University of Alberta Library

0 1620 3448525 8

For Reference

NOT TO BE TAKEN FROM THIS ROOM

THE RELATIONSHIP BETWEEN CERTAIN
DIETARY FACTORS AND SERUM
ALKALINE PHOSPHATASE OF RATS

by

RIDLEY KUSTAA SHAW
B.Sc. (Alberta)

Thesis
1950
#46

Ex LIBRIS
UNIVERSITATIS
ALBERTAENSIS

Digitized by the Internet Archive
in 2018 with funding from
University of Alberta Libraries

<https://archive.org/details/relationshipbetw00shaw>

THE UNIVERSITY OF ALBERTA

THE RELATIONSHIP BETWEEN CERTAIN DIETARY FACTORS AND
THE SERUM ALKALINE PHOSPHATASE OF RATS.

A DISSERTATION

SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE.

FACULTY OF AGRICULTURE

by

RIDLEY KUSTAA SHAW

EDMONTON, ALBERTA.

1950

ACKNOWLEDGEMENT.

I wish to express my thanks to Dr. J. Tuba for his invaluable guidance and assistance throughout this investigation.

I am also indebted to Mr. R. Clelland for his care of some of the experimental animals and his willing assistance in obtaining blood samples. Personal thanks is also given Miss Ruth Renner, of the Animal Science Department, for the use of her animals and data with regard to the cereal protein diets.

This work was conducted with the assistance of a grant to Dr. J. Tuba from the Division of Medical Research, National Research Council, Ottawa (Project MP 156).

CONTENTS.

	Page.
I. Introduction	1
II. Methods.	
1. The Experimental Animal	6
2. The Experimental Diets	6
3. Blood Sugar Determination	7
4. Serum Alkaline Phosphatase Determ.	9
III. Experimental.	
1. The relationship of dietary protein to the serum alkaline phosphatase, growth and food consumption of weanling male rats with regard to;	
(a) The Level of Casein	12
(b) Cereal Proteins	13
(c) Purified Proteins	14
2. The relationship to serum phosphatase, growth, and food consumption of;	
(a) Fat	16
(b) Methionine	17
(c) Cystine	18
3. The Effect of Methionine, and Choline on Alloxan Diabetes.	
(a) Subcutaneous Injection of Methionine.	23
(b) Oral Administration of Methionine.	24
(c) Dietary Methionine and Choline Supplements.	26

Contents cont'd.

4.	Differentiation of Normal, "High-fat" and Alloxan Diabetes Serum Alkaline Phos- phatase by Enzyme Inhibitors.	29
IV.	Discussion	36
V.	Summary	39
VI.	References	41

INTRODUCTION.

There is extensive evidence in the literature that the concentration of alkaline phosphatase in rat serum may be rapidly and significantly altered by a number of dietary factors, or the nutritional state of the animal. Bodansky (1) showed that only carbohydrate increased the fasting levels of alkaline phosphatase in dogs. Weil and Russel (2), working with rats, found that starvation decreased plasma phosphatase activity and that these lowered levels were elevated following the ingestion of certain unsaturated fatty acids, while saturated fatty acids, proteins and carbohydrates had no effect. Hough and Freeman (3) found that removal of protein from the diet (fat level 33%) of dogs caused an increase in the serum alkaline phosphatase level and later (4) showed that this increase could be offset by feeding 0.5 g. of choline chloride daily. This prevented an increase in phosphatase activity during the early weeks only of the protein deficiency. One gram of cystine ingested daily increased the high phosphatase concentration and puppies fed a diet deficient in choline and low in methionine rapidly showed an elevation of the enzyme level. Oral choline supplements prevented or reversed this high level. Their work indicated that the level of the enzyme varied directly with the concentration

in the diet of labile methyl groups in the form of protein, methionine or choline and that cystine displayed an antagonism towards these methyl groups. Choline deficiency studies with dogs, were carried out by McKibben, Thayer, and Stare (5). Puppies fed diets deficient in choline died in three weeks and the addition of 0.7% methionine or 0.1% choline chloride rendered these diets adequate. The choline deficiency caused a rise in the plasma phosphatase and also increased the liver lipides three to four times. Reports (44) from this department showed that diets high in fat caused the fasting levels of serum alkaline phosphatase of adult male rats to increase to values twice those of the controls. Diets high in carbohydrate caused no increase in the fasting levels and diets high in protein caused only a slight increase. In later dietary studies (45), also in this department, there was found a tendency towards the inverse relationship of the level of dietary protein to the serum alkaline phosphatase activity. When essential amino acids were added to a low-protein diet it was found that methionine lowered, while lysine, phenylalanine, and tryptophane increased the serum alkaline phosphatase.

The work of McKibben et al shows that there appears to be a direct correlation between the liver lipides and the serum alkaline phosphatase. While there

is general agreement in the literature on the effect of high-fat diets and supplements of methionine and choline on liver lipides there is no such agreement of the effect of supplementary cystine.

Best and Huntsman (6) reported in 1935 that choline lowered the liver lipides of rats on various diets. Channon et al (7) studied various purified proteins for lipotropic activity and, among others, reported the following in decreasing order of activity; caseinogen, albumin, gliadin, gelatin and zein. Griffith (8) reported that 30% casein provided sufficient methyl groups to overcome fatty livers in rats. Young rats on 18 - 24% casein diets required 4 - 6 mg. of choline daily to offset fat infiltration of the livers of young rats.

Györgyi and Goldblatt (9) maintained that choline alone was not as effective as choline plus cystine in preventing hepatic injury in rats. They found that 25 - 50 mg. cystine greatly aggravated the hepatic injury caused by a low-protein, high-fat diet. This injurious effect of cystine was reported earlier by Curtis and Newburgh (10)(11).

Clark, Eilert, and Dragstedt (12) reported that 0.5% cystine had no effect on the liver lipides of rats

on high-fat, low-protein diets. Supplee et al (13) also reported that cystine had no effect in offsetting the action of choline. Griffith however felt that cystine had a definite effect but this effect was one of a nutritional deficiency in the diets used (14)(15). The diets used to study the lipotropic factors are low in protein (5 - 12%) and as a result the main deficiency that developed was a lack of cystine. When cystine was added to these diets the nutritional state was improved so that more food was consumed and as a result there appeared an increase in the liver lipides or in the incidence of renal damage.

Channon et al (16) and Salmon (17) attributed to cystine a definite antilipotropic effect although their data revealed that there was an increase in the consumption of these diets. Treadwell (18) reported in early work that 1017 mg.% cystine would offset the beneficial effect of 0.7% methionine in a high-fat, low-protein diet. In later work, Treadwell (19) said that if the protein was lowered from nine to five percent, then the addition of cystine would not produce an increase in growth and yet it would exhibit an antilipotropic effect.

Recent work by Canepa et al (20) with pancreatic duct ligated dogs showed that oral administration of

"Dragstedt's lipocaic" and "Chaikoff's pancreatic fraction C 27" lowered the abnormally high serum alkaline phosphatase values. Best (21), Huntsman (22), and Chaikoff (23) all reported that choline was effective in lowering the increased liver lipides of depancreatized dogs.

On the basis of this work it was felt that a further study of the effect of protein on serum alkaline phosphatase was warranted. Also an attempt was made to clarify further the relationship of the main lipotropic factors to the serum alkaline phosphatase of normal and alloxan diabetic rats.

II. METHODS.

1. The Experimental Animal.

Immature and adult male albino rats (Wistar Strain) were used in all experiments. They were kept in all-metal cages and except in one experiment were fed and watered ad lib. Food consumption and rates of growth of all animals were noted. Bleeding for estimation of serum alkaline phosphatase and blood sugars was from the tail. Serum was stored at 5°C, when necessary, but enzyme levels were determined within two to three days after the blood was collected.

2. The Diets.

All diets for alloxan diabetic animals consisted of ground Purina Fox Checkers with addition of the required supplements. All other diets, excepting those with cereal grains, contained 4% McCollum's Salt Mixture, 2% cod liver oil, and adequate supplements of thiamine, pyridoxine, pantothenic acid, nicotinic acid, and riboflavin. Except when choline-free diets were fed, 0.1% choline chloride was added. The remaining 94% was made up with sucrose, Crisco, and purified protein. The cereal grain diets contained; 3% Crisco, 4% McCollum's Salts, 4 g. fish oil (400 units D, 2400 units A), and adequate supplements of choline, thiamine, pyridoxine, pantothenic acid, nicotinic acid, riboflavin, biotin and tocopherol.

3. Blood Sugar Determination (47).

Reagents.

(a) Ferric iron-gum ghatti solution: Suspend gum ghatti tears (20 g./liter water) for 18 hours at the top of a large beaker filled with water. Filter. Add 7 g. ferric sulfate hydrate dissolved in 75 ml. 85% phosphoric acid (per liter). Add small amounts of 1% KMnO_4 solution until a trace of pink persists for 15 minutes. This destroys certain reducing materials present in the gum ghatti.

(b) Cyanide-carbonate buffer: Add 4 g. sodium carbonate dissolved in 20 - 25 ml. water to 0.75 g. sodium cyanide dissolved in 75 ml. water. The combined solutions are then diluted to 500 ml. with water.

(c) Potassium ferricyanide solution: 250 mg. potassium ferricyanide are dissolved in 500 ml. water.

(d) Dilute tungstic acid: 10 ml. of 0.67N sulfuric acid and 10 ml. of 10% sodium tungstate are added separately to 480 ml. water with shaking.

Collection of Blood Samples.

A 0.020 ml. hemoglobin pipette is filled by suction. The pipette is wiped clean with Kleenex and the blood level is adjusted to the mark. The blood is discharged into 5 ml. of the tungstic acid reagent and

the pipette is rinsed by drawing the tungstic acid up several times. By blowing through the solution with the pipette the sample is thoroughly mixed. After 15 minutes the mixture is centrifuged.

Procedure.

1.0 ml. of the supernatant solution is transferred to a tube (15 x 100 mm.). 1.0 ml. of the potassium ferricyanide solution is then added and the tube is placed for 15 seconds in a boiling water bath. It is then removed from the bath, 1.0 ml. of the buffer is added, the tube is covered with large marbles and immediately placed in the boiling water bath for 15 minutes. After this time has elapsed the tubes are cooled in an ice bath to 30°C, then 1.0 ml. of the ferric iron-gum ghatti solution is added. 1.0 ml. water is added to bring the total volume to 5.0 ml. and the contents are thoroughly mixed. After 30 minutes the amount of ferri-cyanide is estimated in a Coleman Universal spectrophotometer set at 640 millimicrons. A blank, of 1 ml. water, treated in the same manner as the sample is used in setting the photometer at zero. A calibration curve determined from glucose samples of known concentration

is used to change the photometer readings to sugar concentration. When the blood glucose exceeds 200 mg. %, 0.5 ml. samples are used.

4. Serum Phosphatase Determination.

Reagents.

(a) Substrate - buffer mixture: (pH 9.8)

0.4240 g. sodium diethylbarbiturate
(Veronal, Merck)

0.5000 g. sodium beta-glycerophosphate
(Merck)

0.2464 g. magnesium sulfate . 7 H₂O

Dissolved in 100 ml. double distilled C ϕ ₂-

free water. The substrate was prepared

every ten days and stored in the refrigerator.

(b) Trichloroacetic acid: 10 g. trichloroacetic acid made up to 100 ml. with double distilled water.

(c) Molybdic acid: Prepared daily by adding one part 7.5% sodium molybdate to one part cold 10 N sulfuric acid, with constant shaking.

(d) Stannous chloride: Stock solution - 6.0 g. SnCl₂ + 10 ml. conc. HCl. This is covered with a toluene layer and stored in the refrigerator. Dilute solution - 0.2 ml. of the stock reagent is diluted to 100 ml. with cold, double distilled water and used within four hours.

Procedure (24)(25)(26).

Inorganic phosphorus was determined on 0.2 ml. of serum diluted 1:10 and the alkaline phosphatase was determined on 0.2 ml. of serum diluted 1:100. 0.4 ml. water is added to the phosphorus tube and after warming in a constant temperature bath ($37^{\circ}\text{C} + 0.01$) 0.4 ml. of the substrate solution is added to the other. After exactly one hour 0.4 ml. of trichloroacetic acid is added to both tubes. The tubes are then centrifuged and 0.5 ml. of the supernatant is pipetted into two other tubes containing 0.7 ml. 0.1 N NaOH. The color is developed by adding 0.4 ml. molybdic acid and then 0.4 ml. stannous chloride, shaking both times to insure thorough mixing of the contents of the tubes. The intensity of the color which develops due to the formation of reduced oxides of molybdenum is read after twenty minutes in the Coleman Universal spectrophotometer set at 600 millimicrons. A blank using 0.5 ml. of double distilled water instead of the original supernatant is treated as ^{were} the samples and is used in setting the photometer at zero.

The percent transmittance obtained from the spectrophotometer is converted into concentration of phosphorus by the use of a standard graph which is

prepared by plotting the values of a number of different known values of phosphorus concentrations against their percent transmittance.

The unit of phosphatase activity is defined by Shinowara and associates (25) as, "equivalent of one milligram of phosphorus, as phosphate ion, liberated during one hour of incubation at 37°C , with a substrate containing sodium beta-glycerophosphate, hydrolysis not exceeding ten percent of the substrate and optimum pH of the reaction mixture 9.3 ± 0.15 ."

III. EXPERIMENTAL.

1. The relationship of serum alkaline phosphatase, growth, and food consumption of weanling, male rats with regard to:

- (a) The Level of Casein.

Groups of ten weanling male rats, twenty-one days old, were placed on diets containing; 0%, 5%, 10%, 30%, and 91% casein for six weeks. All these diets contained 5% fat. The average daily consumption and average weights as well as the average serum alkaline phosphatase values are given for each group at different intervals in Table I. The standard deviations are indicated for the terminal phosphatase values.

The results show that as the level of casein was lowered from the optimum of 30%, the consumption, growth and serum alkaline phosphatase also decreased. It appears that the lowering of protein subjects the animals to partial starvation and previous work in the Department has shown that as a result phosphatase activity is lowered. There was no indication ~~here~~ of the inverse ratio between the level of protein in the diet and the phosphatase values, reported by Tuba, Cantor, and Richards (45) on a diet consisting of low-protein barley and casein supplements. However the 91% casein diet did show a lowered

TABLE I:

The effect of dietary protein concentration on average serum alkaline phosphatase levels (units/100 ml.)(P), average daily food consumption (g.)(C) and average weight (g.)(W). (Ten rats in each group.)

% Casein.	Two weeks.			Four weeks.			Five weeks.			Six weeks.		
	P	C	W	P	C	W	P	C	W	P	C	W
90	109	5.0	69	135	9.8	124	96	11.4	142	108±24*	10.3	162
30	115	8.0	98	121	11.2	155	--	12.5	175	105±20	14.6	199
10	74	5.6	59	95	9.1	74	76	11.4	77	82±14	8.9	82
5	71	4.8	44	56	5.7	46	57	7.3	45	29±4	8.4	46
0	43	3.5	34	31	2.5	30	--	--	--	--	--	--

*Standard deviation.

growth rate as compared to the optimal diet. This is accounted for by the lowered consumption by these animals; apparently the diet was much less palatable than those containing carbohydrate.

(b) Cereal Proteins.

Six groups of six weanling, male rats, twenty-one days old, were placed on diets containing 93%; low-protein oats (LPO), barley (LPB), or wheat (LPW) and high-protein oats (HPO), barley (HPB), or wheat (HPW). The consumption, weights, and alkaline phosphatase values for the first two weeks are reported in Table II.

TABLE II.

The effect of different cereal proteins, in diets containing 93% of the grain, on the average serum alkaline phosphatase levels (units/100 ml.)(P), and the average weight (g.)(W) of weanling rats.

Diet.	Two weeks.	
	P.	W.
LPO	174	62
HPO	148	82
LPB	146	59
HPB	162	77
LPW	149	53
HPW	164	62

Six rats in each group.

Although it was possible to report the results for only the first two weeks of the experiment it does show that the higher protein cereals gave better growth than the corresponding low-protein grains. The interesting point is that the phosphatase values are much higher than with the diet containing 10% casein (74 units/100 ml.)(Table I) and the value of 108 units/100 ml. reported by Tuba et al (45) after three weeks on standard laboratory diet of checkers. The phosphatase values for LPB of 146 are lower than the three week values of 186 units found by Tuba et al (45), but this can be accounted for on the basis of a lower fat content in the above LPB diet (see below for influence of dietary fat). This also agrees indirectly with the work of Huntsman and Best (6) who ~~they~~ found that "mixed-grain" diets tend to produce fatty livers in rats.

(c) Purified Proteins.

Diets containing 10% casein (Smaco) + 10% raw egg albumin (Nutritional Biochemical Corp.), 10% casein + 10% gluten (N.B.C.), 10% casein + 10% gelatin (B.D.H.), 10% casein + 10% zein (N.B.C.) and a control diet of 20% casein were fed to groups of ten weanling rats for six weeks. The animals receiving the albumin diet were given five micrograms of biotin daily. All diets contained five percent fat. The weights and alkaline phosphatase values at the end of the experiment are reported in Table III.

TABLE III.

The effect of different purified proteins on the average serum alkaline phosphatase levels (reported as a percentage of the basal value)(P) and the average weights (percent of original weight)(W) of weanling rats.

Diet.	Six weeks.	
	P.	W.
Basal*	100	367
Albumin	90	367
Gluten	88	550
Zein	116	319
Gelatin	90	255

*20% casein diet. Ten rats in each group.

There was no marked variation in phosphatase values with these different protein diets, although the growth rates were markedly affected. The basal level of 10% casein is apparently sufficient to take care of amino acid deficiencies or imbalances in the supplementary proteins, since previous work had shown that diets containing 10% or 30% gelatin or 10% or 30% zein killed all animals within one week. 30% gluten diets did afford subnormal growth and the alkaline phosphatase values were correspondingly low.

2. The relationship to serum alkaline phosphatase, growth and food consumption of;

(a) Fat.

Groups of ten weanling male rats were placed on diets containing 10% protein and fat as indicated in Table IV. Each diet was fed for six weeks and the average daily consumption and average serum alkaline phosphatase values are given at two week intervals.

TABLE IV.

The effect of dietary fat concentration on average serum alkaline phosphatase levels (units/100 ml.)(P) and average daily food consumption (g.)(C).

Percent fat in diet.	Two weeks.		Four weeks.		Six weeks.	
	P	C	P	C	P	C
5.0-Diet I	74	5.6	95	9.1	82+13*	8.9
8.5-Diet II	98	5.9	103	6.2	99+12	7.0
25.0-Diet III	252	4.7	244	5.3	237+41	5.9

*Standard deviations are indicated for the terminal Phosphatase values.

Ten rats in each group.

The results in the above table show clearly that increased fat concentration enhanced the serum alkaline phosphatase activity, although at the same time the daily food consumption tended to decrease.

Because both methionine and fat have been shown to influence serum alkaline phosphatase levels, it seemed likely that the ratio of these two in the diet might be of critical importance in determining the concentration of the enzyme. In the low-protein barley diet, fed by Tuba et al (45), the methionine to fat ratio (M:F) was 1:70 and this produced abnormally high enzyme activity. The 25% fat diet, Diet III, above was designed to have a ratio of M:F = 1:70 and again very high phosphatase values resulted. In the 8.5% fat diet, Diet II, M:F = 1:25 and this ratio resulted in phosphatase values comparable to those obtained on the stock laboratory diet of fox checkers. Diet I had M:F = 1:14 and resulted in abnormally low phosphatase values.

(b) Methionine.

Methionine was added to diets with a normal (Diet IV) and an increased (Diet VI) level of fat. Diet V had a supplement of 0.35% DL-methionine which, taking into consideration the methionine content of the casein resulted in M:F = 1:7. A group of eight weanling rats was fed this diet for the usual six week period. The increased M:F ratio produced, by the fifth week, a very pronounced lowering of the enzyme activity which remained at this level until the termination of the experiment.

A group of ten weanling rats was fed a diet containing 25% fat and 10% protein (M:F = 1:70) for three weeks and then 1.45% methionine (Diet VII) was added as a supplement (M:F = 1:14). The high phosphatase values produced by the three week feeding period were lowered 45% in 14 days by the methionine supplement. However the high fat concentration counteracted the methionine effect to such an extent that enzyme activity was not restored to normal, much less to the subnormal values obtained with Diet I, which also had M:F = 1:14, but only contained 5% fat. Higher levels of methionine fed in conjunction with the high-fat diets resulted in loss of weight so these were discontinued. In Table V the effect of methionine supplements is shown.

(c) Cystine.

The methionine-opposing action of cystine is apparently most marked in a high-fat, low-protein diet. It was shown with the 25% fat diet that weanling rats subjected to this regime would in three weeks manifest serum alkaline phosphatase levels as much as 200% above normal. Methionine supplements introduced at the end of the three week lipogenic period had a potent influence in diminishing phosphatase activity. It seemed probable that counter effects of cystine and methionine on the enzyme might also be demonstrated when concentrations

TABLE V.

The effect of methionine supplements on the average serum alkaline phosphatase (units/100ml) (P) and daily food consumption (g.)(C) of diets high in fat content and low in fat content.

Diet.	Two weeks.		Four weeks.		Five weeks.		Six weeks.	
	P	C	P	C	P	C	P	C
10%Casein 5%Fat (Diet IV) (10 rats)	74	5.6	95	9.1	--	--	82+13*	8.9
+0.35 % methionine (8 rats)(Diet V)	98	7.7	90	8.7	50	7.7	45+10	7.7
	P	C	P	C	P	C	P	C
25% Fat	244	5.3	---	---	---	---	237+41	5.9
10% Casein (10 rats) (Diet VI)								
+1.45 % methionine. (10 rats)(Diet VII)	296	6.0	195	4.4	171	4.6	167+35	5.0

*Standard deviation.

in serum were at high levels.

Twenty weanling rats were fed a basal diet of 10% casein and 25% fat for three weeks. A supplement of 1.8% L-cystine was then fed to ten of the animals and the remaining ten received supplements of 1.8% L-cystine + 1.45% DL-methionine. Consumption and serum phosphatase activities are recorded in Table VI at the termination of the three week period on the basal diet and at intervals for three weeks while the amino acids were being fed. The supplementary cystine did not in this three week period produce an enhancement of enzyme activity.

Actually there was a slight decrease in activity and although the results are not shown the cystine supplement caused the deaths of the animals in five weeks. Cystine showed no opposition to the effect of supplementary methionine.

Two groups of eight immature (70 - 80 g.) rats were fed choline-free diets (25% fat, 10% casein) for fourteen days. As seen in Table VII the addition of cystine to a high-fat diet deficient in choline increases the consumption 50% and as a result the phosphatases show a similar increase.

TABLE VI.

The effect of supplementary cystine and methionine on average serum alkaline phosphatase (units/100 ml.)(P) and average food consumption (g.)(C) of rats previously fed a diet containing 25% fat and 10% protein.

Ten rats in each group.

Supplement.	Zero days. P C	Two days. P C	One week. P C	Two weeks. P C	Three weeks. P C
1.8% cystine	235 6.0	254 7.4	235 7.0	206 7.6	198±24* 9.5
1.8% cystine +1.45% meth.	250 7.4	167 4.6	174 6.6	161 6.6	165±27 7.4
1.8% meth- ionine.	296 6.0	195 4.4	171 4.6	167±35 5.0	

*Standard deviation.

TABLE VII.

The effect of supplementary cystine on average serum alkaline phosphatase (units/100 ml.)(P) and average food consumption (g./day)(C) of rats on choline deficient diets. (25% fat, 10% casein).

Diet.	9 days.		14 days.	
	P	C	P	C
No cystine.	158	5.4	172+26*	5.0
+0.9% cystine.	215	9.4	216+23	7.9

*Standard deviation.

Eight rats in each group.

When the consumption of the cystine-supplemented animals was lowered to that of the controls there was a marked drop in the phosphatase activity of the animals. This was attributed to the fact that the supplemented diet was higher in nutritional value and any restriction of consumption subjected the animals to partial starvation.

3. The Effect of Methionine and Choline on Alloxan Diabetes.

It was shown above that methionine supplements; (1) lowered the high level of phosphatase of animals fed diets high in fat; and (2) lowered the subnormal level of phosphatase of animals fed diets low in protein and with normal content of fat. Best (21) and others (22)(23) have reported that choline was effective in lowering the liver

lipides in depancreatized dogs. It was felt that methionine might also show the same effect on alloxan diabetic rats (which presumably show increased liver lipides) and manifest this by a decrease in the very high serum phosphatase concentration. To achieve this purpose methionine was administered in various ways.

(a) Subcutaneous Injection of Methionine.

Fifteen adult, male rats which had been diabetic* for at least three weeks were used. The first day they were bled at zero time (8a.m.), the food was removed and six hours later they were bled again to get the normal diurnal variation. The second day they were bled at zero time and were then given a subcutaneous injection of 50 mg. methionine dissolved in distilled water. They were then treated as ^{on} the previous days. The phosphatase values are shown for the two day period in Table VII.

TABLE VII.

The effect of subcutaneous injections of 50 mg. methionine on the average serum alkaline phosphatase of alloxan diabetic rats. Fifteen rats in each group.

	Control Period.		Test Period.	
	Zero.	Six hours.	Zero.	Six hours.
Serum Phosphatase.	291	267	288	260
Percent variation.	8%		10%	

* Subcutaneous injection of 160 mg. alloxan / kg. body weight.

The results of this short term experiment show that subcutaneous methionine injections had no effect on the serum alkaline phosphatase of alloxan diabetic rats.

(b) Oral Administration of Methionine.

500 mg. methionine was suspended in 1.5 ml. propylene glycol and was fed orally from a pipette by force-feeding. Blood samples were taken at zero time then the methionine suspension was fed and after six hours without food another blood sample was taken. Food consumption until the next morning was noted and the procedure was then repeated. A control group of animals was treated similarly, except that these were fed propylene glycol only. Six alloxan diabetic males were used in each group. Table VIII shows the average daily consumption for the three days preceding the experiment in addition to the average weights, food consumptions, and serum alkaline phosphatase for the test period.

Both groups responded in somewhat the same manner, and there was no lowering of the alkaline phosphatase by the methionine administration. The first day there was a decided drop in the phosphatase values and then as the consumption of food returned to normal the phosphatase rose to approximately the original values.

TABLE VIII.

The effect of oral administration of 500 mg. methionine on average serum alkaline phosphatase (units/100 ml.)(P), average daily food consumption (g.)(C) and average weight

(g.)(W) of alloxan diabetic, male rats.
Six rats in each group.

Group.	Zero days. P C W 0 6	First day. P C W 0 6	Second day. P C W 0 6	Third day. P C W 0 6	Fourth day. P C W 0 6
+ meth.	277 203 34 252	172 150 19 249	183 162 17 241	--- --- 22 243	245 206 24 240
Control.	263 196 35 253	193 195 12 239	--- --- 17 222	221 186 20 227	--- --- --- ---

(c) Dietary Methionine and Choline Supplements.

Supplements of methionine or choline were added to the normal laboratory diet, by mixing these lipotropic substances with ground fox checkers, ^{these diets were} and fed to alloxan diabetic, male, adult rats. Choline chloride was fed at the level of 0.4% and the amino acid was added to the extent of 0.7%, 1.8%, and 3.6%. The first two diets were fed to groups of ten animals and ~~to~~ the last two to groups of six animals. The average weights and alkaline phosphatases are reported in Table IX.

TABLE IX.

The effect of dietary methionine and choline supplements on the average weight (g.)(W) and average serum alkaline phosphatase (units/100 ml.)(P) of diabetic, male adult rats.

Supplement.	Zero time.		Two days.		One week.		Two weeks.	
	P	W	P	W	P	W	P	W
3.6% meth.	371	244	260	241	271	240	279+35	237
1.8% meth.	366	268	287	261	328	266	341+54	271
	Zero time.		Four weeks.		Five weeks.			
	P	W	P	W	P	W		
0.7% meth.	203	251	266	250	251	255		
0.4% choline.	226	257	251	243	258	256		

First two groups of ten rats each.

Second two groups of six rats each.

The average daily consumption figures were unavailable due to excessive wastage by the animals and the difficulty of estimating this amount because of the absorption of urine, everpresent in the litter trays of such animals. However it was felt that with adult animals any abnormal decrease in the food consumption would be reflected by a drop in weight.

The results show that ~~only~~ 3.6% methionine was capable of lowering the phosphatase of the diabetic animals and the decrease in two weeks was 25%. However the significance of this decrease is to be questioned. The animals in all groups showed an improvement in their general condition. This is especially true where the diets were fed for five weeks. Generally, diabetic rats over such a period of time show severe weight losses, becoming quite emaciated and inactive.

The supplements of 0.7% methionine or 0.4% choline appeared to increase the phosphatase values. However it was felt that this was due to the fact that the animals used here had only been diabetic for one week and generally the serum alkaline phosphatase activity tends to increase up to three weeks after injection of alloxan. In addition the supplements may improve the nutritional value of the diets.

The high methionine supplements undoubtedly make the diet less palatable to the rats. This is indicated by the sharp drop in phosphatase activity and then the gradual return to normal concentrations on feeding 1.8% methionine supplements. It is suspected that these animals ate very little the first two days and then as they became accustomed to the diet their consumption increased, keeping their weight approximately constant, and the phosphatases varied accordingly. With the 3.6% methionine supplementation there is still the problem as to whether the phosphatase decrease is real or whether it would return to the original value with a longer period of time. Although the consumption has apparently remained constant, as judged by the weights of the animals, it may be that these rats would behave as the others, but only a longer feeding period would show this. If the weights and phosphatase values remained at a constant level the effect would be real. If the weights decreased further then the consumption would have been lowered and the alteration in phosphatase concentration would be meaningless. In addition there may be a delayed return to the original values because of the increased unpalatability due to the higher methionine supplement. Further work on these diets is warranted.

4. The Differentiation of Serum Alkaline Phosphatase of Normal, "High-fat", and Alloxan Diabetic Rats by Enzyme Inhibitors.

There is a wealth of information in the literature pertaining to the differentiation of the alkaline phosphatases of various tissues. It was felt that such an approach to this work would show whether or not different enzymes were being encountered in the increased levels of serum phosphatase of animals on high^{-fat} diets as compared to the enhanced activity of serum phosphatase in diabetic rats. If this were true then it would be one explanation as to why methionine lowered the phosphatase readily in one instance and only slightly, if at all, in the other.

According to Tauber (29), "cysteine in concentrations above 0.0005 M strongly inhibited the phosphomonoesterase of all organs, inhibited the pyrophosphatase of intestine and kidney to some extent, and had no effect on liver pyrophosphatase. Oxalate and fluoride had no effect on any of the pyrophosphatases at pH 7.8. Oxalate at 0.0001 M to 0.01 M concentration strongly inhibited the phosphomonoesterase of bone and white corpuscles, slightly inhibited the phosphomonoesterase of liver and kidney, and had no effect on phosphomonoesterase of intestine. Sodium fluoride slightly inhibited the phosphomonoesterase of liver and white corpuscles and

did not affect the phosphomonoesterase of bone, kidney, or intestine. Bile salts in concentrations of 0.002 M to 0.1 M strongly inhibited the phosphomonoesterase of liver, bone, and kidney and the pyrophosphatase of kidney. They did not inhibit the phosphomonoesterase of intestine or the pyrophosphatase of intestine and bone, and they activated liver pyrophosphatase."

Drill and Riggs (27) reported that NaCN in concentrations from 0.0001 to 0.1 M reduced the increased human alkaline phosphatases associated with liver damage, to normal levels. Gutman and Jones (28) went further and showed that CN inhibited the serum alkaline phosphatase of normal human subjects and the increased levels of patients with obstructive jaundice and skeletal diseases. They believe that most of the serum alkaline phosphatase is of osseous origin since bone phosphatase is inhibited by CN and liver alkaline phosphatase is CN insensitive. Dubois et al (46) found that beryllium chloride inhibited all phosphatases activated by magnesium and calcium.

The six inhibitors mentioned above were used in in vitro studies on serum from normal and diabetic rats as well as from animals fed a high-fat diet for three weeks prior to the experiment. The latter two groups have abnormally high alkaline phosphatases. The results of these experiments are shown in Table XI.

TABLE XI.

The effect of various inhibitors* on the serum alkaline phosphatase (results are given in percent of values for substrate containing no inhibitor) of normal rats, diabetic rats, and rats fed a diet high in fat.

<u>Inhibitor.</u>	<u>Normal.</u>	<u>"High-fat".</u>	<u>Diabetic.</u>
<u>Cysteine.</u>			
0.0	100	100	100
0.0001M	85	114	114
0.0005M	65	82	83
0.001M	54	75	83
0.01M	14	11	5
<u>Cyanide.</u>			
0.0	100	100	100
0.0001M	107	100	100
0.0005M	53	56	73
0.001M	22	25	23
0.01M	5	0	1
<u>Beryllium.</u>			
0.0	100	100	100
0.0001M	33	28	13
0.001M	15	11	11
<u>Taurocholate.</u>			
0.0	100	100	100
0.0001M	92	98	107
0.001M	87	102	107

* pH adjusted to 9.8 after addition of inhibitor to substrate.

cont. Table XI.

<u>Inhibitor.</u>	<u>Normal.</u>	<u>"High-fat"</u>	<u>Diabetic.</u>
<u>Fluoride.</u>			
0.0	100	100	100
0.0001M	119	100	96
0.0005M	100	113	93
0.001M	108	96	93
0.01M	58	76	70
<u>Oxalate.</u>			
0.0	100	100	100
0.0001M	93	100	93
0.001M	93	100	93
0.01M	82	105	86
0.1M	21	36	43

The results with the three different types of sera for cysteine, cyanide, and beryllium show no differences in the inhibition of the enzyme. However the other three inhibitors do tend to show a difference between the three types of sera. Tauber mentions that bile salts 0.002-0.1M strongly inhibit the phosphomonoesterase of liver, bone, and kidney but not if intestinal in origin. From this it would appear that the enzyme in diabetic and "high-fat" rats is mainly intestinal in origin while the normal enzyme is of liver, bone, and kidney origin. The fluoride inhibition

indicates that in diabetes the enzyme originates in the liver ⁱⁿ and the other two from bone, kidney, and intestines. The most significant indication appears to be in the inhibition with oxalate. This shows ~~rather clearly~~ that in the high-fat animals the enzyme is of intestinal origin and the diabetic and normal enzyme is of kidney and hepatic origin.

These results however have no statistical significance since only one animal of each type was used for each inhibitor. Nevertheless it was felt that a possible explanation was indicated as to why methionine lowers the serum enzyme readily in animals fed a diet high in fat and did not do so, as readily in diabetic animals.

Therefore it can be said that normally the serum alkaline phosphatase arises in the bone, kidney, liver, and intestine. When a diet high in fat is fed to normal animals there are certain enzymatic changes which adapt the animal to the altered diet. Moog (30) in her review describes work that indicates that phosphatase is active in the transport of glucose and fatty acids molecules through the intestinal wall. It is quite feasible that the phosphatase secretion through the biliaries into the intestine is increased. There ^{also be} may be an increase in the secretion of alkaline phosphatase from the salivary glands (42),

by diets high in fat. As a result more phosphatase is absorbed and so more of the enzyme is present in the blood. Flock and Bollman (31) have shown that the phosphatase of the intestinal lymph does increase markedly after the ingestion of a meal high in fat. Whether or not the increase of the enzyme is due to actual production in the intestinal wall is very difficult to say. Furthermore, Verzář (32) has shown that mono-iodoacetic acid, which inhibits the enzyme phosphatase, also inhibits the absorption of fatty acid. This inhibition of fat absorption was still evident when the fatty acid was mixed with bile acids and glycerophosphate, so ~~that~~ it was concluded that iodo-acetic acid acts by preventing in some way the resynthesis of neutral fat in the mucosal cells rather than by preventing phosphorylation.

Now when methionine or choline are added they assist in fat transportation. As shown by Perlman and Chaikoff (35) and Horning and Eckstein (34) methionine and choline increase the phospholipide activity of the liver, and Stetten and Salcedo (33) have shown that choline increases the migration of fat through the body. The possibility then exists that methionine and choline assist in fat transport through some system, which may be increased phospholipide activity, and that in the absence of enough choline and methionine the phosphatase is increased to move the fat.

In the case of diabetic animals we have a different problem. The amount of fat moving through the intestinal wall is no more than with normal animals, but there is a marked increase in the metabolism of fat. Since the diabetic rat mainly uses fat as a source of energy there is presumably an increase in the phosphatase to assist in this increased metabolism of fat. That phosphatase plays a vital role in diabetes seems likely since the enzyme activity increases so soon after the injection of alloxan. Presumably methionine and choline cannot replace phosphatase in such a role. These assumptions are partially borne out by the work of Canepa et al (20). They found that the methionine, choline and inositol content of "Dragstedt's lipocaic" and "Chaikoff's pancreatic fraction C27" did not account for the lowering of the serum phosphatase of pancreatic duct ligated dogs; some unknown and vital substance was at work.

IV. DISCUSSION.

The effects of nutritional factors on serum alkaline phosphatase can be discussed under two broad headings. First, those which exert a nutritional effect and, second, those which alter some vital function within the body.

Work on the dietary level of protein shows that as the level of protein is lowered from the optimum, growth, daily food consumption, and serum alkaline phosphatase decrease. This disagrees with previous work (45) done in this laboratory as well as with the conclusions of Hough and Freeman (3). However there is an explanation for these differences. The diets used by Hough were high in fat and the removal of protein also removed the source of labile methyl groups. As a result there was an increase in enzyme concentration due to the lipogenic effect of the diet and not ^{to} a lack of protein. In the work reported from this laboratory the ⁽⁴⁵⁾ basal diet was comprised largely of barley. The protein content of this diet was increased by replacing part of the barley with casein. ^{for the high phosphatase values} Again the explanation appears to lie with the level of methionine in the diet. Russel et al (36) working with legumes ^{showed} that such diets were deficient in methionine; as a result it would be

expected that the phosphatases would be high. As the barley was removed and casein was added there was an addition of methyl groups and so the phosphatase decreased. There was the added effect of lowering the fat content of the diet as barley was replaced with casein.

The addition of cystine to high-fat, low-protein diets showed that this factor exerts a nutritional effect. When such diets have adequate methionine or choline the addition of cystine actually has a toxic effect on the animals as death resulted soon after, (10% cystine killed young rats within three days). This is in agreement with findings of Curtis and Newburgh (10)(11). However when cystine is added to diets low in methionine and choline there is a definite nutritional improvement of such diets. Consumption is increased, the growth rate is doubled and the phosphatase is increased. As a result it was concluded that cystine showed no antilipotropic effect, as measured by serum alkaline phosphatase.

The effect of fat and methionine is due to an alteration of body function. When diets high in fat are fed to rats there is an accumulation of lipides in the liver. This phenomenon affords another explanation

as to why the serum alkaline phosphatase increases in the serum.

Work on human patients (37)(38) and dogs (40) (3) showed that serum phosphatase is an excellent indication of impaired liver function. As a result one would expect that as the fat content of the liver increased and its function was impaired, the serum phosphatase would rise. On the addition of methionine or choline the liver lipides are lowered and the phosphatase returns to normal.

V. SUMMARY.

1. The level of casein in the diet had a nutritional effect on the serum alkaline phosphatase of normal, weanling rats. That is, as the level of casein was lowered from the optimal of 30%, the phosphatase decreased and paralleled diminished food consumption. 10% casein was the minimal amount which did allow some growth in weanling rats and produced phosphatase activity slightly below normal. 5% casein did not afford growth and phosphatase levels were near starvation values of adult rats.
2. The purified proteins, egg albumin, wheat gluten, gelatin, and zein showed no effect on the serum alkaline phosphatase of growing rats. These were fed as supplements to 10% casein which is generally considered as a critical value to demonstrate the effect of supplements of amino acids or proteins. The biological value of these proteins was manifested in the growth curves.
3. The cereal grains produced an increase in the serum alkaline phosphatase of growing rats. This increase was apparently due to the increased methionine to fat ratio (approximately 1:70 in all cases).

4. 25% fat + 10% casein diets showed an increase in the serum alkaline phosphatase of young rats and this was lowered by the addition of methionine. The phosphatase concentration associated with various diets appeared to be related to the methionine to fat ratio.
5. Cystine supplements did not demonstrate an "anti~~lip~~ lipotropic" effect as measured by serum alkaline phosphatase activity.
6. 3.6% DL-methionine supplements effected a 25% decrease in serum alkaline phosphatase of alloxan diabetic rats in two weeks. The significance of the results is discussed.
7. Preliminary studies with various inhibitors is reported and a possible interpretation of the results is presented.

VI. REFERENCES.

- (1) Bodansky A., J.Biol.Chem. 104, 473, 1934.
- (2) Weil L., and M.A. Russel, J.Biol.Chem. 136, 9, 1940.
- (3) Hough V.H. and S. Freeman, Am.J.Physiol. 138, 184, 1942.
- (4) Hough V.H., E.P. Monahan, Tsan-Wen Li and S. Freeman,
Am.J.Physiol. 139, 642, 1943.
- (5) McKibben J.M., S. Thayer and F.J. Stare,
J.Lab.Clin.Med. 29, 1109, 1944.
- (6) Best C.H. and M.E. Huntsman, J.Physiol. 83, 255, 1935.
- (7) Channon H.J., J.V. Loach, P.A. Loizides and M.C. Manifold,
Biochem.J. 32, 976, 1938.
- (8) Griffith W.H., J.Nut. 22, 239, 1941.
- (9) Györgyi P. and H. Goldblatt, J.Exptl. Med. 75, 355, 1942.
- (10) Curtis A.C. and L.H. Newburgh, Arch.Int.Med. 39, 828, 1927.
- (11) Curtis A.C. and L.H. Newburgh, Arch.Int.Med. 39, 817, 1927.
- (12) Clark D.E., M.L. Eilert and L.R. Dragstedt,
Am.J.Physiol. 144, 620, 1945.
- (13) Supplee G.C., L.S. Gall, J.F. Caul and L.C. Babcock,
J.Dairy Sc. 28, 435, 1945.
- (14) Mulford D.J. and W.H. Griffith, J.Nut. 23, 91, 1942.
- (15) Griffith W.H., J.Nut. 21, 291, 1941.
- (16) Channon H.J., M.C. Manifold and A.P. Platt, Biochem.J. 32,
969, 1938.
- (17) Salmon W.D., J.Nut. 33, 155, 1947.
- (18) Treadwell C.R., M.Groothius and H.C. Eckstein,
J.Biol.Chem. 142, 653, 1942.
- (20) Canepa J.F., M.I. Grossman and A.C. Ivy, Am.J.Physiol. 156,
387, 1949.
- (19) Treadwell C.R., J.Biol.Chem. 176, 1149, 1948.

- (21) Best C.H., G.C. Ferguson and J.H. Hershey, J.Physiol. 79,
94, 1933.
- (22) Huntsman M.E. and F.G. Young, J.Physiol. 85, 8P, 1935.
- (23) Chaikoff I.L., C. Entenman and M.L. Montgomery,
J.Biol.Chem. 168, 177, 1947.
- (24) Bodansky A., J.Biol.Chem. 101, 93, 1933.
- (25) Shinowara G.Y., L.M. Jones and H.L. Reinhart,
J.Biol.Chem. 142, 921, 1942.
- (26) Gould B.S. and H. Schwachman, J.Biol.Chem. 151, 439, 1943.
- (27) Drill V.A. and D.S. Riggs, J.Biol.Chem. 162, 21, 1946.
- (28) Gutman A.B. and B. Jones, Proc.Soc.Exptl.Biol.Med. 71, 572,
1949.
- (29) Tauber H., Chemistry and Technology of Enzymes, p 33.
John Wiley & Sons Inc. 1949.
- (30) Moog F., Proc.Cam.Phil.Soc. June 1945.
- (31) Flock E.V. and J.L. Bollman, J.Biol.Chem. 175, 439, 1948.
- (32) Verzář F. and E.J. McDougall, Absorption from the Intestine
1936, Longman's, London (quoted).
- (33) Stetten D.W. and J. Salcedo Jr., J.Biol.Chem. 156, 27, 1949.
- (34) Horning M.G. and H.C. Eckstein, J.Biol.Chem. 166, 711, 1946.
- (35) Perlman I. and I.L. Chaikoff, J.Biol.Chem. 127, 211, 1939.
- (36) Russel W.C., M.W. Taylor, T.G. Mehrhof and R.R. Hirsch,
J.Nut. 32, 313, 1946.
- (37) Greene C.H., H.F. Shattuck and L. Kaplowitz,
J.Clin.Invest. 13, 1079, 1934.
- (38) Freeman S., Y.P. Chen and A.C. Ivy, J.Biol.Chem, 124, 79, 1938.
- (40) Bodansky A. and H.L. Jaffe, Proc.Soc.Exptl.Biol.^{Med} 31, 1179, 1942.
- (41) Cinelli A.P., Semana med.(Buenos Aires), II, 1292, 1942.
(quoted from C.A. 6643¹, 1942)
- (42) Deame H.W., Am.J.Anat. 80, 321, 1947.

- (44) Cantor M.M., P.A. Wight and J. Tuba,
Tran.Roy.Soc.Can. 52, III, 51, 1948.
- (45) Tuba J., M.M. Cantor and A.G. Richards,
Can.J.Res. E, 27, 25, 1949.
- (46) Dubois K.P., K.W. Cochran and N. Nazer, Science 110, 420, 19
- (47) Reinecke R.M., J.Biol.Chem. 143, 351, 1942.

... ..

... ..

... ..