EBONTA, TETA, DTPA and CDTA had only slight or no effect on the growth of poults. A stability constant for zinc between 13 and 17 was most satisfactory.

The improvement of the availability of zinc by the addition of the disodium salt of ethylenediaminetetraacetic acid (EDTA) from diets that contained isolated soybean protein has been established for turkey poults by Kratzer et al. (1) and for chickens by Davis et al. (2) and Scott and Zeigler (3). Kratzer and Starcher (4) reported that 100 mg/kg of the disodium salt of EDTA gave growth in turkey poults equivalent to approximately 8 mg/kg of zinc when added to a zinc-deficient, purified diet containing isolated soybean protein.

A number of synthetic chelating agents, besides EDTA, are known to improve the availability of zinc for plants, as reviewed by Wallace.[2] Since similar information was lacking for poultry, the present study was undertaken to compare a large number of chelating agents to EDTA in improving the availability of zinc for turkey poults.

EXPERIMENTAL

The Broad Breasted Bronze turkey poults used in this investigation were fed either a practical poult starter diet or a zinc-deficient, purified diet for 5 days and then were divided into groups of approximately the same weight. These groups, each of which contained 10 poults, were housed in electrically heated battery cages which had been coated with an epoxy resin. They were fed the experimental diets for about 20 days and were weighed 2 times each week. The composition of the zinc-deficient diet is shown in table 1. These diets are estimated to contain 25.5 mg/kg zinc.

TABLE 1
Zinc-deficient basal diet

	g/kg
Cornstarch	463.4
Isolated soybean protein [1]	330.0
Cellulose, powdered [2]	50
$CaHPO_4$	30
$CaCO_3$	25
Mineral mixture [3]	23.6
Vitamin premix [4]	20
Erythromycin [5]	1
Soybean oil	35
DL-Methionine	4.5
Choline chloride [6]	10
Vitamin A, dry (10,000 IU/g)	0.5
Vitamin D_3, dry (1,500 IU/g)	3
Vitamin E, dry (44 IU/g)	2
Inositol	1
Butylated hydroxytoluene	1

[1] ADM C-1 Assay Protein, Archer-Daniels-Midland, Minneapolis.
[2] Solka Floc, Brown Company, New Hampshire.
[3] Supplied following minerals: (in mg/kg of diet) NaCl (uniodized), 10.0; $MnSO_4 \cdot H_2O$, 0.3; $FeSO_4 \cdot 7H_2O$, 0.65; $CuSO_4 \cdot 5H_2O$, 0.08; cobalt acetate tetrahydrate, 0.02; KI, 0.009; $Al_2(SO_4)_3 \cdot 18H_2O$, 0.25; $MgSO_4 \cdot 7H_2O$, 4.0; KCl, 3.0; K_2HPO_4, 5.0; Na_2MoO_4, 0.009.
[4] Premix supplied the following vitamins: (in mg/kg diet) riboflavin, 10; thiamine·HCl, 10; pyridoxine·HCl, 10; Ca pantothenate, 30; niacin, 120; folic acid, 5; menadione, 10; biotin, 0.4; and vitamin B_{12}, 10 μg.
[5] Gallomycin, Abbott Laboratories, North Chicago.
[6] Twenty-five per cent in wheat middling carrier.

Each trial contained a group fed the basal diet and groups fed other diets supplemented with 15 mg of zinc/kg or 200 mg (0.684 mmole) EDTA (free acid form)/kg, or other chelating agents at levels equimolar with EDTA. The quantities of chelating agents were small enough to be

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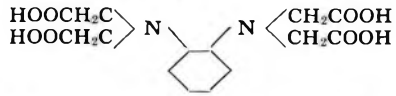
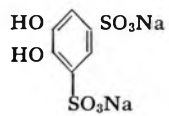
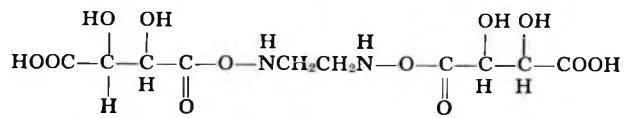
[1] Supported in part by grant number A-5334 from the National Institutes of Health, U.S. Public Health Service.
[2] Wallace, A. 1962 A decade of synthetic chelating agents in inorganic plant nutrition (Arthur Wallace, 2278 Parnell Avenue, Los Angeles 64).

TABLE 2

The chelating agents, their structures, abbreviated names, molecular weights, and stability constants for zinc

No.	Chelating agent	Structure	Molecular weight	Abbreviation	Stability constant for zinc
1	1-Amino-2-naphthol-4-sulfonic acid [1]	$NH_2C_{10}H_5(OH)SO_3H$	239	ANS	
2	1,2-Diaminocyclohexanetetraacetic acid [2]		340	CDTA	18.67
3	Dihydroxyethylethylenediamidiacetic acid [3]	HOH_2CH_2C / $HOOCH_2C$ > N—CH_2—CH_2—N < CH_2CH_2OH / CH_2COOH	264	DHEEDA	
4	Diethylenetriaminepentaacetic acid [5]	$(HOOCCH_2)_2NCH_2CH_2$—N(—CH_2COOH)—$CH_2CH_2N(CH_2COOH)_2$	393	DTPA	18.14
5	4,5-Dihydroxy-*m*-benezenedisulfonic acid disodium salt [1]		314	DHBDS	10.41
6	N-N-Di(β-hydroxyethyl) glycine (sodium salt) [5]	$(HOH_2CH_2C)_2NCH_2COONa$	185	DHEG	5.36
7	[Ethylenebis(oxyethylenenitrilo)] tetraacetic acid [1]	$(HOOCH_2C)_2$—N—CH_2CH_2—O—CH_2—CH_2—O—$(HOOCH_2C)_2NH_2CH_2C$	380	EBONTA	11.0
8	Ethylenediaminebitartrate [2]		324	EDBT	
9	Ethylenediamine-N,N′-diacetic acid [2]	$HOOCH_2C$—N(H)—CH_2CH_2—N(H)—CH_2COOH	176	EDDA	11.1
10	Ethylenediamine-N,N′-diacetic acid-N,N′-dipropionic acid [6]	$HOOCH_2C$ / $HOOCH_2CH_2C$ > N—CH_2CH_2—N < CH_2COOH / CH_2CH_2COOH	318	EDDADP	14.5

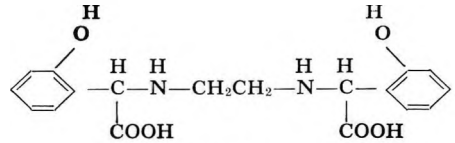
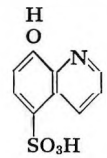
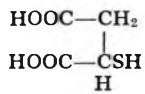
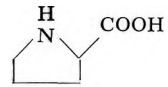
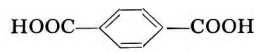
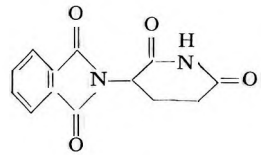
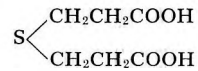
11	Ethylenediamine (di(O-hydroxy-phenylacetic acid))		361	EDDHA	9.26
12	Ethylenediaminetetraacetic acid [1]	$(HOOCH_2C)_2NCH_2CH_2N(CH_2COOH)_2$	292	EDTA	16.5
13	Ethylenedinitrilotetraethanol [1]	$(HOH_2CH_2C)_2NCH_2CH_2N(CH_2CH_2OH)_2$	236	EDTE	
14	Ethylenediaminetetrapropionic acid (tetrasodium salt)	$(NaOOCH_2CH_2C)_2NCH_2CH_2N(CH_2CH_2COONa)_2$	436	EDTP	7.8
15	Glutamic acid	$HOOCH_2CH_2C{-}CH(NH_2){-}COOH$	147	GA	5.45
16	(2-Hydroxyethylimino)diacetic acid [1]	$HOH_2CH_2C{-}N(CH_2COOH)_2$	177	HEIDA	8.57
17	Hydroxyethylethylenediaminetriacetic acid [1]	$(HOH_2CH_2C)(HOOCH_2C)N{-}CH_2CH_2N(CH_2COOH)_2$	278	HEDTA	14.5
18	2-Hydroxypropylenediaminetetraacetic acid [6]	$(HOOCH_2C)_2N{-}CH_2CH(OH)CH_2N(CH_2COOH)_2$	322	HPDTA	
19	8-Hydroxy-5-quinoline-sulfonic acid [1]		225	HQS	8.72
20	Iminodiacetic acid disodium salt monohydrate [1]	$HN(CH_2COONa)_2 \cdot H_2O$	195	IDA	7.02
21	Mercaptosuccinic acid [1]		150	MSA	
22	Nitriotriacetic acid disodium salt	$HOOCH_2C{-}N(CH_2COONa)_2$	235	NTA	10.45
23	Propylenediaminetetraacetic acid tetra sodium salt	$(NaOOCCH_2)_2NCH_2CH_2CH_2N(CH_2COONa)_2$	394	PDTA	

TABLE 2 (*Continued*)

The chelating agents, their structures, abbreviated names, molecular weights, and stability constants for zinc

No.	Chelating agent	Structure	Molecular weight	Abbreviation	Stability constant for zinc
24	Proline		115	P	10.2
25	Terephthalic acid		166	TP	
26	Thalidomide		258	TA	
27	Thiodipropionic acid [7]		178	TPA	
28	Triethylenetetraamine		146	TETA	12.1

[1] Eastman Organic Chemicals, Rochester, New York.
[2] K and K Laboratory, Inc., Jamaica 33, New York.
[3] Courtesy of Dr. A. Wallace, UCLA, Los Angeles.
[4] Courtesy of Geigy Chemical Corporation, Ardsley, New York.
[5] Courtesy of Dow Chemical Company, Midland, Michigan.
[6] Aldrich Chemical Company, Inc., Milwaukee 10, Wisconsin.
[7] Courtesy of American Cyanamid Company, New York.
[8] Laboratory synthesis carried out in the investigators' laboratory.

added directly to the diet without making any adjustments in composition.

The list of chelating compounds[3] tested is shown in table 2 along with their abbreviated names, structural formulae, molecular weights and stability constants for zinc if available from the literature. The method of preparation of some of the compounds was as follows:

Propylenediaminetetraacetic acid (PDTA)

This compound was prepared according to the procedure of Schwarzenbach and Ackerman (5). The free acid formed a supersaturated solution but the tetrasodium salt crystallized from dilute (80%) ethanol.

Ethylenediaminetetrapropionic acid (EDTP)

The tetrasodium salt of this acid was prepared by the procedure of Martel and Chaberek (6).

Ethylenediamine-N,N′-diacetic acid-N,N′-dipropionic acid (EDDADP)

About 5 g ethylenediamine-N,N′-diacetic acid (28 mmole) were neutralized with 56 mmole of NaOH in 30 ml of water and 8.9 g (81.5 mmole) of chloropropionic acid which had been neutralized with NaOH in 20 ml of water were added by drops over a 10-minute period. The solution was refluxed for 2 hours and left overnight at room temperature. On the next day, the solution was adjusted to a pH between 2 and 3 with 50% H_2SO_4 when the crude product precipitated. It was filtered, triturated with ether, redissolved in NaOH and reprecipitated by adjusting the pH to 2 to 3; yield, about 3 g.

Calculation of relative gain. Two treatments were repeated in each experiment to serve as controls; one group was fed the zinc-deficient diet and the other group received this diet supplemented with 200 ppm EDTA. The test group received diets containing the other chelating agents. The relative gain was calculated as the gain of the experimental group less the gain of the basal group compared with the gain of the EDTA-fed group less the gain of the basal set equal to 100.

RESULTS AND DISCUSSION

The data of the trials as presented in tables 3 and 4 indicate that chelating agents such as aminonaphtholsulfonic acid (ANS), diaminocyclohexanetetraacetic acid (CDTA), dihydroxyethylglycine (DHEG), dihydroxybenzenedisulfonic acid (DHBDS), ethylenediaminetetrapropionic acid (EDTP), ethylenediaminetetraethanol (EDTE), glutamic acid (GA), ethylenediaminebitartrate (EDBT), iminodiacetic acid (IDA), hydroxyquinolinesulfonic acid (HQS), mercaptosuccinic acid (MSA), proline (P), thalidomide (TA), triethylenetetraamine (TETA), terephthalic acid (TP), and thiodipropionic acid (TPA) gave growth responses either poorer than the zinc-deficient basal diet or of the same order.

Compounds such as nitrilotriacetic acid (NTA), hydroxyethylethylenediaminetriacetic acid (HEDTA), propylenediaminetetraacetic acid (PDTA), hydroxypropylenediaminetetraacetic acid (HPDTA), dihydroxyethylethylenediaminediacetic acid (DHEEDA), ethylenediaminediacetic acid-dipropionic acid (EDDADP) and ethylenediaminediacetic acid (EDDA) gave definite improvement in the growth of the poults. The relative growth was of the same order as for EDTA for some of these compounds.

Definite but inferior growth than for EDTA was obtained for ethylenebis (oxyethylenenitrilo) tetraacetic acid (EBONTA), hydroxyethyliminodiacetic acid (HEIDA), and diethylenetriaminepentaacetic acid (DTPA). A high degree of mortality was observed for ethylediamine (dihydroxyphenyl) acetic acid (EDDHA) in a single experiment in which it was used and it is difficult to determine whether the response is meaningful.

The growth response was influenced by the diet which was fed to the poults for the initial 5 days before the start of the trials. The birds fed the zinc-deficent diet had a higher overall mortality and were somewhat lighter than the poults started with stock diet.

The relative growth values obtained by the feeding of some of the chelating agents were compared with their stability constants for zinc (fig. 1). The growth response appeared to be equally good over the range of stability constants 13 to 17

[3] We are grateful to Dr. A. Wallace, University of California, Los Angeles; Geigy Chemical Corporation, Ardsley, New York; Dow Chemical Company, Midland, Michigan; and American Cyanamid Company, New York for gifts of certain of the chelating agents.

TABLE 3

Comparison of the relative gain in weight and survival for poults fed a zinc-deficient diet supplemented with various chelating agents

Starting feed	Zn-deficient		Stock mash		Stock mash		Zn-deficient		Stock mash	
Trial duration	19 days		21 days		20 days		22 days		19 days	
Treatment	Relative growth	Survival	Relative growth	Survival	Relative growth	Survival	Relative growth	Survival	Relative growth	Survival
Basal	0	7	0	8	0	7	0	2	0	6
15 ppm Zn	96	10	85	10			79	10		
EDTA	100	10	100	10	100	10	100	10	100	10
EDBT	0	9	−15	10						
DTPA	8	10	45	10						
EDDA	42	10	87	10						
ANS	−3	10	6	9						
TA	6	9	6	10						
DHEG	−2	9	9	10						
CDTA	3	9	14	10						
HEDTA					106	9	—	—	92	9
HQS					5	9	3	3		
DHBDS					19	7	−4	2		
MSA					6	5	11	5		
TP					−8	8	−15	2		
EDTA-Na solution					100	10	—	—		
P					15	8	2	2		
Ca-EDTA-Na							65	10	80	10
GA					−3	5	2	10		

TABLE 4

Comparison of the relative gain in weight and survival for poults fed a zinc-deficient diet supplemented with various chelating agents

Starting feed	Stock mash		Stock mash		Zn-deficient		Stock mash		Stock mash	
Trial duration	21 days		22 days		21 days		19 days		20 days	
Treatment	Relative growth	Survival	Relative growth	Survival	Relative growth	Survival	Relative growth	Survival	Relative growth	Survival
Basal	0	10	0	8	0	4	0	6	0	8
15 ppm Zn	100	9	123	10	87	10			92	10
EDTA	100	10	100	10	100	10	100	10	100	9
NTA	70	10	66	10	—	—				
TETA	17	8	2	9	—	—				
EDTE	−2	9	−1	7	—	—				
HEDTA	150	10	121	10	159	10			101	9
EBONTA	20	10	32	9	—	—				
HEIDA	33	8	13	10						
IDA	12	8	8	7						
56 ppm Zn					193	10			115	9
56 ppm Zn + HEDTA					208	10			98	9
TPA					27	5	−8	9		
PDTA					82	9	58	10		
HPDTA					81	10	89	10		
EDDHA					20	3				
EDTP							−1	10	−2	8
DHEEDA							117	10	95	10
EDDADP							125	10	107	9

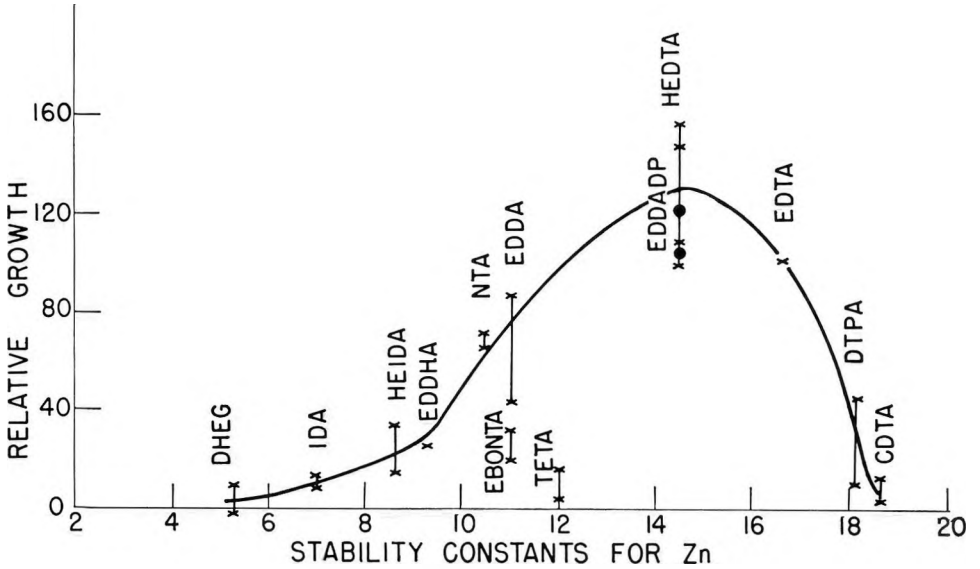


Fig. 1 Relation of stability constant for zinc of several chelating agents to their growth promoting effect in poults when added to a zinc-deficient diet.

with an optimal value at 14.5. The compounds EDTA, EDDADP, and HEDTA, have stability constants of 16.5, 14.5 and 14.5, respectively. It is to be understood that in an in vitro system the chelating agents used in the study are capable of complexing with a variety of metals with various affinities, which in turn depend upon the pH of the system. These interactions have been neglected in the present discussion.

The NTA with a stability constant of 10.45 gave a definite growth response. The reduction of one or more of its carboxyl groups (HEIDA, DHEG) reduced the stability constants and also the effectiveness for improving the availability of zinc. EDDA improved the availability of zinc more than NTA for poults. A modification of EDDA to EDTA by the addition of 2 more acetic acid molecules increased the stability constant for zinc from 11.1 to 16.5. The reduction of one of the acetic acids of EDTA to ethanol gave rise to HEDTA which has a stability constant value of 14.5 for zinc. Attachment of 2 molecules of propionic acid to EDDA gives rise to EDDADP with a stability constant of 14.5. These 2 different compounds with stability constants of 14.5 for zinc appeared to be most effective for increasing its availability in this study. Acetic acid molecules attached to ethylenediamine tend to give chelating agents with stability constants for zinc higher than compounds containing propionic acid. EDTP has a value of only 7.8 and was a poor chelating agent for zinc availability.

When all the acetic acid molecules of EDTA were reduced to ethanol, EDTE was obtained which did not improve availability of zinc. However, the compound containing 2 alcohols and 2 acetic acid molecules attached to ethylenediamine (DHEEDA) gave a relative growth response of the same order as EDTA. No data are available about its stability constant for zinc but it may be predicted that the value will be lower than 14.5 and probably near to 13.

Calcium-disodium EDTA was less effective than free EDTA or EDTA-disodium salt (table 3) in improving zinc availability. This may be due to failure of the zinc to displace the calcium in the EDTA complex in the gastrointestinal tract and to come to equilibrium as it would in an in vitro system.

Compounds like EBONTA and TETA were less effective for improving zinc availability than could be predicted on the basis of their stability constants. The exact mechanism for this discrepancy is not

known but may be attributed to a definite requirement for a certain size of the molecule, steric considerations or toxicity of these compounds. This is being investigated further.

One mechanism by which a chelating agent might improve mineral availability depends upon the chelating agent having a stronger stability constant for the metal than the metal binding substance in the feed so that the metal is complexed with the chelating agent in the gastrointestinal tract. The metal chelate can then be absorbed,[4] if it is a relatively small molecule. After absorption the metal might be available for specific body functions if it can be removed from the chelating agent. This means that the various systems in which the metal is required for proper functioning (e.g., enzymes) should have higher stability constants for the metal than the chelating agent with which it is absorbed. To satisfy the requirements of this mechanism, a chelating agent, to be effective in improving metal availability, would need to have a stronger stability constant than the binding agent in the feed and a weaker stability constant than the tissue system.

CDTA and DTPA with stability constants of more than 18 for zinc also were not very suitable for improving the availability of zinc. Our data lead us to speculate that chelating agents with stability constants below 13 may not be able to release zinc effectively from its "bound" form from the diets containing isolated soybean protein. The stability constant of the chelating factor in this protein must be higher than 13 but lower than 14.5. Once this zinc has been released from the dietary factor, another chelating substance may be involved in the living organism with a stability constant lower than 16.5 but higher than 14.5 which transports zinc to the various tissues where it is needed. EDTA may be interfering slightly in releasing its complexed zinc to the "transporting" chelating system of the poult. This appears to be definitely the case for CDTA and DTPA, which complex zinc so strongly that its availability to the tissue transport system is of a low order.

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Reproductive Performance of Rats Receiving Various Levels of Dietary Protein and Fat[1]

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ABSTRACT Female rats fed practical diets containing 18 or 25% protein produced and weaned a few more young than those fed 30 or 35%. Females fed purified diets containing 14, 17 and 20% soybean protein in different periods of the reproductive cycle produced as many young as the control group which consumed 25% soybean protein. With 10% protein throughout the cycle, the number of young and weaning weight were reduced to less than those of the controls. With 10% protein during mating and gestation period, the number weaned and their weight was essentially equal to that of the control group. With 14% protein during 2 or more periods, the weight was less than that of the young in the control group. The number of young weaned by females fed a practical diet containing 3 to 18% fat was essentially the same but the number weaned was less with 25% fat. With a purified diet reproduction by females consuming 3 or 20% fat was superior to those fed 36 and 50%. When the protein-metabolizable energy was adjusted to a constant ratio (0.074 to 0.070) the effect of a high fat diet on the number of young weaned and the weaning weight was very small.

During the past decade, several laboratories (1–5) have determined qualitatively the nutrients required for satisfactory reproduction and lactation in rats. Comparatively few investigations have been carried out to determine quantitatively the amount of dietary protein or fat required for female rats to produce and wean the maximal number of young when the same diets are fed for several generations.

Cessation of estrus or long and irregular cycles occurred (6) with diets containing 3.5 to 5.0% protein, and diets (7) containing 10% protein (8) were adequate for gestation but were not satisfactory for lactation. Diets containing different levels of amino acid mixtures (2, 3, 5) as the sole source of protein were not completely adequate for optimal reproduction and lactation. The effect of high and low calorie diets on reproductive performance has been investigated in a few species of animals. In swine, high calorie diets[2,3] appeared to produce early puberty and a high ovulation rate, whereas low calorie diets tended to increase the number of live embryos and the number of young born and weaned (9–11). In chickens, low calorie diets during the growing period increased the percentage of hatching eggs (12).

After satisfactory reproductive performance had been obtained in this laboratory with female rats fed a highly purified diet, studies were initiated to determine the effect of levels of dietary protein and dietary fat on reproductive performance in rats. The results are summarized in the present report.

EXPERIMENTAL

Animals from the Texas A&M colony were used in all tests and the care of animals and litters were essentially the same as that described previously (1).

Ten females and 2 males from the parent, F_1 and F_2 generations were used to determine the effect of levels of dietary protein and 10 females and 2 males from the parent and F_1 generations were used to determine the effect of levels of dietary fat on reproductive performance. Each female was given an opportunity to produce and wean 3 litters with the exception

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[1] Contribution from Texas Agricultural Experiment Station.

[2] Christian, R. E., and J. C. Nofziger 1952 Puberty and other reproductive phenomena in gilts as affected by plane of nutrition. J. Animal Sci., *11:* 789 (abstract).

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of one group that had the opportunity to produce and wean 4 litters. Litters with more than 10 young were reduced to 10 young at birth. The number of young shown in the "observed" column in the tables include these 10 young per litter and all those alive in other litters with a smaller number of young. The young were weaned and the total weight of the young in each litter was recorded at 21 days of age.

Diets. Two general diets were used. One contained sorghum grain and soybean meal and is referred to as a practical diet. The other contained purified ingredients and is referred to as a purified diet. The composition of typical diets is given in table 1.[4]

TABLE 1

Composition of typical diets

	Diets	
	Practical	Purified
	g	*g*
Sorghum grain (milo)	44.7	
Glucose monohydrate[1]		61.3
Soybean meal	48.7	
Soybean protein [2]		25.0
Wood pulp [3]		3.0
Corn oil [4]	3.0	5.5
Steamed bone meal	3.0	
NaCl	0.5	
Mineral mixture [5]		5.0
DL-Methionine	0.1	0.2
Vitamins [6]	+	+

[1] Cerelose, Corn Products Company, Argo, Illinois.
[2] ADM C-1 Assay Protein, analyzed 82.3 (N × 6.25) % protein, Archer-Daniels-Midland, Minneapolis.
[3] Solka-Floc BW 40, Brown Company, Berlin, New Hampshire.
[4] Mazola, Corn Products Company, Argo, Illinois.
[5] Reference (13).
[6] Vitamins supplied/100 g of diet were: (in IU) vitamin A, 3000; vitamin D, 400; and (in mg) menadione, 0.75; α-tocopherol, 3.0; thiamine·HCl, riboflavin and pyridoxine·HCl, 1.0 each; Ca pantothenate, 3.0; niacin, 2.0; folic acid, 0.2; vitamin B_{12}, 0.001; choline chloride, 200; and inositol, 10.

Dietary protein. The practical diet was fed throughout the experimental period. Protein content of the various diets was obtained by adjusting the proportion of soybean meal and sorghum grain. The purified diets containing soybean protein at 5 different levels in various combinations were fed at 4 different periods in the reproductive cycle. Various protein levels were obtained by substituting soybean protein for glucose monohydrate.[5]

The periods were growth (G) from weaning until the first mating; mating and gestation (MG) from mating to the birth of litter; lactation (L) from birth until the young were weaned at 21 days; and maintenance (M) from weaning of the litter to 7 days later when the female was returned to the stock cage for mating.

Dietary fat. To determine the effect of the level of dietary fat on reproductive performance the amounts of sorghum grain and soybean meal in the practical diet were adjusted so that the final diet with the various amounts of fat always supplied 25% protein. In one series with the purified diet, purified soybean protein was supplied at 25% regardless of the level of dietary fat. In the second series the amounts of soybean protein and glucose monohydrate were adjusted so that the soybean protein-metabolizable energy ratio of the diet remained essentially constant (0.074 to 0.070). As the fat was increased, it was necessary to add 10% wood pulp to some of the purified diets to maintain the desired metabolizable energy (14, 15) content. This increase in wood pulp did not change the reproductive performance. Corn oil [6] and lard were used as the source of added fat.

The amount of protein and fat that the various diets contained is shown in the tables along with the combined data for all generations used to determine the reproductive performance with a given diet.

RESULTS AND DISCUSSION

Dietary protein. The combined reproduction data for 3 generations of females that received 18, 25, 30 and 35% protein in a practical diet are summarized in table 2. A diet containing 18% protein was essentially equal to one containing 25% protein for reproduction. When the protein was increased to 30 or 35%, the number of young weaned was still high but the average total number both produced and weaned per female was less than when the diet contained 18 or 25% pro-

[4] The authors are indebted to Merck and Company, Rahway, New Jersey, for generous supplies of vitamins; to Lederle Laboratories, Division of American Cyanamid, Pearl River, New York, for folic acid; and to Dow Chemical Company, Freeport, Texas, for DL-methionine.
[5] Cerelose, Corn Products Company, Argo, Illinois.
[6] Mazola, Corn Products Company, Argo, Illinois.

TABLE 2

Summary of reproduction data of female rats fed different levels of protein in a practical diet [1]

Protein	Energy [2]	Litters		Young					
						Weaned [3]			
		No. born	No. weaned	No. born	No. observed	No.		Avg no./ litter	Avg wt
%	*kcal/100 g*						%		*g*
18	290	116	111	891	826	757	91.6	6.8	35.6
25	267	115	107	911	806	739	91.6	6.9	33.5
30	250	110	105	729	674	637	94.5	6.2	31.5
35	234	108	99	741	698	612	87.6	6.2	30.4

[1] This group of females had the opportunity to produce and wean 4, rather than 3, litters.
[2] Metabolizable energy references (14, 15).
[3] % Weaned = $\frac{\text{no. weaned}}{\text{no. observed}}$.

tein. The calculated metabolizable energy decreased from 290 to 234 kcal/100 g as the protein content of the diet increased from 18 to 35%. Since the energy values of these diets was relatively low, it is possible that the ratio of metabolizable energy to protein may account for the inferiority of the high level of dietary protein.

The combined reproduction data for 3 generations of females receiving different amounts of purified soybean protein in a purified diet during 4 separate periods in the reproduction cycle are summarized in table 3. Data for group 1, table 3, include the combined results of 2 different lots of females. One lot was composed of 3 generations which were tested concurrently with the females in groups 2 to 10 inclusive. The other lot was composed of 2 generations which were tested concurrently with the females in groups 11 to 15 inclusive. The reproductive performance of the females in the first lot was below that reported previously (1, 16) for a diet of the same composition. The reproductive performance of the 2 generations in the

TABLE 3

Summary of reproduction data of female rats fed a purified diet supplying different levels of soybean protein during various stages of the reproductive cycle

Group	Soybean protein				Litters		Young					
	Period [2]								Weaned [1]			
	G	MG	L	M	No. born	No. weaned	No. born	No. observed	No.		Avg no./ litter	Avg wt
	%	%	%	%						%		*g*
1	25	25	25	25	129	120	929	889	768	86.4	6.4	36.4
2	25	10	10	10	83	67	589	566	404	71.4	6.0	22.4
3	25	10	25	25	85	79	625	584	502	85.9	6.4	35.6
4	25	10	20	20	80	71	542	511	430	84.1	6.1	32.2
5	25	10	17	17	84	76	576	559	455	81.4	6.0	31.9
6	20	20	20	20	84	79	598	579	514	88.9	6.5	34.8
7	20	10	20	20	88	84	686	643	571	88.9	6.8	32.9
8	20	17	20	20	83	79	675	643	582	90.5	7.4	33.9
9	20	17	17	20	86	76	689	639	522	81.7	6.9	33.3
10	20	17	17	17	86	78	652	627	504	81.0	6.5	33.5
11	17	17	17	17	80	72	648	622	527	84.7	7.3	33.4
12	20	14	25	20	86	78	715	697	574	82.1	7.4	35.6
13	20	14	14	20	84	76	662	620	546	88.1	7.2	31.2
14	20	14	14	14	82	75	631	599	539	90.0	7.2	29.8
15	17	14	14	14	90	87	762	730	644	87.7	7.4	27.6

[1] % Weaned = $\frac{\text{no. weaned}}{\text{no. observed}}$.

[2] G indicates growth — from weaning until the first mating; MG indicates mating and gestation — from mating to birth of the litter; L indicates lactation — from birth until young were weaned at 21 days; and M indicates maintenance — from weaning of the litter to 7 days later.

second lot was essentially equal to that obtained in the earlier studies. There were no obvious changes in the experimental procedure to account for the relatively poor performance of the first lot of females in group 1.

Some variation in reproductive performance occurred in the various groups, which could not be attributed to the level of dietary protein. However, the data show that a diet containing 10% protein for all periods of the reproductive cycle after growth (group 2) was inadequate for females to rear the maximal number of young. Reproductive performance (group 3) was normal when a diet containing 10% protein was fed only during the mating and gestation period. The number of young weaned and their average weaning was reduced slightly when 10% protein was fed during the mating and gestation period and 20% (group 4) or 17% (group 5) was fed during the lactation and maintenance periods. When the diet contained 17% (groups 8, 9, 10 and 11) or 14% (groups 12, 13, 14 and 15) protein during the mating and gestation period, the number of young weaned was in a normal range regardless of whether it contained 14, 17, 20 or 25% protein during the lactation and maintenance periods. The weaning weight of the young was reduced slightly when the diet contained only 14% protein during the mating and gestation and lactation periods. These data show that diets containing 14 to 25% soybean protein are adequate for female rats to produce and wean a maximal number of young over 3 generations provided all other nutrients are supplied in optimal amounts.

Dietary fat. The combined data showing the reproductive performance of females receiving different amounts of dietary fat are summarized in series A, B and C of table 4.

With a practical diet (series A) the total number of young produced was larger with diets containing 5, 10 and 15% corn oil (groups 2, 3 and 4) than it was with 3% corn oil (group 1). Lard was used as the major fat component in diets containing 18% (group 5) and 25% (group 6) total fat as the physical structure of

TABLE 4

Summary of reproduction data of female rats fed various amounts of dietary fat

Group	Fat added			Energy [1]	Litters		Young					
									Weaned [2]			
	Corn oil	Lard	Total		No. born	No. weaned	No. born	No. observed	No.		Avg no./ litter	Avg wt
	%	%	%	*kcal/100 g*						%		*g*
Series A, practical diet												
1	3	—	3	266	52	51	415	391	352	90.2	6.9	34.5
2	5	—	5	278	57	54	506	411	379	92.2	7.0	34.9
3	10	—	10	305	54	54	524	444	407	91.7	7.5	36.1
4	15	—	15	333	59	53	546	437	396	90.6	7.5	39.1
5	3	15	18	349	54	51	444	415	375	90.4	7.4	39.5
6	3	22	25	394	57	54	456	427	352	82.4	6.5	42.4
Series B, purified diet												
7	3	—	3	339	52	48	426	384	335	87.2	6.9	34.8
8	3	17	20	404	57	50	470	425	353	83.1	7.1	40.2
9	3	33	36	525	47	41	356	332	276	83.1	6.7	36.9
10	3	47	50	596	48	35	385	361	235	65.1	6.7	33.1
Series C, purified soybean protein-to-calorie ratio constant [3]												
11	3	—	3	339	54	54	460	426	401	94.1	7.4	35.1
12	3	12	15	413	54	50	427	401	344	85.8	6.9	39.5
13	3	28	31	493	54	48	459	423	341	80.6	7.1	44.5
14	3	42	45	569	56	48	449	417	343	82.3	7.1	40.8

[1] Metabolizable energy references (14, 15).

[2] % Weaned = $\frac{\text{no. weaned}}{\text{no. observed}}$.

[3] Groups 11, 12, 13 and 14 received 25, 30, 35 and 40% purified soybean protein, respectively.

the diet was very greasy when high levels of corn oil were used. Females receiving diets that contained 18 and 25% dietary fat produced a larger number of young than those receiving the control diet (group 1), but the number was less than with diets containing 5, 10 and 15% corn oil. Females consuming 18% or less dietary fat weaned over 90% of their young, whereas those consuming 25% fat weaned only 82.4%. The addition of fat to the practical diet increased the number of young produced and the average weaning weight, but levels of fat within the range normally occurring in practical diets had essentially no influence on the number of young weaned.

The reproductive performance of females consuming the purified diet that contained 3, 20, 36 and 50% total dietary fat is summarized in series B, group 7 to 10 inclusive. Females receiving 3 and 20% fat produced 426 and 470 young, whereas those fed 36 and 50% fat produced 356 and 385 young, respectively. Increasing the fat to 20 or 36% decreased the percentage of young weaned from 87.2 to 83.1 and increasing fat to 50% decreased the percentage weaned to only 65.1. Most of this mortality occurred within 3 days after the litter was born. Only 69.8% of the young born to females fed this diet were alive on the third day. With 50% fat the average number of young weaned per female was reduced from 21.3 to 11.0. Young from females consuming 20 and 36% total dietary fat weighed an average of 40.2 and 36.9 g, whereas young from females fed 3 and 50% fat weighed an average of 34.8 and 33.1 g, respectively. The purified diet in series B contained 25% protein and rats fed the high fat diets consumed relatively less protein in proportion to metabolizable energy than those fed the low fat diet. It appeared possible that this difference in ratio of protein to metabolizable energy may have accounted for the smaller number of young weaned by females fed high dietary fat. To test this possibility, a third series of tests was run to determine the influence of the protein-metabolizable energy ratio on reproductive performance. These data which are summarized in series C, groups 11 to 14, inclusive, show that when the protein-to-metabolizable energy ratio was constant, the number of young produced was affected very little, if any, by levels of dietary fat between 3 and 45%. The number weaned was less with 31 and 45% than with 3 and 15% dietary fat. However, since females consuming 45% dietary fat weaned 82.3% of their young, whereas those consuming 36% fat weaned 80.6% of their young, it is doubtful whether the level of fat per se had any major effect on the number of young produced or weaned by females fed different levels of fat when the protein-metabolizable energy ratio was constant. Young from females fed 15, 31 and 45% dietary fat were an average of 4.4 to 9.4 g heavier than young from females fed 3% fat. These data show that when there is a balance between protein and total metabolizable energy any injurious effect of extremely high fat diets in reproduction in rats was markedly reduced.

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Effect of Vitamin A and Vitamin A Acid on Cerebrospinal Fluid Pressure and Blood and Liver Vitamin A Concentrations in the Pig[1,2]

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ABSTRACT Thirty-six pigs, weaned at 3 weeks of age, were depleted until their blood plasma vitamin A concentrations had decreased to less than 12 μg/100 ml. They were then supplemented for 75 ± 1 days with vitamin A or vitamin A acid, or both. Blood plasma vitamin A increased with vitamin A intake but decreased and was unrelated to any level of vitamin A acid fed. Liver vitamin A increased with vitamin A and vitamin A acid intake although values for the pigs fed vitamin A acid were low. In addition, those pigs fed a combination of vitamin A and vitamin A acid had considerably higher liver vitamin A concentrations than those pigs fed vitamin A alone, indicating that vitamin A acid had a sparing effect on liver vitamin A in the pig. No vitamin A acid could be detected in the plasma or liver. A similar decrease in cerebrospinal fluid pressure occurred with an increase in vitamin A or vitamin A acid intake, suggesting that vitamin A acid was effective in meeting this physiological requirement. Cerebrospinal fluid pressure proved to be an adequate criterion in measuring the vitamin A status of the pig when fed either vitamin A or vitamin A acid.

Arens and van Dorp (1, 2) and Sharman (3) reported that vitamin A acid was biologically active but, whether injected or given orally as the sodium salt to vitamin A-deficient rats, no vitamin A could be detected in the liver. Dowling and Wald (4) further observed that rats maintained with vitamin A acid grew normally and remained in good condition except for the development of nyctalopia which progressed to total blindness. However, rats fed vitamin A acid as the only source of vitamin A were depleted of their liver vitamin A stores at the same rate as rats fed a vitamin A-deficient diet and those rats maintained with a vitamin A acid-supplemented diet stopped growing within a few days upon its removal. Therefore, it was suggested that vitamin A acid had no sparing action on liver vitamin A in the rat.

Growth rate was observed to be an insensitive criterion for establishing the vitamin A status of the pig (5, 6). The first measurable change to occur in vitamin A-deficient pigs after a decrease in plasma vitamin A, was an increase in cerebrospinal fluid (CSF) pressure.

The present experiment was designed to obtain quantitative data on the effect of feeding vitamin A palmitate and vitamin A acid, singly and in combination, on CSF pressure and plasma and liver vitamin A concentrations in the pig.

EXPERIMENTAL

Thirty-six Duroc pigs from 4 litters were weaned at 3 weeks of age and self-fed a vitamin A-deficient semi-purified diet (6). After the blood plasma vitamin A concentration of each pig had decreased to less than 12 μg/100 ml the pigs were randomly assigned, within sex and litter, to one of 9 lots of 4 pigs each as described in table 1. Each pig was then individually fed the basal diet as previously reported (6). The daily amount of vitamin A[5] or

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[2] Presented in part at the annual meeting of the American Institute of Nutrition, Atlantic City, New Jersey, 1962. Federation Proc., 21: 474 (abstract).

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[5] Rovimix A-325; vitamin A palmitate stabilized in gelatin beadlets assayed to contain 116.5 mg of vitamin A/g. Kindly supplied by Dr. R. H. Bunnell, Hoffmann-LaRoche, Inc., Nutley, New Jersey.

TABLE 1

Effect of supplemental vitamin A and vitamin A acid on growth rate and feed conversion following depletion [1]

Lot	Intake		Avg initial wt	Avg final wt	Avg daily gain	Avg daily feed [2]	Feed/ 454 g gain
	Vitamin A	Vitamin A acid					
	μg/454 g live weight/day		*kg*	*kg*	*kg*	*kg*	
1	—	—	15.6	44.5	0.39	0.97	1.13
2	1	—	16.4	58.9	0.57	1.22	0.97
3	10	—	14.6	55.5	0.55	1.15	0.95
4	100	—	13.2	52.3	0.53	1.11	0.97
5	—	1	15.8	57.4	0.56	1.20	0.98
6	—	10	15.1	56.7	0.56	1.16	0.94
7	—	100	16.7	58.3	0.56	1.24	1.01
8	1	100	15.3	55.2	0.54	1.18	1.01
9	10	100	15.1	55.5	0.54	1.18	0.99
Mean and SD/pig			15.4 ± 2.0	55.2 ± 5.6 [3]	0.54 ± 0.20 [3]	1.16 ± 0.10	0.99 ± 0.08

[1] Values are averages of 4 pigs/treatment except lots 1, 4, 6 and 8 which contained 3 pigs/treatment.
[2] Daily allowance of feed was predetermined by each pig's initial weight.
[3] Average final weight and average daily gain of lot 1 were significantly less ($P < 0.01$) than any other lots. However, there were no significant differences among any of the other lots.

vitamin A acid [6] was mixed with a small portion of each pig's daily basal allowance and fed first each morning. After the supplemental mixture was completely consumed, the remaining basal allowance was fed. Each pig was weighed to the nearest 250 g every 7 days and the daily allowance of vitamin A and vitamin A acid was adjusted to meet the next 7 days' expected growth rate calculated from the previous 2 weeks' growth.

Oxalated blood samples were taken at 2-week intervals and blood plasma vitamin A determined according to the method of Kimble (7).

Terminal plasma vitamin A and CSF pressure were determined after 75 ± 1 days of supplementation. Cerebrospinal fluid pressure was measured with a saline manometer as described by Nelson et al. (6). Approximately 5 days after the CSF pressure was measured, cerebrospinal fluid was withdrawn, the animals electrocuted and blood collected for serum analysis. The livers were removed, cooled at 4°C, homogenized, and frozen at −20°C until liver vitamin A was measured (8). Moisture was determined by drying duplicate samples of the homogenate at 100°C.

An attempt was made to detect vitamin A acid in terminal blood plasma and livers by using the antimony-trichloride reaction and measuring absorption at 565, 580 and 620 mμ. Blood serum and cerebrospinal fluid were analyzed for total protein and inorganic phosphorus by the method of Lowry et al. (9) and Fiske and Subbarow (10), respectively. Sodium, potassium and calcium concentrations were measured utilizing a Beckman model DU flame photometer.

RESULTS

Data reported were collected from 32 of the 36 pigs. A pig in lot 1 died on the seventy-second day of the supplementation period. Gross necropsy revealed no lesions and the cause of death was not determined. One pig in lot 4 died during the administration of the anesthetic prior to the CSF pressure determination. A pig in lot 6 died shortly after CSF pressure was measured. Necropsy revealed the cause of death to be a ruptured liver. A fourth pig (lot 8) received the wrong supplement midway in the supplementation period and the data from this pig were therefore omitted.

Animal performance. The mean and standard deviation of the weight of the 32 pigs at the start of depletion was 5.7 ± 0.7 kg and at the end of depletion 15.4 ± 2.0 kg. Depletion time ranged from 28 to 32 days.

Performance data for the 75 ± 1 days while animals were fed one of the vitamin A or vitamin A acid supplements are sum-

[6] Vitamin A acid stabilized in gelatin beadlets, assayed to contain 103 mg of vitamin A acid/g, kindly supplied by Dr. R. H. Bunnell, Hoffmann-LaRoche, Inc., Nutley, New Jersey.

marized in table 1. There were no significant differences in initial weight, daily feed or feed conversion among treatments. The final weights and average daily gains of lot 1, which received only the basal diet, were significantly less ($P < 0.01$) when compared with the supplemented groups. However, there were no significant differences among other treatments.

Vitamin A and vitamin A acid concentrations in blood plasma and liver. The mean and standard deviation of the plasma vitamin A concentration for all the pigs at the start of the depletion period was 28.6 ± 5.8 μg/100 ml. Initial values prior to supplementation are shown in table 2. Terminal plasma vitamin A concentrations increased linearly ($P < 0.01$) with the logarithm of vitamin A intake. However, when vitamin A acid was fed, plasma vitamin A concentrations were very low and unreleated to the intake of vitamin A acid. This lack of relationship between vitamin A acid intake and plasma vitamin A is further shown by comparing the values obtained from pigs fed only vitamin A with those pigs fed a comparable level of vitamin A in addition to vitamin A acid (table 2, lot 2 vs. 8 and 3 vs. 9). No vitamin A acid was detected in any of the plasma samples.

Terminal liver vitamin A concentrations (table 2) were very low in those pigs fed only the basal diet (lot 1). The logarithmic relationship between vitamin A intake and liver vitamin A is illustrated in figure 1. The micrograms of vitamin A fed per 454 g live weight/day are shown in parentheses. Although the response was not linear in the present experiment, the increase in liver vitamin A was highly significant ($P < 0.01$) over the 3 levels of vitamin A fed.

The liver vitamin A values for those pigs fed only vitamin A acid were very low when compared with those of pigs fed similar levels of vitamin A (table 2). However, when expressed as micrograms per

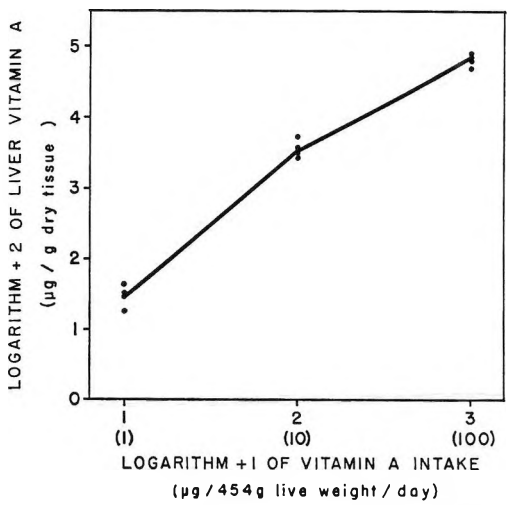

Fig. 1 Effect of vitamin A intake on liver vitamin A in the pig.

TABLE 2

Effect of vitamin A and vitamin A acid intake on cerebrospinal fluid pressure, plasma vitamin A and liver vitamin A concentrations following depletion [1]

Lot	Intake		Plasma vitamin A		Liver vitamin A	Cerebrospinal fluid pressure
	Vitamin A	Vitamin A acid	Initial	Terminal	Terminal	Terminal
	μg/454 g live weight/day		*μg/100 ml*		*μg/g dry tissue*	*mm of saline*
1	—	—	8.40	1.60	0.14	221
2	1	—	5.64	5.52	0.31	202
3	10	—	7.14	18.30	37.89	117
4	100	—	8.68	26.04	658.75	92
5	—	1	6.87	3.12	0.28	198
6	—	10	7.84	3.24	0.83	124
7	—	100	6.54	2.52	1.46	111
8	1	100	9.12	6.12	5.10	87
9	10	100	6.00	16.41	58.31	102

[1] Values are averages of 4 pigs per treatment except lots 1, 4, 6 and 8 which contained 3 pigs/treatment.

gram of dry tissue, liver vitamin A increased with the logarithm of vitamin A acid intake (fig. 2). The increase was linear and highly significant ($P < 0.01$). A missing value was calculated (11) for the 10-μg level in figure 2 but is not included in the mean value in table 2. The liver vitamin A values were greater when vitamin A acid was fed in combination with vitamin A than when vitamin A was fed singly at the same respective level. This is shown by comparing the liver vitamin A concentrations of lots 2 and 8 (0.31 vs. 5.10) and lots 3 and 9 (37.89 vs. 58.31). An absorption spectra of the vitamin A acid supplement revealed no other vitamin A-related compounds.

Cerebrospinal fluid pressure. Terminal cerebrospinal fluid (CSF) pressures after 75 ± 1 days of the supplementation period are shown in table 2. The CSF pressures were highest for the pigs in lot 1. As either vitamin A or vitamin A acid intake was increased, a corresponding and highly significant ($P < 0.01$) decrease in CSF pressure occurred (fig. 3). Here, as in figures 1 and 2, vitamin A and vitamin A acid intake are expressed as the logarithm with the actual intake levels (micrograms per 454 g live weight/day) in parentheses below the logarithmic values. Missing values were calculated (11) for the zero- and 100-μg level of vitamin A acid and are included in figure 3 but are not included in the mean cerebrospinal fluid pressure values as expressed in table 2.

Constituents of blood serum and cerebrospinal fluid. The mean and standard deviation of the constituents measured in blood serum and cerebrospinal fluid are shown in table 3. There were no significant differences for any of the constituents measured attributable to a level of vitamin A or vitamin A acid fed.

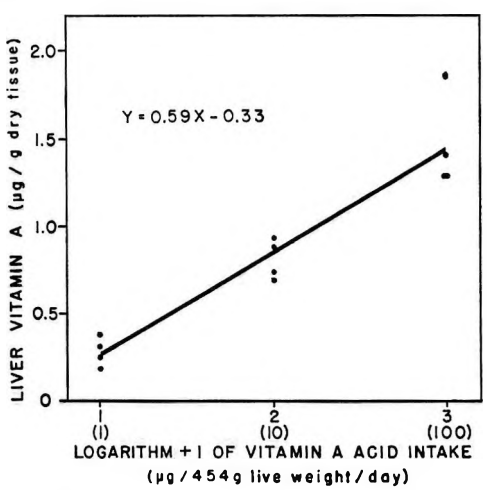

Fig. 2 Effect of vitamin A acid intake on liver vitamin A in the pig.

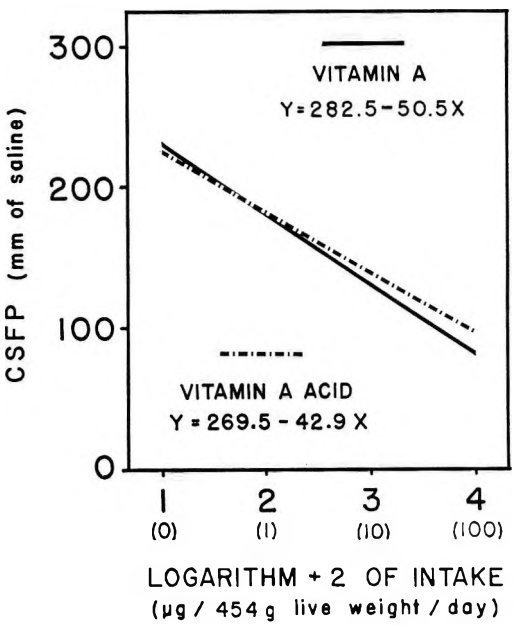

Fig. 3 Effect of vitamin A and vitamin A acid intake on the cerebrospinal fluid pressure of the pig.

TABLE 3

Constituents measured in blood serum and cerebrospinal fluid

	Serum	Cerebrospinal fluid
	mg/100 ml	*mg/100 ml*
Total protein	6881 ± 472 [1]	36 ± 7
Inorganic phosphorus	7.9 ± 0.7	1.8 ± 0.4
Sodium	324 ± 12	329 ± 36
Potassium	32.2 ± 5.7	10.8 ± 2.9
Calcium	11.0 ± 0.4	— [2]

[1] Mean ± SD.
[2] Value not obtained.

DISCUSSION

The highly significant linear response of liver vitamin A to the 3 levels of vitamin A acid fed indicates that vitamin A acid had a sparing effect on liver vitamin A in the pig. A similar sparing effect has been reported by Krishnamurthy et al. (12) in the chick. As further evidence, all pigs fed vitamin A acid had higher liver vitamin A concentrations than those pigs fed only the basal diet. In addition, the liver vitamin A concentrations were greater for those pigs fed 100 μg of vitamin A acid in combination with either 1 or 10 μg of vitamin A than for those pigs fed only 1 or 10 μg of vitamin A. The differences between these results and those of Dowling and Wald (4), who observed no sparing action in the rat, may be due to a species difference. The low liver and plasma vitamin A concentrations of those pigs fed vitamin A acid suggests that vitamin A acid was probably not converted to vitamin A in the pig, which is in agreement with the observations of the above investigators.

Cerebrospinal fluid pressure has been a very sensitive criterion in establishing the vitamin A status of various species (5, 6, 13–19). The ability of vitamin A acid and vitamin A to reduce CSF pressures at the same rate suggests that the biological potencies of these 2 substances may be equal in the pig. Even at the low level of 1 μg of vitamin A or vitamin A acid/454 g live weight/day the CSF pressures were less than those pigs fed only the basal diet. Thus, CSF pressures may provide a sensitive measurement for evaluating the state of the deficiency of the intact animal being fed only vitamin A acid.

The lack of changes in the concentrations of the constituents measured in blood serum and cerebrospinal fluid are not in agreement with previous results. Following a depletion period, Dehority et al. (19) observed a slight decrease in cerebrospinal fluid potassium with increased carotene intake in calves and Nelson et al. (6) reported an increase in serum potassium in pigs with an increase in vitamin A intake. Further investigations involving greater replication and the measurement of these and other constituents might provide more conclusive information and possibly explain the reasons for an increase in CSF pressure during a vitamin A deficiency.

In conclusion, although no relationship existed between vitamin A acid intake and plasma vitamin A, vitamin A acid did have a sparing effect on liver vitamin A in the pig. Cerebrospinal fluid pressure decreased in a linear manner as either vitamin A or vitamin A acid intake increased and was the most sensitive criterion for establishing the vitamin A status of those pigs fed graded levels of vitamin A acid.

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Chemical Pathology of Acute Amino Acid Deficiencies

VI. INFLUENCE OF FAT INTAKE ON THE MORPHOLOGIC AND BIOCHEMICAL CHANGES IN YOUNG RATS FORCE-FED A THREONINE-DEVOID DIET[1]

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ABSTRACT Young Sprague-Dawley rats were force-fed for 3 days a purified diet devoid of threonine and containing 0.6, 5, or 25% corn oil. All animals developed fatty liver with a periportal distribution, increased hepatic glycogen, and atrophy of the pancreas, submaxillary gland, stomach, spleen and thymus. The results indicate that a decrease in dietary fat intake from 5 to 0.6% corn oil does not alter the pathologic changes due to the deficient diet. An increase in dietary fat intake from 5 to 25% corn oil did not protect against the pathologic changes, but actually accentuated some of the changes due to the deficient diet.

This investigation is the sixth of a series concerned with the study of the morphologic and biochemical tissue changes in young rats force-fed purified diets devoid of single essential amino acids. In the preceding studies (1–5) we have found that young rats force-fed purified diets devoid of methionine, threonine, histidine, valine or lysine develop pathologic changes that closely resemble many of those noted in infants with kwashiorkor (6). In these studies, the quantity of diet consumed (1–3) as well as the dietary content of carbohydrate (4) and amino acids (5) were important in influencing the induction and severity of pathologic changes.

Surveys on the dietary history of children in whom kwashiorkor develops reveal a dietary intake low in fat content as well as deficient in protein (7). It has been suggested that kwashiorkor may in part be due to an "essential fatty acid deficiency" (8). Others (9, 10) have stressed the possible importance of dietary fat intake in the pathogenesis of kwashiorkor and have speculated that more calories from fat instead of carbohydrate would be beneficial in children developing kwashiorkor. Recently a great deal of interest has been focused on reports suggesting that the addition of fat to the diet may accelerate recovery (11).

Prompted by these clinical reports, we have investigated the influence of the quantity of fat intake on some of the morphologic and chemical changes noted in young rats force-fed a threonine-devoid diet. The results of this study, showing that the amount of corn oil in the diet was not important in the development of the pathologic changes resembling kwashiorkor, are reported in this communication.

METHODS

Male and female rats of the Sprague-Dawley strain, one month old, and weighing on the average 73 g were used. The animals were maintained with a commercial chow[2] for 4 days before the experiments were begun. In all experiments several groups of rats, each of the same sex, age and weight, were used.

The basal regular calorie diet was based upon that used by Forbes and Vaughan (12) and was similar to that used in our own earlier experiments (4). The percentage composition was as follows: Essential amino acids, 9.2; nonessential amino acids or L-glutamic acid, 8.1; salt mixture (13), 4; vitamin sucrose mixture, 5; corn oil,[3] 5; cod liver oil, 1.5; and dextrin, 67.2. Animals in the threonine-devoid group received the basal ration devoid

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[2] Wayne Lab-Blox, Allied Mills, Inc., Chicago.
[3] Mazola, Corn Products Company, New York.

of threonine with dextrin substituted for the missing amino acid. L-Glutamic acid was substituted for the nonessential amino acids in some experiments without any change in the results.

Animals were divided into 3 major groups (table 1) according to the fat intake: 1) High fat, animals received 1.65 g corn oil/day; 2) normal fat, animals received 0.35 g corn oil/day; 3) low fat, animals received 0.04 g corn oil/day. Subgroups consisted of animals fed a high, regular or low calorie complete or threonine-devoid diet. Animals fed the regular calorie diet received 29 to 31 kcal/day. The caloric intake was maintained by decreasing the dextrin content as corn oil was increased or vice versa. Animals fed the high calorie diets received 41 kcal/day, the additional calories being due to extra corn oil (1.65 g/day). Animals fed the low calorie diet received 27 kcal/day, the decrease in calories being due to less corn oil (0.04 g/day). To keep the fat intake very low in the low fat group, cod liver oil was replaced by oleum percomorphum (4 mg/day). The rations were blended with distilled water so that each milliliter of diet mixture contained from 0.4 to 0.6 g diet depending on the type of diet.

Rats were force-fed according to the method of Shay and Gruenstein (14) using plastic tubes. All rats were force-fed the complete diet with normal fat and regular calorie content for one day before beginning the experimental diets. The rations were fed 3 times daily, at 8:30 AM, 1 PM, and 5:30 PM for 3 days. The animals received, depending on the type of diet, an average daily feeding of from 0.75 to 1.14 g ration/10 g initial body weight. All rats had free access to water. Rats were housed in individual wire cages with raised bottoms and kept in an air conditioned room maintained at 25.5°C. On the morning of the fourth day the rats were anesthetized with ether and exsanguinated, approximately 16 hours after the last feeding. In one experiment animals were killed after they were fed the experimental diets for one day.

Rats were weighed at the beginning and end of each experiment. The organs were weighed fresh. In paired organs, the right organ was weighed. For histologic study pieces of tissue from certain organs were fixed in Zenker-formol solution and in 10% formalin. Paraffin sections were routinely stained with hematoxylin and eosin. Frozen sections of liver after formalin fixation were stained with oil red O.

Collection of material

Liver. The whole liver was weighed. For glycogen determination, 2 equal pieces, one from the median and one from the left lateral lobes, that weighed approximately 1 g were rapidly placed in 30% KOH. For protein determination, one piece was homogenized in distilled water and was then frozen. For lipid determination, a large piece of liver was frozen.

Pancreas. After removing surrounding lymph nodes and adipose tissue, the whole organ was weighed. Part of the organ was homogenized in ice-cold 0.02 M phosphate buffer, pH 7.6 and then frozen at − 15°C. This homogenate was later used for enzyme analysis and for protein determination.

Gastrocnemius muscle. The right muscle was removed, weighed and then stored at − 15°C. After thawing the tissue was homogenized in distilled water and the homogenate was used for protein determination.

Chemical analysis

Tissue protein. Protein was determined by the biuret method, according to Layne (15).

Liver glycogen. The method of Seifter et al. (16) was used.

Liver lipid. After thawing the frozen aliquot, it was ground with anhydrous sodium sulfate to a dry powder. This powder was extracted with chloroform for 24 hours. Chloroform was then evaporated and the residue was extracted with petroleum ether. The lipid remaining on evaporation of this second solvent was weighed.

Pancreatic enzymes. Aliquots of pancreatic homogenates were assayed for amylase activity by the method of Smith and Roe (17) and for trypsin activity by the method of Schwert and Takenaka (18). For the latter determination, the enzyme was activated by enterokinase[4] (19).

[4] Enterokinase, Pentex Biochemicals, Inc., Kankakee, Illinois.

TABLE 1

Diet intake, body weight changes and organ weights of rats force-fed complete or threonine-devoid diets containing high, normal or low fat

Group [1]	Daily diet intake		No. rats	Changes in body wt [2]	Liver	Gastrocnemius muscle	Spleen	Testis
	Calories	Corn oil						
	kcal	*g*		*g*	*g*	*mg*	*mg*	*mg*
High fat								
High calorie C	41	1.65	24	+8.3 ± 0.2 [3]	3.05 ± 0.04 [3]	417 ± 10 [3]	278 ± 11 [3]	447 ± 17 [3]
High calorie TD	41	1.65	17	+1.8 ± 0.9 [4]	3.88 ± 0.13 [4]	356 ± 10 [4]	156 ± 11 [4]	365 ± 26 [5]
Regular calorie C	30	1.66	9	+6.9 ± 0.5	3.04 ± 0.06	418 ± 9	234 ± 11	—
Regular calorie TD	30	1.66	8	+2.7 ± 0.7 [4]	3.82 ± 0.07 [4]	358 ± 10 [4]	161 ± 5 [4]	—
Normal fat								
Regular calorie C	29	0.35	18	+4.8 ± 1.0	2.91 ± 0.19	418 ± 10	256 ± 9	461 ± 25
Regular calorie TD	29	0.35	14	−0.3 ± 0.2 [4]	3.55 ± 0.11 [4]	358 ± 13 [4]	174 ± 13 [4]	367 ± 23 [5]
Low fat								
Regular calorie C	31	0.04	10	+4.6 ± 1.4	2.80 ± 0.16	449 ± 17	270 ± 25	457 ± 10
Regular calorie TD	31	0.04	10	−3.3 ± 1.5 [4]	3.93 ± 0.28 [4]	398 ± 18	172 ± 11 [4]	449 ± 22
Low calorie C	27	0.04	11	+5.1 ± 0.9	2.78 ± 0.15	448 ± 20	264 ± 11	488 ± 33
Low calorie TD	27	0.04	13	−1.6 ± 0.6 [4]	3.66 ± 0.13 [4]	401 ± 14	189 ± 15 [4]	426 ± 12

[1] C = complete; TD = threonine-devoid.
[2] Initial body weights ranged from 72.9 to 80.6 g.
[3] Mean ± SE of mean.
[4] $P < 0.01$ (highly significant).
[5] P between 0.01 and 0.05 (probably significant).

RESULTS

In table 1 the changes in the weights of the whole body and the weights of the liver, the gastrocnemius muscle, the spleen and the testis in rats of the different groups force-fed for 3 days are summarized. Rats of the groups fed the complete diets with high fat gained somewhat more weight than animals of the groups fed the same diet with normal or low fat. However, rats fed the threonine-devoid diets with high fat gained significantly more weight than animals fed the same deficient diets with normal or low fat even when the deficient diets were kept isocaloric with each other. In all cases rats fed the threonine-devoid diets gained significantly less weight than rats fed the comparable complete diets. In each experiment the pancreas, the kidney and the adrenal gland of the animals were weighed. Since there were no significant differences between the weights of these organs in the control and experimental groups, they were not included in table 1. The mean weight ranges of these organs were as follows: pancreas, 356 to 449 mg; kidney, 392 to 424 mg; and adrenal gland, 15.8 to 19.3 mg.

The mean liver weight of animals of all groups force-fed the threonine-devoid diet was significantly heavier than that of animals fed the complete diet. The spleen of animals of all groups weighed less in animals fed the threonine-devoid diets than in those fed the complete diets. The gastrocnemius and testis of animals of the high and normal fat groups weighed less in animals fed the deficient diets than in those fed the complete diets.

Biochemical changes. A summary of the lipid, glycogen and protein content of the liver of animals in the different groups is presented in table 2. In all of 3 major groups according to dietary fat intake, the liver lipid, glycogen and protein were greater in the animals fed the threonine-devoid diet than in the animals fed the complete diet. The largest increase of liver lipid (56 to 67%) developed in rats of the high fat group fed the threonine-devoid diet in comparison with those fed the complete diet. The livers of control animals in the high fat group contained more lipid (approximately 54%) than control animals in the other 2 groups (normal and low fat).

The protein content of the gastrocnemius muscle was significantly decreased in animals fed the threonine-devoid diet in comparison with animals fed the complete diet in the low fat group.

Pancreatic protein was significantly lower in animals fed the regular calorie threonine-devoid diet with normal fat than in comparable animals fed the control complete diet. In the other groups pancreatic protein was similar in control and experimental animals. Although the trypsin activity of the pancreas of animals of all groups showed only minor variations, there was some decrease of amylase activity in the pancreas of animals fed the threonine-devoid diet of all groups. This decrease was statistically significant in the experimental animals fed the high calorie diet with high fat and in those fed the regular calorie diet with normal fat. The amylase levels of the control and experimental animals fed the high fat diets were lower than those of comparable animals of the other 2 groups (normal and low fat).

In view of the increase in liver lipid and glycogen of animals fed the threonine-devoid diets for 3 days, an experiment was performed to determine whether similar changes would be observed after one day. Table 3 summarizes the data of this experiment. Liver lipid and glycogen were elevated in the rats fed the threonine-devoid diet in comparison with those fed the complete diet containing high fat. Liver lipid and glycogen were only slightly elevated in animals fed the threonine-devoid diet in comparison with animals fed the complete diet with normal fat. To determine whether an additional feeding on the morning of the second day would alter this response, some rats were killed 5 hours after such a feeding. The results (table 3) indicate an increase in liver glycogen in rats of all groups over those killed 5 hours earlier. Rats fed the threonine-devoid diet still had a much higher level of liver glycogen than those fed the complete diet with high fat.

Morphologic changes. Normal fat: The animals fed the regular calorie complete and threonine-devoid diets developed changes which were identical to those described in earlier reports (1, 4, 5). In brief, the findings consisted of periportal

TABLE 2

Analyses of liver, right gastrocnemius muscle and pancreas of rats force-fed complete or threonine-devoid diets containing high, normal or low fat

Group [1]	No. rats	Liver			Gastrocnemius muscle protein	Pancreas		
		Total lipid	Glycogen	Protein		Protein	Amylase	Trypsin
		mg/liver	*mg/liver*	*mg/liver*	*mg/muscle*	*mg/pancreas*	*units* $\times 10^{-3}$/*pancreas*	*units* $\times 10^{-3}$/*pancreas*
High fat								
High calorie C	24	194 ± 8 [2]	33 ± 7 [2]	649 ± 16 [2]	91.6 ± 2.7 [2]	63.1 ± 1.9 [2]	6.0 ± 0.4 [2]	11.8 ± 0.8 [2]
High calorie TD	17	303 ± 20 [3]	90 ± 13 [3]	743 ± 44 [4]	81.7 ± 4.1	54.5 ± 4.0	3.0 ± 0.6 [3]	8.9 ± 1.0 [4]
Regular calorie C	9	203 ± 9	55 ± 8	632 ± 15	93.5 ± 4.1	75.0 ± 8.8	4.5 ± 0.5	13.5 ± 1.8
Regular calorie TD	8	338 ± 19 [3]	103 ± 8 [3]	771 ± 38 [3]	84.5 ± 7.5	81.8 ± 9.2	3.9 ± 0.6	16.4 ± 1.2
Normal fat								
Regular calorie C	18	137 ± 8	35 ± 4	624 ± 21	95.4 ± 5.5	71.1 ± 3.8	12.6 ± 1.6	16.3 ± 1.9
Regular calorie TD	14	188 ± 16 [3]	86 ± 18 [3]	739 ± 40 [4]	83.5 ± 4.7	55.6 ± 3.6 [3]	7.6 ± 1.7 [4]	15.6 ± 1.7
Low fat								
Regular calorie C	10	138 ± 14	20 ± 7	652 ± 51	109.6 ± 2.7	67.7 ± 4.5	9.7 ± 0.9	16.0 ± 0.8
Regular calorie TD	10	221 ± 22 [3]	117 ± 27 [3]	847 ± 63 [4]	95.9 ± 4.4 [4]	64.8 ± 4.0	7.4 ± 1.1	17.9 ± 1.8
Low calorie C	11	128 ± 7	20 ± 9	653 ± 52	111.1 ± 3.6	71.3 ± 1.7	10.2 ± 0.9	16.9 ± 1.7
Low calorie TD	13	192 ± 9 [3]	64 ± 10 [3]	785 ± 44	95.9 ± 2.5 [3]	73.7 ± 4.2	8.9 ± 0.9	19.7 ± 1.6

[1] C = complete; TD = threonine-devoid.
[2] Mean ± SE of mean.
[3] $P < 0.01$ (highly significant).
[4] P between 0.01 and 0.05 (probably significant).

TABLE 3

Liver lipid and glycogen of rats force-fed complete or threonine-devoid diets containing high or normal fat for one day

Group	No. rats	Liver	
		Total lipid	Glycogen
		mg	*mg*
	24 Hours [1]		
High fat			
Complete	3	175 ± 8 [2]	24 ± 2 [2]
Threonine-devoid	3	223 ± 13 [3]	73 ± 10 [4]
Normal fat			
Complete	6	148 ± 13	51 ± 15
Threonine-devoid	6	170 ± 15	89 ± 31
	29 Hours [5]		
High fat			
Complete	4	173 ± 12	182 ± 21
Threonine-devoid	5	254 ± 30 [3]	338 ± 12 [4]
Normal fat			
Complete	3	156 ± 7	127 ± 24
Threonine-devoid	5	158 ± 13	184 ± 27

[1] Animals were force-fed at 9AM, 1 PM and 5 PM and killed 9 AM the following day.
[2] Mean ± SE of mean.
[3] P between 0.01 and 0.05 (probably significant).
[4] $P < 0.01$ (highly significant).
[5] Animals were force-fed the first day at 9 AM, 1 PM and 5 PM and on the second day at 9 AM and killed at 2 PM.

fatty liver, excess hepatic glycogen, and atrophy of the pancreas, submaxillary gland, stomach, thymus and spleen in animals fed the threonine-devoid diet. No pathologic changes were observed in the animals fed the complete diet.

High fat: The changes observed in the animals fed the high calorie and regular calorie diets were similar, and for this reason the two groups are described together. The livers of the animals fed the threonine-devoid diets had a marked degree of fatty change with a periportal distribution, whereas the livers of the animals fed the complete diets had a mild degree of fatty change with a periportal distribution. The pancreas and submaxillary gland of most of the experimental animals showed moderate atrophy of the glandular cells while the same organs of the control animals showed mild glandular atrophy in only a few animals. The thymus, spleen and stomach showed moderate atrophic changes in the experimental animals but these organs were normal in the control animals. The changes described in the liver and pancreas of the experimental animals appeared to be somewhat more marked in animals fed the high calorie than those fed the regular calorie deficient diet.

Low fat: The changes observed in the animals fed the regular calorie and low calorie diets were identical and are therefore described together. Animals fed the threonine-devoid diets showed the following morphologic changes: mild-to-moderate fatty liver with a periportal distribution and mild atrophy of the pancreas, submaxillary, stomach, thymus, and spleen in most of the animals. The same organs were all normal in the animals fed the complete diets.

DISCUSSION

The results of this study indicate that young rats force-fed for 3 days a threonine-devoid diet containing 0.6, 5, or 25% (20 to 30%) corn oil develop fatty liver with a periportal distribution, increased hepatic glycogen, and atrophy of the pancreas, submaxillary, stomach, spleen, and thymus. From these results, 2 points stand out clearly: First, a decrease in dietary fat intake from 5 to 0.6% corn oil does not alter the pathologic changes due to the deficient diet. Also, an increase in dietary fat intake from 5 to 25% corn oil not only

does not protect against the pathologic changes, but if anything, accentuates the changes due to the deficient diet.

Since a fatty liver of similar severity develops in animals force-fed the deficient diet with either low fat (0.6% corn oil) or normal fat (5% corn oil) content, it appears unlikely that the level of dietary fat, in the range from 0.6 to 5%, is a major determinant in the development of the lipid accumulation. In an earlier study (2) using a purified diet devoid of threonine and containing 5% corn oil, we observed that the elevated liver lipid in the experimental animals could not be accounted for by an accelerated conversion of the carbon chains of amino acids to lipids. The increment in liver lipid is therefore most probably derived from other precursors in the diet or from the depots. Studies by Macdonald et al. (20, 21) with animals and on children with kwashiorkor suggested that the increased liver lipid is derived from dietary carbohydrate.

In contrast with the experimental results with animals fed diets containing 0.6 or 5% corn oil, the fatty liver induced in animals fed the deficient diet but containing high fat (25% corn oil) does appear to be related in part to the dietary lipid level. This is supported by the increase in liver lipid observed in the control animals fed the complete diet containing 25% corn oil. The livers of animals fed the deficient diet with 25% corn oil were much more fatty histologically and contained more lipid than those of the controls and also more than the livers of animals fed the deficient diets with 0.6 or 5% corn oil. This increase in liver lipid in animals fed the diets with 25% corn oil was not due to an increase in calorie intake since it occurred even when the high (25% corn oil) fat diet was kept isocaloric with the normal (5% corn oil) or the low (0.6% corn oil) fat diets.

Earlier workers (22) have reported that the composition of the diet influences the levels of pancreatic excretory enzymes. It has been found that rats fed ad libitum a high fat diet adequate in protein show a decrease in activity of pancreatic amylase. Our present force-feeding experiments, likewise, indicate a decrease (approximately 50%) in pancreatic amylase activity in rats fed the complete diet containing high fat in comparison with rats fed the isocaloric complete diet containing low fat. The pancreases of animals fed the threonine-devoid diets, regardless of fat content, had a mild-to-marked decrease in amylase activity in comparison with those of animals fed the control diets. On the other hand, pancreatic trypsin activities showed little difference between the control and experimental animals. Similar results with pancreatic amylase and trypsin activity have been reported in other experiments (4, 5, 23) where animals were force-fed purified diets.

Our experimental results that low dietary fat intake is not of major importance in the induction of pathologic changes in rats fed a threonine-devoid diet are in agreement with experimental results of others using another species (24, 25). Young swine fed low protein, low fat diets developed pathologic changes in many organs. However, even more severe pathologic changes developed in similar animals fed low protein, high fat diets. Based upon these observations and also our own, there appears to be no interrelationship between low fat intake and protein or amino acid deficiency in the induction of the pathologic changes. Actually, the experimental results with rats and pigs suggest that a high fat intake superimposed on protein or amino acid deficiency is deleterious rather than beneficial.

In man kwashiorkor is considered to be a disease primarily due to protein deficiency. Clinical reports from many areas of the world have stressed that the diets of children with kwashiorkor generally contain low protein, high carbohydrate and also low fat (7). Several workers (9, 10) have suggested that low fat intake was of major importance and that an increased fat intake would be beneficial to infants who develop kwashiorkor. This view is supported by observations of others (cf. 26) that a high intake of fat, especially the quantity found in milk, is important in normal growth and development of infants and young children. However, further clinical studies are necessary to determine whether an interrelationship between low fat intake and protein deficiency exists in the pathogenesis of kwashiorkor.

If such an interrelationship is established for man, it would appear that dietary lipid has a different effect upon the pathologic manifestations of protein deficiency in different species.

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Exclusion of the Exocrine Pancreatic Secretion: Effect on Digestibility of Soybean and Milk Protein by Baby Pigs at Various Ages[1,2,3]

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ABSTRACT This study was undertaken to evaluate the role of the pancreas in the digestion of protein from soybean and milk sources as a function of age by the baby pig. Protein and dry matter digestibility were determined using baby pigs with ligated pancreatic ducts and sham operated control animals. Milk protein produced faster and more efficient gains than soybean protein in the diets of baby pigs. Protein and dry matter digestibility were significantly reduced by the exclusion of pancreatic secretion into the duodenum. The reduction in digestibility was of a greater magnitude for pigs fed a soybean protein diet than pigs fed a milk protein diet. The evidence indicates that the relative role of the pancreas in the overall digestive process decreased very slightly in the case of the soybean protein with advancing age but remained relatively unchanged in the case of the milk protein, and that the efficiency of the whole digestive system except the pancreas improves considerably with advancing age in the case of soybean protein and improves to a lesser degree in the case of milk protein.

Mutually supporting evidence has been accumulated in recent years, demonstrating that dried skim milk is superior to processed soybean meal as the primary source of protein in baby pig diets (1–3).[6,7,8] The report of their research observations indicates that rate of body weight gain, efficiency of feed utilization, protein and dry matter digestibilty, and protein biological value mutually support the nutritional superiority of dried skim milk as compared with processed soybean meal in the baby pig diet. Wherever the above criteria were evaluated at various intervals during the early post-weaning period, it was found that in the case of soybean meal, a consistent improvement was apparent up to the age of approximately 8 weeks. It has been reported that both casein and lactose of dried skim milk contribute to its nutritional superiority.[9] Concurrently, evidence was reported indicating that the difference of performance between animals fed the 2 diets could be reduced by dietary supplementation with crude enzyme preparations or by predigestion of the protein (4). The effect was attributed to a greater utilization of the soybean meal protein without appreciable effect on the milk protein.

It has been proposed that the baby pig under 8 weeks of age cannot utilize soybean protein because it is not adequately digested owing to an underdeveloped digestive system (5); the basic supposition was that the underdeveloped digestive system was adequate for digestion of dried skim milk protein. Others (6–8) have demonstrated no beneficial effects from dietary supplementation of baby pig diets with digestive enzymes.

Enzymic activity of extracts of digestive gland tissue of the embryonic and newborn pig have been investigated extensively (9–

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15).[6] Whereas the proteolytic enzymes were found to increase, other enzymes, particularly lactase, were found to decrease in concentration with advancing age during the early postpartum periods studied.

Although the observed trends may be of significance, they could be misleading as measures of digestive ability. Of the complex of physiological parameters that affect the dietary as it traverses the gastrointestinal tract, one of the most important is the total quantity of the respective enzymes secreted into the lumen of the gut. The latter cannot be estimated by tissue enzyme concentration until a reliable correlation can be demonstrated between the tissue enzyme concentration of the digestive glands and the total enzyme output of the respective glands in the various species of animals. It has already been demonstrated that pancreatic secretion in the baby pig is highly irregular and unpredictable.[10,11,12] From the results of these investigations into the spontaneous pancreatic secretion over sustained periods (up to 4 days) of time, it appears doubtful that a measure of the tissue enzyme concentration predicts reliably the quantitative digestive capacity of the secretions; emphasizing the need for studies of the intralumenal output of each of the digestive secretions. Exclusion of a secretion by experimental devices and studying the effect of such exclusion on the overall digestive process can be considered a sufficiently direct approach to study the role of the secretion in the gut.

The investigations reported herein demonstrate the effects of excluding pancreatic digestion, by ligation of the pancreatic duct, upon protein and dry matter digestibility and protein biological value of solvent extracted soybean protein and dried skim milk protein fed in carefully balanced semipurified diets to pigs between 2 and 8 weeks of age. The procedure permits a relatively direct study of the digestive role of pancreatic juice in the digestion of the 2 basic dietary proteins.

EXPERIMENTAL

Two experimental diets were prepared (table 1) so that 91.4% of the protein was supplied by dried skim milk or solvent-extracted soybean meal (50% protein) in the respective diets, referred to hereafter as the milk protein diet and soybean protein diet. The remaining 8.6% of the protein was provided in each of the 2 diets by ground yellow corn.

TABLE 1

Composition of experimental diets

	Milk protein diet	Soybean protein diet
	%	%
Ground yellow corn	20.00	20.00
Cornstarch	15.83	3.05
Sucrose	4.90	5.66
Lactose	—	26.90
Dried skim milk	53.50	—
Soybean meal (50% protein)	—	36.70
Stabilized lard	2.00	2.10
Cellulose [1]	1.16	—
Vitamin premix	2.00 [2]	2.00 [3]
Iodized salt	0.10	0.74
Calcium carbonate	—	0.21
Dicalcium phosphate	—	1.94
Trace minerals [4]	0.20	0.20
KCl	—	0.50
$MgCl_2 \cdot 6H_2O$	0.31	—

[1] Solka Floc, Brown Company, Chicago.

[2] Provided the following per kilogram of diet: vitamin A, 5,724 IU; vitamin D, 875 IU; riboflavin, 0.176 mg; pantothenic acid, 3.37 mg; niacin, 55.66 mg; choline, 0.461 g; vitamin B_{12}, 14.56 μg; α-tocopherol, 21.13 IU; vitamin K, 1.10 mg; thiamine, 4.05 mg; pyridoxine, 2.27 mg; folic acid, 0.70 mg; butylated hydroxytoluene, 62.48 mg.

[3] Provided per kilogram of diet: vitamin A, 5,940 IU; vitamin D, 1,093 IU; riboflavin, 9.81 mg; pantothenic acid, 16.19 mg; niacin, 53.90 mg; vitamin B_{12}, 44.0 μg; α-tocopherol, 21.05 IU; ascorbic acid, 37.4 mg; vitamin K, 1.10 mg; thiamine, 5.06 mg; pyridoxine, 0.44 mg; folic acid, 0.81 mg; biotin, 0.006 μg; butylated hydroxytoluene, 62.48 mg.

[4] Provided the following per kilogram of diet: (in milligrams) Fe, 139.8; Cu, 9.49; Co, 3.32; Zn, 161.8; Mn, 113.5; Ca, 105.5; K, 15.0.

The calculated nutrient compositions of the 2 diets are essentially identical. Lactose was added to the soybean protein diet to equal the lactose provided by the dried skim milk in the milk protein diet. Cornstarch was added to the milk protein diet in an amount approximating the plant starches provided by the soybean meal to the soybean protein diet. The total fiber of the 2 diets was balanced by the addition of cellulose to the milk protein diet. Ground yellow corn was added only to give the diet a texture more acceptable to the pig. An

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effort was made to formulate 2 diets comparable in every nutrient except the source of protein, the variable of prime concern in this study.

The 2 basic diets were tested in experiment 1 by measuring the growth rate and feed efficiency of baby pigs between 10 and 45 days of age. Before more extensive experiments could be justified using these diets, it had to be demonstrated that the meticulous balance of the 2 diets did not equalize their nutritional quality and thereby invalidate the protein source hypothesis. Forty baby pigs, weaned at 7 days of age, were used in a randomized block design. Pairs of littermate pigs were randomly assigned across the 5 blocks (replications) with one member of each pair going to one of the 2 diet subgroups. In brief, each diet was represented by 20 pigs in 5 pens. All animals were weighed, and feed consumption was evaluated at 7-day intervals. At the conclusion of the experiment, the pigs fed the soybean protein diet displayed unusually large abdomens, suggesting considerably more diet and water retention. Thus, feed and water were removed from all animals receiving both diets for 24 hours, and the animals were weighed again to insure that absolute body gain was measured rather than gain plus gastrointestinal "fill."

In experiment 2, 4 littermate pigs were selected from each of 4 litters. Two groups of 4 littermate pigs were selected for experimental investigation at 25 days of age; the other 2 groups, for investigation at 56 days of age. The animals were weaned at 21 days of age and placed in a pen with their respective experimental diets. After the animals had learned to eat the dry diets, they were assigned to their experimental treatments and placed in galvanized metabolism stalls designed to separate feces and urine. The experimental design consisted of 8 treatments: 2 diet treatments, soybean and dried skim milk protein diets; 2 pancreas treatments, pancreatic duct ligated (PDL) and sham operated control (SOC); 2 age periods, 25 and 56 days of age. Individual pigs in each group of 4 littermate pigs were randomly assigned across the 4 treatments within each age period (2 diet $\times$ 2 pancreas); thus, each experimental treatment was represented by 2 unrelated animals, but littermate pigs were represented in each of the diet $\times$ pancreas subgroups. The group of animals studied at 25 days of age were prepared surgically at 23 days of age. Feces and urine collection were initiated 5 days after the surgical operation. The group of animals studied at 56 days of age was prepared surgically at 55 days of age, and the feces and urine collection were initiated one day later. A 6-day collection period was used for the 56-day-old pigs, and a 4-day collection period was used for the 25-day-old pigs. After termination of the collection period, all animals were examined for gross lesions and histopathological alterations of the pancreas.

In experiment 3, 48 baby pigs were weaned from their dams at 7 days of age. After a 12-hour fast, the 2 experimental diets were offered to the animals. The animals were maintained in floor pens in groups until 5 days before surgery was to be performed, at which time they were placed in the individual digestibility stalls. A $2 \times 2 \times 3$ factorial design was used, consisting of the 2 experimental diets, 2 pancreas treatments (PDL and SOC), and the 3 age groups (2, 4 and 6 weeks). The basic experimental design was replicated 4 times. Each animal was randomly assigned to one of the 12 treatments and 4 replicate groups. Surgery was performed 24 hours before the feces and urine collections were initiated. A 7-day collection period was used with the 2-week-old pigs because of the small quantity of excreta obtained during the first 5 days and a 5-day collection for the 2 older groups of animals. The 2-week-old pigs were killed immediately after the collection period to evaluate gross and histopathological alterations.

Preparation of pancreatic duct ligated (PDL) and of sham operated control (SOC) animals. The baby pigs selected for the study were fasted 24 hours and then anesthetized with sodium pentobarbital by an initial intraperitoneal injection followed by a very slow intravenous infusion (external jugular) to the surgical level of anesthesia. The pancreatic duct was exposed by an incision approximately 10 cm in length on a line parallel with the posterior-ventral margin of the rib cage on the right side.

Two separate nylon ligatures were placed tightly around the pancreatic duct, and the laparotomy was closed. Sham operated control animals were prepared by identical procedure, except that no pancreatic duct ligatures were placed. The general surgical procedures used were those outlined by Markowitz et al. (16).

Evaluation of protein and dry matter digestibility, protein biological value and nitrogen retention. In experiment 2, the collection period was initiated and terminated by feeding the animals a very small portion of a "marked" diet prepared by thoroughly mixing 454 g of chromium oxide into 45 kg of the respective experimental diets. After the animals consumed the "marked" feed, the normal experimental diets (no marker) were again offered. Urine was collected over a period which coincided exactly with the period between the 2 diet "markers."

In experiment 3, the animals were fed their respective experimental diets which contained 0.50% chromium oxide thoroughly mixed into the diet. Because of the small quantities of feces excreted per day by pigs of this age, a diet with a known amount of chromium oxide per 100 g of dry weight was used throughout the collection period so that it could be used as a quantitative index instead of a visual marker, to reduce the errors encountered.

The diets were prepared fresh for each experiment. Feces were collected twice daily, and frozen. Urine was collected daily and stored in closed containers under a layer of toluene.

The nitrogen content of the feces, urine and diet was determined by the standard micro-Kjeldahl distillation procedure. The dry matter content of the diet and feces was determined by drying the feed samples and the total collection of feces to a constant weight at 80°C in a constant temperature oven. The chromium content of the diet and feces was determined by the colorimetric method of Kimura and Miller (17).

Histopathological and gross observation of experiment animals. All the animals from experiment 2 and all the animals studied at 2 weeks of age from experiment 3 were observed for gross lesions and histopathologic alterations.

RESULTS

Experiment 1. Data for average pig gains for each 7-day period, total 35-day gains, and the feed required per kilogram of gain are summarized in table 2. The

TABLE 2

Summary of body weight gains and feed efficiency [1] *(exp. 1)*

	Experimental period (days)					Total 35-day period
	1–7	8–14	15–21	22–28	29–35	
	Body weight gain, kg					
Soybean protein diet (SPD)	0.003 ± 0.022 [2]	0.33 ± 0.150	1.39 ± 0.249	2.18 ± 0.239	3.51 ± 0.412	7.41 ± 0.282
Milk protein diet (MPD)	0.28 ± 0.062	0.52 ± 0.167	1.72 ± 0.203	2.93 ± 0.435	3.69 ± 0.418	9.13 ± 0.995
(SPD):(MPD) ratio	0.01	0.63	0.81	0.74	0.95	0.81 [3]
	Feed (kg) required/kg of gain					
Soybean protein diet (SPD)	292.67 [4]	3.43 ± 2.49	1.62 ± 0.187	1.70 ± 0.255	1.54 ± 0.142	1.75 ± 0.050
Milk protein diet (MPD)	2.79 ± 0.566	1.91 ± 0.564	1.31 ± 0.050	1.48 ± 0.071	1.68 ± 0.100	1.58 ± 0.100
(SPD):(MPD) ratio	104.90	1.80	1.24	1.15	0.92	1.11 [3]

[1] All values are on a per animal basis and are the means of 5 replications with 4 animals per replicate.
[2] SD of mean.
[3] Significantly different ($P < 0.01$).
[4] Calculated from total gain and feed consumption values for 5 replicates because 2 replicates lost weight during this period.

pigs fed the soybean protein diet lost significantly more ($P < 0.01$) weight during the terminal 24-hour feed and water deprivation period than pigs fed the milk protein diet, which suggests that before deprivation, the soybean protein-fed pigs had appreciably more feed and water in their digestive tracts than the pigs fed the milk protein diet.

The pigs fed the milk protein diet grew significantly faster and required significantly less feed per kilogram of gain than the pigs fed the soybean protein diet. The results further demonstrate that the pigs fed the milk protein diet gained more weight and required less feed per kilogram of body weight gain during each of the 5 weekly intervals, with one exception (table 2). Although the pigs fed the milk protein diet surpassed the pigs fed the soybean protein diet in both expressions of performance, it can be seen, from the soybean-to-milk protein ratios in table 2 at each weekly interval, that the relative difference was greatest the first week and decreased each subsequent week so that, at the end of the fifth week, the animals fed the 2 diets performed very similarly. With the exception of the fourth week of the experimental period, the performance of animals fed the soybean protein diet improved markedly relative to the animals fed the milk protein diet so that, at the end of the fifth week of the experiment, the ratios were near unity, indicating diet equality. The average values in table 2 show that this trend was slightly affected by a decline in the performance of the milk protein fed pigs.

Experiment 2. Consumption, excretion, digestibility, nitrogen retention, and biological value data are summarized in table 3. The consumption of dry matter and nitrogen were similar for each of the 4 treatments within the 2 age groups. Protein digestibility was significantly higher for the milk protein diet than the soybean protein diet, was significantly reduced by pancreatic duct ligation, and was significantly higher for pigs at 56 days than at 25 days of age. The age × dietary protein interaction was statistically significant due to the higher digestibility of the soybean protein by pigs at 56 days of age as compared with 25 days of age, whereas the digestibility of milk protein remained relatively unchanged. The percentage of nitrogen retained was significantly lower for pigs fed the soybean protein diet as compared with that of the pigs fed the milk

TABLE 3

Summary of consumption, excretion, digestibility, biological value, and nitrogen retention [1] *(exp. 2)*

Parameter	Age	Soybean protein diet		Milk protein diet	
		SOC [2]	PDL [3]	SOC	PDL
	weeks				
No. of animals	4	2	2	2	2
	8	2	2	2	2
Dry matter consumption, g	4	976.20	965.21	968.21	936.64
	8	4114.73	4849.50	4241.17	4777.90
Nitrogen consumption, g	4	34.30	33.94	34.68	33.55
	8	144.58	170.40	151.89	171.11
Dry matter digestibility, %	4	84.11	74.42	92.71	88.37
	8	92.04	86.63	95.17	84.02
Protein digestibility, %	4	71.42	38.86	89.89	77.16
	8	88.12	72.04	95.72	73.38
Nitrogen retention, %	4	50.30	26.27	76.12	49.49
	8	56.08	46.04	57.68	52.45
Biological value (apparent)	4	69.80	66.57	84.70	64.12
	8	63.58	63.91	60.25	70.55

[1] All values are averages of the values of 2 animals for each treatment.
[2] Sham operated control.
[3] Pancreatic duct ligated.

protein diet, and the percentage of nitrogen retained was significantly reduced by pancreatic duct ligation. Biological value of the dietary proteins was not significantly affected by any of the treatments. However, the biological value of the protein for the sham operated control pigs fed the milk protein diet decreased from 84.7 at 25 days of age to 60.3 at 56 days of age. Hays et al. (2) reported a similar result using nonoperated pigs.

Gross observation of the animal carcasses and their viscera revealed that the wounds healed normally. The only striking difference between the 2 groups of animals, on gross observation, was that the pancreas of the pancreatic duct ligated pigs had atrophied. Bacterial examination of the tissues were negative with one exception.

Histopathological examination of the pancreatic tissues revealed extensive atrophy and fibrosis of the tissues and dilation of the pancreatic ducts of the ligated pigs. Lymphocytic infiltration of the pancreas and the *lamina propria* of the small intestine were observed in all the pigs regardless of the pancreas treatment, with the exception of 2 sham operated control animals. Myelogenic emboli of unknown origin were apparent in the pulmonary arteries of 2 of the sham operated control animals. The islets of Langerhans were more prominent in the 56-day-old pigs than in the 25-day-old pigs, and the islets of Langerhans were undergoing hydropic degeneration in all 4 of the 56-day-old pigs with ligated pancreatic ducts.

Experiment 3. The results of experiment 3 are summarized in table 4. It was necessary to drop 5 pigs from the experiment. The data accumulated in this experiment were analyzed by the approximate method of analysis of variance for disproportionate subclass numbers in a factorial design (18). There were no significant treatment or interaction effects for the percentage nitrogen retention or protein biological value. The average value for each of the cells does suggest that both

TABLE 4

Summary of consumption, excretion, digestibility, biological value, and nitrogen retention[1] *(exp. 3)*

Parameter	Age	Soybean protein diet		Milk protein diet	
		SOC[2]	PDL[3]	SOC	PDL
	weeks				
No. of animals	2	4	3	2	4
	4	4	3	4	4
	6	4	4	3	3
Dietary dry matter (chromium equivalent in feces),g	2	608.4	413.2	347.8	386.6
	4	619.8	519.4	690.5	567.2
	6	958.4	1163.4	1344.8	1159.5
Dietary nitrogen (chromium equivalent in feces), g	2	20.43	10.72	11.82	12.54
	4	20.81	17.44	23.49	19.30
	6	32.26	39.06	45.75	39.44
Protein digestibility, %	2	65.64	30.38	68.27	55.80
	4	69.23	29.92	88.16	53.48
	6	74.58	46.18	89.22	59.05
Dry matter digestibility, %	2	80.62	67.89	79.60	73.72
	4	82.06	67.24	88.22	77.25
	6	85.36	75.58	90.12	80.56
Nitrogen retention, %	2	12.49	−12.79	2.74	−17.28
	4	23.50	−5.85	31.34	12.49
	6	35.34	7.12	23.08	13.03
Biological value (apparent)	2	17.55	−63.03	−29.76	−135.65
	4	33.15	−56.54	36.09	14.04
	6	47.69	15.63	25.98	23.89

[1] All values are averages of the individual values calculated from the pigs of each treatment.
[2] Sham operated control.
[3] Pancreatic duct ligated.

the biological value and nitrogen retention are generally reduced by ligation of the pancreatic duct as was found in experiment 2. In this experiment, as in experiment 2, protein digestibility was affected appreciably. There was a significant difference in protein digestibility between the 3 age groups of animals. The data indicate that the digestibility increased for both diets each subsequent 2-week period. Two of the 4 sham operated control pigs fed the milk protein diet at 2 weeks of age did not eat, and consequently, no digestibility values were obtained. A third animal (no. 7261S) from the same group ate a very small quantity of feed, and the protein digestibility value was very low in comparison with values calculated in other studies. It is probable that the fourth value is most representative.

With the exception of the 2-week age group with which reliable data were not obtained, milk protein was significantly more digestible than soybean protein, and the digestibility of both proteins was significantly reduced by ligation of the pancreatic duct. Also, there was a significant interaction between the dietary protein source and the pancreas treatment. The data indicate that this was due to the greater reduction of protein digestibility by ligation of the pancreatic duct of pigs fed the soybean protein diet than pigs fed the milk protein diet. However, there was no significant interaction between age and dietary protein or between age and pancreas treatment. These results agree well with the results of experiment 2, and support the proposition that the pancreas plays a more prominent role in the digestion of soybean protein than in the digestion of milk protein. Although these data are difficult to interpret, they do not clearly support the concept that the pancreas gains a more prominent digestive role with advancing age in the case of pigs fed the soybean protein or a decreasing role in the case of pigs fed the milk protein.

Dry matter digestibility values reflect the same experimental influences as described for protein digestibility. Dry matter digestibility increased significantly with age, was significantly higher for pigs fed milk protein than pigs fed soybean protein and was significantly reduced by ligation of the pancreatic ducts.

The report of the gross and histopathological examination of the 16, two-week-old pigs indicated that the pancreas was severely affected by ligation of the pancreatic duct. The islets of Langerhans were not observed in 14 of the 16 animals, with only few immature islets present in the remaining 2 animals. All the pigs with ligated pancreatic ducts revealed severe atrophy and fibrosis of the pancreas tissue; the pancreatic ducts were dilated, and only few acini were observed. The pancreas of 3 of these animals were infiltrated with lymphocytic cells. With the exception of animal no. 7261S, all of the sham operated control animals had normal appearing pancreas glands. The pancreatic tissue from animal no. 7261S very closely resembled the tissues of pancreatic duct ligated animals. This observation unquestionably explains the unusually low protein and dry matter digestibility values contributed by this animal. Perhaps this condition resulted from slight trauma to the gland during the surgical procedure.

DISCUSSION

Using a method of pancreatic duct ligation to exclude the exocrine pancreatic secretion, it was possible to measure the digestive accomplishment of non-pancreatic secretions and to demonstrate the relative digestive role of the pancreas. Although the pancreas participates in both endocrine and exocrine functions, only the latter was of concern in this study. However, the possibility of a secondary pathological involvement of the endocrine function due to ligation of the pancreatic duct with subsequent influence on other digestive functions should not be overlooked. The 2 functions of the gland are under intricate control, demonstrating nervous stimulation and inhibition and hormonal influences. The exocrine functions of the gland are complex, the gland extruding a spectrum of enzymes and inorganic buffers into the lumen of the duodenum which participate in digestion and absorption. The composition of pancreatic juice has been discussed by Thomas (19). The dietary influence on secretion and composition of pancreatic juice was first studied

in the classical experiments of Pavlov and Babkin which led to the "purposive adaptation" and "parallelism" theories, respectively. More recently a body of knowledge has been accumulated by numerous investigators, which suggests that the dietary influences pancreatic secretion (19). Chernick et al. (20), Lyman and Lepkovsky (21), Lyman (22) and Lepkovsky et al. (23) have reported results which indicate that the trypsin inhibitor of raw soybean has a marked stimulatory effect on the pancreas of chicks.

Our own study of the pancreatic secretion of the baby pig using chronic pancreatic fistulae has been mentioned before.[10,11,12]

In experiment 1, the milk protein diet was consistently superior to the soybean protein diet, but the difference in performance of the animals on the 2 diets decreased until it was negligible at 45 days of age. In experiments 2 and 3, pancreatic duct ligation and the associated elimination of pancreatic digestion consistently reduced protein and dry matter digestibility and nitrogen retention and had a variable effect on protein biological value. The percentage digestibility of protein or dry matter of the PDL pigs is taken as the digestive capability of the whole gastrointestinal system, minus the pancreas; whereas the digestibility of the nutrients by the SOC pigs is taken as the overall digestive capability, including the pancreas contribution. Therefore, the difference in digestibility between the 2 groups of animals (SOC − PDL) can be taken as a direct indication of the digestive role of the pancreatic secretion. Protein and dry matter digestibilities were always reduced to a greater degree by excluding pancreatic digestion in pigs fed the soybean protein diet than in those fed the milk protein diet. In the absence of pancreatic secretion, milk protein was approximately twice as digestible as soybean protein (tables 3 and 4), indicating that milk protein can be more completely degraded by intestinal and gastric secretions than soybean protein. The additional protein digested by the pancreas, in excess of the digestion accomplished by non-pancreatic secretions, was much greater with the soybean protein diet than with the skim milk protein diet (although the total digestibility was greater with the skim milk protein diet). These results can probably be explained by one of the following 3 possibilities, or a combination of them: the soybean protein diet effectively elicited a superior pancreatic secretion (either quantitatively or qualitatively); the soybean protein substrate which remained after gastric and intestinal digestion was more effectively degraded by pancreatic enzymes than the milk protein substrate because of specific peptide linkages in the soybean protein which demanded the specific enzymes secreted by the pancreas; a greater quantity of the soybean protein than of the milk protein was not degraded by the non-pancreatic secretion and as a consequence a similar quantity of pancreatic secretions accomplished a greater amount of digestion with the soybean protein. It is possible that the results are unrelated to the enzyme components and instead reflect a lack of the abundant inorganic buffers of pancreatic juice. Indeed the digestive accomplishment is the result of a complex integration of many simultaneous processes.

In general, the pigs tolerated ligated pancreatic ducts well for the period of study used in this investigation. Karvinen et al. (24, 25) observed that exclusion of pancreatic digestion in the rat by ligation of the pancreatic duct significantly reduced the utilization of various fats as compared with sham operated control rats. Pavlov (26) reported that ligation of the pancreatic ducts of dogs caused no detrimental effects; on the contrary, Popper and Sorter (27) reported that ligation of the pancreatic ducts of dogs resulted in death within 12 months.

The combined results of experiment 2 and 3 (table 5) illustrate that soybean protein is very poorly digested by the 2-week-old pig in the absence of pancreatic juice and that the digestibility improves quite markedly to 8 weeks of age. Although milk protein is twice as digestible as soybean protein at 2 weeks of age in the absence of pancreatic digestion, it did not improve to the extent of the soybean protein. The 2 proteins approached equality at 8 weeks of age in the absence of pancreatic juice. These results do not neces-

TABLE 5

Accumulation and summary of protein digestibility data of experiments 2 and 3

Exp.	Age	Soybean protein (SP)			Milk protein (MP)			(SP) SOC / (MP) SOC	(SP) PDL / (MP) PDL
		SOC [1]	PDL [2]	PDL SOC	SOC	PDL	PDL SOC		
	weeks	%	%		%	%			
3	2	65.64	30.38	0.463	68.27 [3]	55.80	0.817 [3]	0.961 [3]	0.544
3	4	69.23	29.92	0.432	88.16	53.48	0.607	0.785	0.559
2		71.42	38.86	0.544	89.89	77.16	0.859	0.795	0.504
3	6	74.58	46.18	0.619	89.22	59.05	0.662	0.836	0.782
2	8	88.12	72.04	0.818	95.72	73.38	0.767	0.921	0.982

[1] Sham operated control.
[2] Pancreatic duct ligated.
[3] See results of experiment 3 for explanation of these questionable values.

sarily indicate quantitative differences in output of pancreatic enzymes in each case but do indicate the relative effects of excluding pancreatic secretion; these results may be due to a net change in the absorption of the protein degradation products rather than to a digestive change, or both digestion and absorption could be involved (conventional digestibility experiments measure only the net absorption); exclusion of pancreatic secretion could physiologically enhance other digestive secretions.

In experiments 2 and 3, 16 sham operated control pigs and 16 pigs with ligated pancreatic ducts were killed for examination of the pancreatic tissue. With one exception, the tissues of the sham operated control animals appeared normal, whereas the tissues of the pigs with ligated pancreatic ducts revealed extensive atrophy and fibrosis and dilation of the pancreatic ducts. The islets of Langerhans were clearly distinguishable in the pancreatic tissues of both the pancreatic duct ligated pigs and the sham operated control pigs at 8 weeks of age; they were less distinct at 4 weeks of age; and they were indistinguishable at 2 weeks of age. This observation does not relate specifically to the exocrine function of the pancreas, but it may reflect that the pancreas of the baby pig has not fully differentiated and matured during the early postpartum period. Acinar cells in the pancreatic tissue of 2-week-old pigs with ligated pancreatic ducts appeared to be less prominent and less numerous than normal. The histological alterations observed in this study are in good agreement with the results reported by Popper and Sorter (27) and Gibbs and Ivy (28), after the ligation of pancreatic ducts of dogs. Gross observations of the carcasses and viscera of these baby pigs did not reveal any differences between sham operated control pigs and the pancreatic duct ligated pigs. The wounds were found to be healing nicely, and no active peritonitis was apparent.

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Effect of Zinc Toxicity on Calcium, Phosphorus and Magnesium Metabolism of Young Rats[1,2]

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ABSTRACT The effects of high levels of dietary zinc on bone mineralization, on growth and on the metabolism of calcium, phosphorus and magnesium of young male rats in the presence and absence of several dietary supplements were studied in a series of experiments. Zinc toxicity resulted in marked decreases in bone calcium and phosphorus levels and marked increases in bone zinc levels at the end of the first week animals were on experiment. Significant decreases in weight gain associated with zinc toxicity did not occur until the second week. Bone magnesium levels were not affected by zinc toxicity. Supplements of calcium and phosphorus alleviated the adverse effects of zinc on weight gain and on the deposition of calcium and phosphorus in the bone. Calcium and phosphorus supplements prevented the marked accumulation of zinc in the bones of young rats fed high levels of zinc. Zinc toxicity resulted in highly significant decreases in the retentions of calcium, phosphorus and magnesium by young rats which occurred at the end of the first week the animals were on experiment.

Although Sadasivan (1) reported more than a decade ago that high levels of dietary zinc (0.5 and 1.0%) prevented the normal deposition of calcium and phosphorus in the bones of young rats, the exact nature of this interference of zinc with bone mineralization has not been determined. Other reports indicate that high levels of dietary zinc are associated with decreases in the retention of phosphorus in rats (2, 3) and decreases in the retentions of both calcium and phosphorus in lambs (4). Whiting and Bezeau (5), however, reported that dietary zinc increased the retention of calcium in pigs, but had no influence on phosphorus retention.

Abelson and Aldous (6) reported that excessive zinc interferred with the metabolism of magnesium in several species of bacteria. Adiga et al. (7) reported that zinc toxicity caused a conditioned magnesium deficiency in *Aspergillus niger* which could be alleviated by supplementing the culture media with magnesium. Recently, Sastry et al. (8) have found that the toxicity of several metals, including zinc, on the growth of *Neurospora crassa* can be alleviated by magnesium supplementation.

The specific objectives of the present study were to determine the effect of zinc toxicity on calcium, phosphorus and magnesium levels of the bones of young rats in the presence and absence of dietary supplements of calcium, phosphorus and magnesium and to investigate the effect of a high level of zinc on the absorption, utilization and retention of calcium, phosphorus and magnesium. Changes in weight gains and bone zinc levels of the zinc-fed rats under the various dietary conditions were observed also.

MATERIALS AND METHODS

Weanling male Sprague-Dawley rats were used in all experiments. The animals were housed in individual wire-bottom cages in an air conditioned room maintained at 24.5°C. Food and water were offered ad libitum. All animals used in a particular experiment were randomized into replications on the basis of initial body weight.

The composition of the basal diet was: (in per cent) casein, 19;[4] starch, 63;[5] vege-

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[2] Supported in part with a grant from The University of North Carolina at Greensboro Research Council.

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[4] Vitamin Test Casein, Nutritional Biochemicals Corporation, Cleveland.

[5] Globe Easy-flow Corn Starch 3366, Corn Products Sales Company, Greensboro, North Carolina.

table oil, 10;[6] mineral mix, 4;[7] vitamin mix, 2;[8] cellulose, 2;[9] and oleum percomorphum.[10] Chemical analyses of samples of the basal diet taken periodically throughout the study revealed that it contained an average of 0.52% of calcium, 0.55% of phosphorus, 0.04% of magnesium and 10 ppm of zinc. Supplements were incorporated into the basal diet at the expense of equal amounts of starch. Zinc was fed as the carbonate; supplements of calcium, phosphorus and magnesium were in the carbonate, potassium dihydrogen phosphate and sulfate forms, respectively.

Bone mineralization and growth experiments

With the exception of experiment 2, rats from randomly selected replications were killed at the end of each experiment, and the femurs of each animal were removed. The bones were cleaned of muscle and fat, dried in an oven and weighed on an analytical balance. The bone samples were wet-ashed with nitric and perchloric acids on a hot plate. The ash of each pair of bones was dissolved in 3 ml of 0.6 N HCl and brought to a volume of 100 ml with distilled water. Five milliliters of the first dilution were rediluted to 100 ml, and appropriate aliquots of the second dilution were taken for the calcium and phosphorus determinations. Appropriate aliquots of the first dilution of each bone sample were used for the magnesium and zinc determinations.

Calcium, phosphorus, magnesium and zinc determinations were made on the bone samples by the methods of Weybrew et al. (9), Simonsen et al. (10, 11) and McCall et al. (12), respectively.

Experiments 1 and 2. The objective of these experiments was to characterize the effect of zinc on bone mineralization with respect to the dietary level of zinc required to produce a change in mineral levels and to the time of onset of the symptoms. Levels of zinc tested in these experiments were 0.0, 0.25, 0.5 and 0.75%. In experiment 1 six animals were maintained with each of the 4 dietary regimens for 4 weeks. Forty-eight animals were divided equally into 4 groups in experiment 2. Each group received one of the dietary regimens previously mentioned. At weekly intervals 4 animals from each group were killed, and the femurs of each animal were removed for subsequent analyses.

Experiments 3 and 4. The primary purpose of these experiments was to determine the effects of calcium, phosphorus and magnesium supplements on bone mineralization of zinc-fed rats. In experiment 3 supplements of 0.4% of calcium or phosphorus and 0.08% of magnesium were added to diets containing 0.75% of zinc (table 1). Supplements of 0.4, 0.8 and 1.2% of calcium or phosphorus were added to diets containing 0.75% of zinc in experiment 4 (table 2).

Mineral balance study

Eight animals, averaging 53 g in weight initially, were divided into 2 groups. One group received the basal diet; the other group received the basal diet containing 0.75% of zinc. The basal diet used in the balance study, by chemical analyses, contained 0.45% of calcium, 0.59% of phosphorus and 0.03% of magnesium; and the diet with added zinc contained 0.46% of calcium, 0.58% of phosphorus and 0.03% of magnesium. Each animal was placed in a wire-bottom metabolism cage which was fixed in such a manner so that feces and urine could be collected separately. Fresh feed was provided daily, and distilled water was available to the animals at all times.

All animals received the basal diet during a 4-day preliminary period prior to the experimental period. Four consecutive 7-day collection periods were used during the experimental period. During a collection period the respective excreta from an individual animal were collected daily,

[6] Crisco, Procter and Gamble Company, Cincinnati.

[7] Salt Mixture W, Nutritional Biochemicals Corporation, Cleveland. The composition of this salt mixture is listed as: (in per cent) $CaCO_3$, 21.000; $CuSO_4 \cdot 5H_2O$, 0.039; $FePO_4 \cdot 2H_2O$, 1.470; $MnSO_4$, 0.020; $MgSO_4$, 9.000; $KAl(SO_4)_2 \cdot 12H_2O$, 0.009; KCl, 12.000; KH_2PO_4, 31.000; KI, 0.005; NaCl, 10.500; NaF, 0.057 and $Ca_3(PO_4)_2$, 14.900.

[8] Each 100 g of vitamin mix contained: (in milligrams) 0.1% vitamin B_{12} (with mannitol), 0.1; biotin, 1; folic acid, 5; thiamine·HCl, 25; pyridoxine·HCl, 25; 2-methyl-naphthoquinone, 50; riboflavin, 50; nicotinic acid, 50; Ca pantothenate, 150; p-aminobenzoic acid, 500; (in grams) inositol, 5; choline chloride, 7.5; DL-methionine, 30; cornstarch, 56.6. All vitamins and methionine were purchased from Nutritional Biochemicals Corporation, Cleveland.

[9] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[10] Each kilogram of diet contained 24 drops of oleum percomorphum, Mead Johnson and Company, Evansville, Indiana.

TABLE 1

Responses of zinc-fed rats [1] to supplements of calcium, phosphorus and magnesium [2]

Diet	Wt gain at 4 weeks [3]	Bone constituent [4]			
		Ca	P	Mg	Zn
	g	*mg/g dry weight*			
Basal	182 ± 8	162.7 ± 8.7	87.0 ± 4.0	2.98 ± 0.13	0.11 ± 0.01
+ 0.75% Zn	97 ± 8	110.9 ± 5.2	77.0 ± 6.4	2.99 ± 0.13	2.69 ± 0.26
+ 0.75% Zn + 0.4% Ca	126 ± 9	133.9 ± 8.4	80.9 ± 1.4	2.92 ± 0.07	1.72 ± 0.29
+ 0.75% Zn + 0.4% P	106 ± 9	109.4 ± 8.4	74.8 ± 3.3	2.77 ± 0.09	1.95 ± 0.24
+ 0.75% Zn + 0.08% Mg	113 ± 7	100.2 ± 6.1	73.3 ± 0.6	3.20 ± 0.18	2.29 ± 0.27
+ 0.75% Zn + 0.4% Ca + 0.4% P	154 ± 5	158.2 ± 8.2	87.5 ± 4.7	3.00 ± 0.14	1.06 ± 0.04
+ 0.75% Zn + 0.4% Ca + 0.08% Mg	129 ± 7	145.3 ± 14.8	85.0 ± 2.4	3.48 ± 0.09	1.43 ± 0.12
+ 0.75% Zn + 0.4% P + 0.08% Mg	128 ± 13	117.2 ± 8.0	85.6 ± 5.7	3.11 ± 0.24	1.54 ± 0.16
+ 0.75% Zn + 0.4% Ca + 0.4% P + 0.08% Mg	139 ± 11	125.9 ± 4.8	83.0 ± 6.0	3.18 ± 0.15	0.84 ± 0.10

[1] Sprague-Dawley rats averaging 49 g in weight initially.
[2] Each figure represents mean ± SE.
[3] Each figure is the mean of 6 animals.
[4] Each figure is the mean of 4 animals.

TABLE 2

Effects of calcium and phosphorus supplements on rats [1] fed high levels of zinc [2]

Diet	Wt gain at 4 weeks	Bone constituent			
		Ca	P	Mg	Zn
	g	*mg/g dry weight*			
Basal	148 ± 5	163.4 ± 4.8	83.4 ± 1.8	2.41 ± 0.25	0.10 ± 0.01
+ 0.75% Zn	104 ± 9	116.4 ± 5.9	73.2 ± 1.4	2.32 ± 0.20	1.29 ± 0.16
+ 0.75% Zn + 0.4% Ca	124 ± 4	132.3 ± 5.4	79.0 ± 2.9	2.18 ± 0.20	1.03 ± 0.20
+ 0.75% Zn + 0.4% P	113 ± 3	118.3 ± 4.4	73.5 ± 1.5	2.28 ± 0.09	0.99 ± 0.15
+ 0.75% Zn + 0.8% Ca	131 ± 2	149.1 ± 3.9	79.9 ± 1.1	2.18 ± 0.18	0.99 ± 0.09
+ 0.75% Zn + 0.8% P	116 ± 5	124.6 ± 3.2	69.9 ± 3.5	2.27 ± 0.10	0.68 ± 0.10
+ 0.75% Zn + 1.2% Ca	120 ± 4	146.7 ± 3.0	78.3 ± 1.3	2.27 ± 0.19	1.13 ± 0.17
+ 0.75% Zn + 1.2% P	119 ± 3	126.0 ± 4.5	72.6 ± 1.9	1.91 ± 0.11	0.40 ± 0.05
+ 0.75% Zn + 0.4% Ca + 0.4% P	131 ± 3	146.0 ± 6.6	74.9 ± 1.7	2.42 ± 0.18	0.76 ± 0.15
+ 0.75% Zn + 0.8% Ca + 0.8% P	132 ± 6	146.6 ± 6.6	80.4 ± 0.9	1.79 ± 0.19	0.28 ± 0.06 [3]
+ 0.75% Zn + 1.2% Ca + 1.2% P	115 ± 4	156.7 ± 3.8	80.5 ± 1.9	1.41 ± 0.20 [3]	0.26 ± 0.06 [3]

[1] Sprague-Dawley rats averaging 47 g in weight initially.
[2] Each figure represents mean of 6 animals with SE unless otherwise indicated.
[3] Mean of 5 animals

pooled and kept in sealed containers in a refrigerator. Samples of the pooled fecal and urine collections from each animal for each 7-day period were prepared for mineral analyses by the wet-ashing procedure previously mentioned. The ash of each sample was dissolved in 3 ml of 6 N HCl and brought to a volume of 100 ml with distilled water. Representative samples of the 2 diets were prepared for mineral analyses by the same procedures. Calcium, phosphorus and magnesium determinations were made on appropriate aliquots of these samples by the methods previously mentioned.

Statistical analyses

All data were subjected to an analysis of variance. Statements of significance are based on odds of at least 19 to 1 ($P \leq 0.05$).

RESULTS

Bone mineralization and growth experiments

Experiment 1. The results of this experiment are shown in table 3. Increasing levels of dietary zinc resulted in significant linear decreases ($P \leq 0.05$) in bone calcium and phosphorus deposition, highly significant linear increases ($P \leq 0.01$) in bone zinc levels and highly significant decreases ($P \leq 0.01$) in weight gains. Dietary zinc, at the levels used in this experiment, had no apparent effect on the deposition of magnesium in the bones of young rats.

Experiment 2. Results of this experiment (table 4) indicated that high levels of dietary zinc resulted in marked and highly significant changes ($P \leq 0.01$) in

TABLE 3

Effect of zinc on growth and bone mineralization of young rats [1,2]

Level of dietary zinc	Wt gain at 4 weeks	Bone constituent			
		Ca	P	Mg	Zn
%	g	*mg/g dry weight*			
None	188 ± 8	146.3 ± 8.2	71.6 ± 2.4	3.45 ± 0.05	0.09 ± 0.01
0.25	197 ± 4	143.8 ± 5.5	69.5 ± 1.5	3.37 ± 0.09	0.58 ± 0.04
0.50	161 ± 6	131.4 ± 10.1	65.5 ± 0.6	3.44 ± 0.08	1.34 ± 0.14
0.75	113 ± 9	107.4 ± 8.8	61.3 ± 2.8	3.44 ± 0.13	2.34 ± 0.15

[1] Sprague-Dawley rats averaging 53 g in weight initially.
[2] Each figure represents mean of 6 animals with SE.

TABLE 4

Mean weight gain and bone mineral values of young rats [1] *fed diets containing high levels of zinc* [2]

Weeks on experiment	Level of zinc	Wt gain [3]	Bone constituent [4]			
			Ca	P	Mg	Zn
	%	g	*mg/g dry weight*			
1	None	39 ± 1	140.8 ± 4.3	91.5 ± 1.3	2.57 ± 0.14	0.13 ± 0.02
	0.25	33 ± 1	131.8 ± 2.4	87.1 ± 1.5	2.72 ± 0.09	0.40 ± 0.04
	0.50	31 ± 1	113.2 ± 3.6	85.0 ± 3.7	2.56 ± 0.10	0.61 ± 0.09
	0.75	27 ± 1	116.1 ± 4.5	84.4 ± 2.2	2.50 ± 0.08	1.01 ± 0.15
2	None	87 ± 2	150.6 ± 1.4	92.5 ± 0.6	2.59 ± 0.15	0.40 ± 0.00
	0.25	76 ± 4	143.4 ± 5.2	89.8 ± 1.3	2.54 ± 0.17	0.54 ± 0.05
	0.50	67 ± 2	122.5 ± 8.3	84.4 ± 1.7	3.21 ± 0.35	0.90 ± 0.10
	0.75	61 ± 2	114.2 ± 3.4	81.4 ± 1.9	2.54 ± 0.02	1.46 ± 0.12
3	None	134 ± 3	156.0 ± 5.6	93.7 ± 0.9	2.81 ± 0.17	0.13 ± 0.01
	0.25	121 ± 4	144.6 ± 5.8	89.4 ± 0.8	2.75 ± 0.05	0.77 ± 0.09
	0.50	107 ± 2	142.3 ± 7.3	88.7 ± 1.2	2.71 ± 0.07	1.77 ± 0.06
	0.75	88 ± 4	125.6 ± 7.0	84.4 ± 1.0	2.83 ± 0.08	2.39 ± 0.12

[1] Sprague-Dawley rats averaging 49 g in weight initially.
[2] Each figure represents mean ± SE.
[3] Mean of 16, 12 and 8 animals for weeks 1, 2 and 3, respectively.
[4] Each figure is the mean of 4 animals.

the levels of calcium and zinc and a significant change ($P \leq 0.05$) in the level of phosphorus in the bones at the end of the first week. Bone magnesium values varied from week to week and from diet to diet, but these differences were not found to be statistically significant. Analysis of the data indicated that weight gains of animals fed 0.75% of zinc were significantly lower ($P \leq 0.01$) than the weight gains of the controls at the end of the second week.

Experiment 3. Analysis of the results (table 1) revealed that supplementing the high zinc diet with calcium resulted in bone calcium levels which were significantly higher ($P \leq 0.05$) than those of rats fed only zinc. Although supplements of phosphorus or magnesium alone did not prevent the marked decreases in bone calcium, the combination of either of these minerals with calcium resulted in increases in bone calcium which were greater than those observed in the zinc-fed rats receiving only calcium. The addition of either phosphorus or magnesium, but not calcium, to the high zinc diet was associated with increases in the accumulation of phosphorus in the bones. Various combinations of these supplements resulted in bone phosphorus values in zinc-fed rats which were essentially the same as those observed in the control animals. The deposition of magnesium in the bones of animals receiving zinc diets supplemented with magnesium was significantly greater ($P \leq 0.05$) than the level of magnesium that accumulated in the bones of animals fed diets not supplemented with magnesium.

The degree to which zinc accumulated in the bones was closely related to the type of supplementation. The addition of either calcium or phosphorus to the high zinc diet was associated with a highly significant reduction ($P \leq 0.01$) in the amount of zinc deposited in the bone, whereas the addition of magnesium had no apparent effect on bone zinc. A combination of all 3 mineral supplements, however, resulted in the least amount of zinc accumulation in the bones of animals receiving added zinc in their diets.

The results indicated that supplements of calcium, but not phosphorus or magnesium, partially alleviated the marked reduction in weight gain associated with zinc toxicity. Greatest improvement in weight gain of zinc-fed rats occurred when a supplement of calcium and phosphorus was included in the diet. A combination of phosphorus plus magnesium or one of calcium, phosphorus and magnesium also resulted in substantial increases in weight gains of rats fed high levels of zinc.

Experiment 4. As noted in the previous experiment, supplements of calcium resulted in a significant improvement in bone calcium levels of zinc-fed rats, whereas the addition of phosphorus to the diet resulted in little improvement in bone calcium levels (table 2). Increasing the calcium supplement from 0.4 to 0.8% resulted in an additional increase in bone calcium levels of zinc-fed rats. A supplement of 1.2% of calcium resulted in the same increase in bone calcium as did the 0.8% supplemental level. The mean bone calcium values of the zinc-fed rats supplemented with either 0.4% of calcium and phosphorus or 0.8% of calcium and phosphorus were no different from the mean bone calcium values of the rats supplemented with either 0.8 or 1.2% of calcium. A supplemental level of 1.2% of calcium and phosphorus completely alleviated the marked decrease in the calcium level of the bones of the zinc-fed animals since the mean calcium value of these rats was not significantly different from that of the control animals.

In this experiment the addition of calcium supplements partially alleviated the decrease in bone phosphorus, whereas phosphorus supplementation had no beneficial effect on bone phosphorus levels. A combination of either 0.8% of calcium and phosphorus or 1.2% of calcium and phosphorus supplemented in the diet resulted in bone phosphorus levels that approached those of the controls.

No significant reduction in the magnesium content of the bone occurred with an intake of 0.75% of zinc, and no significant increases in the levels of bone magnesium resulted with supplementation.

As observed in experiment 3, supplements of calcium or phosphorus effectively prevented the accumulation of excessive amounts of zinc in the bone. In general a combination of 2 supplements was more

effective than either supplement used alone.

Analysis of the weight gain data (table 2) indicated a highly significant increase ($P \leq 0.01$) in weight gain when calcium was supplemented at the 0.8% level or when calcium and phosphorus were given in combination at either the 0.4% or the 0.8% levels. Supplements of only phosphorus at all levels tested resulted in slight increases in weight gains of the zinc-fed rats.

Mineral balance study

Calcium. A summary of the calcium balance data is given in table 5. A highly significant decrease ($P \leq 0.01$) in the retention of calcium by rats fed the high zinc diet was noted at the end of one week. The data also indicated that calcium retention decreased in the zinc-fed rats as the length of time the animals were kept on the dietary regimen increased. With the basal diet the amount of calcium retained by the animals was approximately the same each week of the experimental period.

Although the feeding of zinc was associated, initially, with a highly significant increase ($P \leq 0.01$) in urinary calcium, the amounts of calcium excreted *via* the urine by both groups of animals were essentially the same by the end of the fourth week. The feeding of zinc resulted in a highly significant increase ($P \leq 0.01$) in fecal calcium during the first week of the experimental period. During the first 2 weeks, approximately twice as much calcium was excreted in the feces of the zinc-fed rats as was excreted by the controls. The amount of fecal calcium of the zinc-fed rats was approximately 1.5 times that excreted by the controls during the third and fourth weeks.

Phosphorus. Table 6 contains the results of the phosphorus balance. Analysis

TABLE 5

Calcium balance summary [1]

Weeks on experiment	Diet	Ca intake	Fecal Ca	Urinary Ca	Ca retention
		mg/day	*mg/day*	*mg/day*	*mg/day*
1	Basal	48.46	16.52	0.08	31.86
	+0.75% Zn	45.69	31.11	0.28	14.30
2	Basal	56.24	21.07	0.07	35.10
	+0.75% Zn	51.26	45.00	0.23	6.03
3	Basal	58.49	27.43	0.16	30.90
	+0.75% Zn	54.20	40.30	0.30	13.60
4	Basal	67.40	34.13	0.26	33.01
	+0.75% Zn	57.89	48.49	0.28	9.12

[1] Each figure is the mean of 4 animals.

TABLE 6

Phosphorus balance summary [1]

Weeks on experiment	Diet	P intake	Fecal P	Urinary P	P retention
		mg/day	*mg/day*	*mg/day*	*mg/day*
1	Basal	63.53	8.02	14.80	40.72
	+0.75% Zn	57.99	21.05	7.48	29.46
2	Basal	73.74	8.60	20.40	44.73
	+0.75% Zn	65.06	28.64	14.82	21.59
3	Basal	76.68	11.72	23.56	41.40
	+0.75% Zn	68.80	21.61	14.10	33.09
4	Basal	88.36	14.90	26.71	46.74
	+0.75% Zn	73.48	26.33	14.40	32.74

[1] Each figure is the mean of 4 animals.

TABLE 7

Magnesium balance summary [1]

Weeks on experiment	Diet	Mg intake	Fecal Mg	Urinary Mg	Mg retention
		mg/day	*mg/day*	*mg/day*	*mg/day*
1	Basal	3.35	0.97	0.10	2.28
	+0.75% Zn	2.70	1.16	0.12	1.42
2	Basal	3.88	1.15	0.02	2.71
	+0.75% Zn	3.03	1.44	0.25	1.34
3	Basal	4.04	1.24	0.16	2.64
	+0.75% Zn	3.20	1.55	0.13	1.52
4	Basal	4.65	1.57	0.09	2.99
	+0.75% Zn	3.42	1.47	0.16	1.79

[1] Each figure is the mean of 4 animals.

of the data showed that a dietary level of 0.75% of zinc resulted in a highly significant decrease ($P \leq 0.01$) in phosphorus retention as early as the first week of the experimental period. In contrast with the results obtained on the calcium balance, the amount of phosphorus retained by either the control animals or the zinc-fed animals was essentially the same for the respective groups during the entire experimental period.

Zinc toxicity resulted in a highly significant decrease ($P \leq 0.01$) in urinary phosphorus and an increase in the amount of phosphorus in the feces which was significant at the 1% level of probability. In general, the urinary and fecal excretion of phosphorus remained fairly constant in the zinc-fed rats throughout the entire experimental period. There were slight increases in urinary and fecal excretion of phosphorus in the control animals as the number of weeks increased.

Magnesium. Analysis of the results of the magnesium balance (table 7) indicated that a highly significant decrease ($P \leq 0.01$) in magnesium retention occurred in animals fed the high zinc diet. The significant decrease in magnesium retention in the zinc-fed rats occurred during the first week of the experimental period. The amount of magnesium retained by either the control animals or the zinc-fed animals increased slightly as the number of weeks increased.

In general, the feeding of zinc to young rats was associated with increases in the fecal and urinary excretion of magnesium. The fecal excretion of magnesium of both groups of animals increased as the number of weeks increased.

Covariance analyses. The effects of zinc toxicity on the excretion and the retention of calcium, phosphorus and magnesium were still significant after adjustments were made for food intake by covariance. The results of the covariance analyses indicated that the significant reduction in the retention and the marked increase in the excretion of these minerals were due to dietary zinc and not to food intake.

DISCUSSION

The overall results of this study indicate that zinc has an antagonistic effect on the normal deposition of calcium and phosphorus in the bones of young rats which can be alleviated with calcium and phosphorus supplements. Since the feeding of zinc was associated with an increase in the excretion of calcium and phosphorus, there is a possibility that additional calcium and phosphorus supplements to a high zinc diet supply sufficient amounts of these minerals to replace the amounts lost by the adverse action of zinc. Added dietary supplements of calcium and phosphorus may, on the other hand, facilitate the removal of excess zinc from the animal body so that near normal conditions exist with respect to zinc absorption and utilization. That calcium and phosphorus supplementation prevented the marked increase in the accumulation of zinc in the bone tends to support this possibility. Lewis et al. (13) reported that increases in the level of dietary calcium resulted in decreases in the zinc levels of several or-

gans of swine and proposed that excessive amounts of calcium probably reduced the zinc content of the organs by hindering zinc absorption from the gastrointestinal tract.

Results obtained in this study showing that calcium and phosphorus supplements alleviated the adverse effect of zinc on growth are consistent with the idea that these supplements facilitate the removal of excess zinc from the animal body. Supplementing a high zinc diet with calcium and phosphorus resulted in a marked increase in weight gain. A smaller, but significant, increase in weight gain was evident with calcium alone. Although phosphorus alone was largely ineffective in promoting increases in weight gains of zinc-fed rats, it appears to be essential for establishing and maintaining the proper calcium-to-phosphorus ratio which is conducive to normal growth.

The thesis that the beneficial effect of supplemental calcium and phosphorus is to remove excess zinc is further supported by the results of the mineral balance study. The overall results of this study indicate that the primary effect of zinc toxicity on calcium and phosphorus is to interfere with the normal absorption and to increase the fecal excretion of these minerals in the young rat. Approximately, 42% of the total daily calcium intake and 14% of the total daily phosphorus intake were excreted in the feces by animals receiving the basal diet. In the zinc-fed rats, these figures were 78 and 37%, respectively. Presumably, calcium and phosphorus are utilized to aid in the elimination of excess zinc, and smaller amounts of these minerals are available to the animal for normal absorption. When excessive amounts of calcium and phosphorus are added to the high zinc diet, sufficient amounts of these minerals are then available to remove the excess zinc and to allow for adequate retention of these minerals by the animal body. Results of Newland et al. (14) indicating that increasing the level of calcium in diets containing added zinc resulted in increases in the fecal excretion of zinc support this possibility. In connection with parakeratosis, Hoekstra et al. (15) suggested that calcium might accentuate the disease by either hindering zinc absorption or by promoting the excretion of zinc. Their data (15), however, indicated that calcium was not altering the pH of the intestinal tract and thereby affecting the absorption of zinc.

Since the calcium-to-phosphorus ratios of some of the diets used in this study are fairly extreme, there is a possibility that some of these ratios could have accounted for some of the depressing effects on calcium and phosphorus levels. Wasserman (16), however, has pointed out that although the calcium-to-phosphorus ratio should be considered in assessing dietary adequacy, the absolute levels of these minerals in the diet should be recognized as important factors also. Presumably, the calcium-to-phosphorus ratio of the diet is an important factor when either element is present in deficient amounts. Under some conditions of severe changes of calcium-to-phosphorus ratio, there may be depression of the availability of the ion present in the least amount, but this relationship may not necessarily hold under other conditions (16). The calcium and phosphorus supplied by the basal diet used in this study do not appear to be present in deficient amounts. In most cases those combinations of calcium and phosphorus supplements that did not alleviate the effect of zinc toxicity in this study did not result in weight gains and bone calcium and phosphorus levels which were lower than those observed when zinc alone was added to the basal. Thus, the depressing effect of excessive dietary calcium and phosphorus does not appear to be a major factor under the conditions of this study.

Zinc toxicity did not significantly decrease weight gain until the second week of the experimental period. Since significant changes in bone mineralization occurred in zinc-fed rats during the first week, there is the possibility that the interference of zinc with bone formation precedes the interference with growth.

The data indicate that high levels of zinc have an adverse effect on both the absorption and the utilization of magnesium. In rats receiving the basal diet, an average of about 67% of the total daily magnesium intake was retained, whereas in the zinc-fed rats, the average value was approximately 49%. In some instances supple-

ments of calcium and phosphorus to the high zinc diet resulted in a further reduction in bone magnesium levels. There is a possibility that the excessive calcium and phosphorus supplied by these diets had some depressing effect on magnesium as it has been shown that the magnesium requirement of the rat is increased when the calcium and phosphorus content of the diet is increased (17–19).

The data of this investigation indicate that calcium is affected to a greater extent by high levels of zinc than is phosphorus or magnesium. The effects of zinc on the movements of phosphorus and magnesium appear to be similar. Some of the data also suggest the possibility of a complex interrelationship in the animal involving calcium, phosphorus, magnesium and zinc.

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