TABLE I.
The Choline Content of Various Materials as Determined by the Reineckate and Neurospora Methods.

Material	Choline content, %	
	By reineckate	By Neurospora*
Purified diet†	<0.03	<0.04
Semipurified diet‡	<0.03	<0.04
Simplified diet§	0.04	>0.08
Peanut meal	0.21	>0.26
Range of 5 practical diets	0.10-0.13	0.11-0.15
Practical diet plus 5% liver meal	0.17	0.26

* The values preceded by the greater-than sign are lower limits of ranges having higher mean values.
† Diet 661 of McGinnis and co-workers.[3]
‡ Diet of Lucas and co-workers.[4]
§ Diet 543 of McGinnis and co-workers.[3]

indicate that, except for those compounds which are determined as choline in the reineckate method, the only compounds of significant effectiveness for Neurospora are mono- and dimethylaminoethanol and monoethylcholine.

On the simplified diet here studied, McGinnis and coworkers[3] observed that choline, methionine, and betaine prevent perosis, but on the purified diet choline only was found to be effective. They concluded that choline *per se* is required for the prevention of perosis. The results of Jukes and coworkers[6,9,10] indicate that mono- and dimethylaminoethanol and mono- and diethylcholine have choline activity for the chick. Mono- and dimethylaminoethanol may function in the chick as choline precursors since these workers found that methionine augmented materially the effects of these compounds.[10]

McGinnis and coworkers[3] have suggested that precursors of choline are present in the simplified diet on which methionine and betaine prevent perosis. Jukes and Oleson[6] have suggested that simple precursors of choline might exist in natural foods. The observations reported in the present paper support these views.

[10] Jukes, T. H., Oleson, J. J., and Dornbush, A. C., *J. Nutrition*, 1945, **30**, 219.

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Changes in Alkaline Phosphatase of Kidney Following Renal Damage with Alloxan.

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The alkaline phosphatase which occurs in high concentration in the proximal convoluted tubules of the kidney rapidly disappears during degenerative changes in these structures. The enzyme has been shown to be reduced by nephrotoxic drugs. Alloxan in addition to its injurious action on the islets of Langerhans causes a varying degree of parenchymatous degeneration of the renal tubules and evidence is here reported of an associated depletion of renal alkaline phosphatase in the damaged kidney.

White rats are readily susceptible to alloxan. Bailey, Bailey and Leech[1] produced

[1] Bailey, C. C., Bailey, O. T., and Leech, R. S., *New Eng. J. Med.*, 1944, **230**, 534.

in these animals necrosis of tubular epithelium with a dosage of 200 mg per kg. Dunn and McLetchie,[2] and Goldner and Gomori[3] reported somewhat similar results.

Method. Alloxan was prepared from uric acid by the method of Fischer.[4] White male rats of the Wistar strain in groups of 3 to 19 animals, after withdrawal of food for 48 hours, were injected subcutaneously with a 5% aqueous solution of alloxan in dosage ranging from 25 to 600 mg per kg of rat. Many of the rats died in 20 to 48 hours following injection of 400 mg or more per kg and younger animals did not tolerate injections as well as older ones. The rats which survived were killed by a blow on the back of the neck at 6, 21, 24, 26, 28, 30 and 48 hours after injection. Immediately after death, blood was withdrawn from the heart for glucose determination and the kidneys were quickly removed for alkaline phosphatase studies. One kidney was cut in thin slices which were fixed in Zenker's solution for routine sections, and in acetone and absolute alcohol for histochemical studies of phosphatase.

The technic used for the histochemical preparations was that of the azo dye method,[5] Gomori's method[6] and in many experiments the Kabat and Furth technic.[7] Paraffin sections of kidney tissue fixed in acetone or alcohol were sectioned at 5 μ for routine stains. Sections of both normal and pathologic tissue were affixed to the same slide and subjected to identical procedures in order to obtain comparative values.

The second kidney was used for chemical estimation of alkaline phosphatase which was carried out by a modification of King and Armstrong's[8] method as follows. The capsule was stripped from the kidney and the pelvis and medulla cut away. Duplicate portions of tissue gauged to weigh between 0.4 and 0.5 g were excised from the remaining organ, weighed exactly and each portion was ground in a mortar with alundum. Comparable duplicate results were obtained only when approximately the above amounts of kidney were used. Each lot of ground tissue was washed from the mortar into a 50 cc centrifuge tube with 100 times its weight of 0.85% sodium chloride and centrifuged 20 minutes at 2,000 r.p.m. The clear supernatant fluid was again diluted with the saline, so that the final dilution of the ground kidney was 1 to 500. The reagents required for the tests were (1) buffer substrate containing 1.09 g of disodium monophenylphosphate and 10.3 g of barbital sodium per liter of distilled water, (2) the phenol reagent of Folin and Ciocalteu[9] diluted 1 to 3 and (3) 0.1% stock phenol solution in 0.1 N HCl, the accuracy of which was controlled by titration procedures described by Peters and Van Slyke.[10]

Procedure. Into each of 4 test tubes, 4 cc of buffer were pipetted. Two of these tubes, used as duplicate controls, for the determination of preformed phenolic bodies in kidney tissue were left at room temperature and the other pair of duplicate tubes were heated to 37°C in a water bath. To all 4 tubes were then added 0.2 cc of the 1:500 dilution of ground kidney and 1.8 cc of dilute Folin-Ciocalteu phenol reagent. All 4 were heated for 30 minutes at 37°C, and then centrifuged for 7 minutes at 2,000 r.p.m. to remove protein. A 4 cc aliquot of the supernatant fluid from each tube was pipetted into 4 electrophotometer tubes and 1 cc of 20% sodium carbonate added to each at successive one-minute intervals. After the color had developed for 20 minutes they were read against

[2] Dunn, J. S., and McLetchie, N. G. B., *Lancet*, 1943, **2**, 384.

[3] Goldner, M. G., and Gomori, G., *Endocrinology*, 1943, **33**, 297.

[4] Fischer, Emil, *Introduction to the Preparation of Organic Compounds*, trans. by R. V. Standord, Van Nostrand Co., London, 1917, 8th ed.

[5] Menten, M. L., Junge, J., and Green, M. H., *J. Biol. Chem.*, 1944, **158**, 471; PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 82.

[6] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 23.

[7] Kabat, A. E., and Furth, J., *Am. J. Path.*, 1941, **17**, 303.

[8] King, E. J., and Armstrong, A. R., *Can. Med. Assn. J.*, 1934, **37**, 376.

[9] Folin, O., and Ciocalteu, V. J., *J. Biol. Chem.*, 1927, **73**, 627.

[10] Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry*, Williams and Wilkins, 655, Vol. II.

TABLE I.
Renal Alkaline Phosphatase After Alloxan Injections of 300 to 600 mg per kg Weight of Rat.

Hrs. after injection when rat killed	300 mg alloxan		400 mg alloxan		450 mg alloxan		500 mg alloxan		600 mg alloxan	
	I	II	III	II	III	II	III	II	III	II
6	158	47.5*		24.2	520	23.8	360	22.0	192	16.2
		50.6		24.1		20.8		21.2		16.2
6				22.4		21.7	208	23.8		26.3
				21.7		20.7		25.1		26.9
6				55.2			250	33.0		
				53.3				33.0		
24	174	27.4			500	13.7		12.2	375	12.8
		28.7		23.9		15.0		11.2		13.4
24			280	26.6		20.9			580	27.1
				28.5		26.0				23.9
24			580	28.9	620	29.7				
				25.0		26.0				
26				17.3			305	17.2		
				17.3				18.7		
26			580	19.5			170	16.3		
				18.7				14.9		
26			620	25.0						
				27.0						
28	133	32.6								
		31.3								
30			215		290	14.2		16.9		
				17.9		13.7		16.8		
30				38.0		19.8	224	24.8		
				36.7	580	21.1		25.8		
30			130	21.3			540	16.8		
				21.9				16.0		
48				19.2	230	22.8				
				19.2		24.1				
48				20.1						
				18.5						
Normal control	150	40.5		50.0		55.3		52.6	73	44.8
		42.0		52.3						
Normal control				46.6		58.4		62.6	93	53.8
				47.1						

I Weight of rat in mg.
II Units phosphatase in duplicate per g wet kidney.
III Blood glucose in mg %.
* Duplicate figures are included because of the fairly wide range of variation obtained in the two values.

a standard in a Fisher electrophotometer. The standard was prepared by adding 15 cc of the 1:3 dilution of the Folin Ciocalteu reagent to 5 cc of a dilute standard phenol solution (containing 0.1 mg of phenol per cc) and diluting to 50 cc. To 4 cc of this mixture was added 1 cc of 20% sodium carbonate and the color allowed to develop for 20 minutes. All tubes were read exactly 20 minutes after the addition of the carbonate. For calculation of the alkaline phosphatase the following formula was used, *viz.*

$\frac{0.04}{RS} \times \frac{6}{4} \times 500 \times \frac{RU}{Wt}$ = units of alkaline phosphatase in 1 g of wet kidney; where $\frac{0.04}{RS}$ = number of mg of phenol in 4 cc of standard divided by the reading of the standard on the electrophotometer, (in our experiments RS = 100); $\frac{6}{4}$ = fraction correcting for aliquot used; 500 = dilution of ground kidney; RU = colorimetric reading of unknown kidney material less the colorimetric reading of control; Wt = weight of kidney tissue used.

Results and Discussion. The general response of our animals to alloxan was similar to that reported by other workers. The intensity of the symptoms usually increased

TABLE II.
Renal Alkaline Phosphatase in Rats Dying After Alloxan Injection.

Mg of alloxan injected per kg of rat	Wt in g of rat	No. of hr after injection when rat died	Units of phosphatase (in duplicate) per g of wet kidney
300	165	48	23.9*
			23.9
400	82	25	14.7
			14.7
400	171	40	13.7
			14.2
450	169	40	14.9
			14.8
450	205	12+	15.1
			15.3
450	139	54	20.4*
			20.1
450	131	60	9.6
			9.3
450	132	120	19.2*
			19.2

* Considerable amount of blood in kidney may be responsible for high values.

with the dosage, although variations occurred in individual rats. Alloxan dosage of 100 mg or less per kg of rat produced practically no symptoms. After a dosage of 200 to 300 mg per kg the animals remained hunched up in their cages for about 24 hours but did not appear ill, and only an occasional rat succumbed to the injection. In 4 to 10 hours after the injection of 400 mg of alloxan per kg the rats appeared listless and ill. After 24 hours they were acutely ill and the excreted urine was tinted pink. Those which survived 30 hours could not stand. Those which received 500 to 600 mg per kg did not live beyond 28 hours, appeared acutely ill and their blood sugars ranged from 200 to 600 mg % at the time of death. The kidneys on excision appeared yellow or a mottled yellow and red. There was a progressive decrease in the survival rate as the dosage increased. Three of the 4 rats receiving 300 mg per kg, 14 of 19 rats of the 400 mg group and only 50% of those which received the 500 or 600 mg dosage survived.

Renal alkaline phosphatase was not decreased when the dosage was 200 mg or less per kg, as shown in 4 groups of rats, each consisting of 4 animals. Rats of each group were given subcutaneously respectively 25, 50, 100 and 200 mg per kg and killed 6, 24, 28 and 48 hours after injection. In all of the treated, as well as the normal control rats, we found the renal alkaline phosphatase to vary between 40 and 51 King and Armstrong units per g of wet kidney. Doses of 300 mg or more per kg caused an appreciable reduction in the phosphatase. The injection of 400 to 600 mg of alloxan per kg was constantly followed by a 50% or greater fall in the enzyme. With high dosage, considerable variation in individual animals in amount of residual enzyme was noted and generally the deleterious effect of the drug was enhanced with prolongation of time after injection up to 21 and 28 hours, when the maximum effect of the drug appeared to be reached. In rats surviving 2 to 3 times this period, little further diminution of phosphatase occurred. The action of the alloxan did not result in the complete removal of the enzyme because phosphatase was present in appreciable amounts in kidneys excised after death from the drug. In 4 rats dying from alloxan the residual enzyme averaged 14.7 units per g of wet kidney, a loss of nearly 70% of the total enzyme as shown in Table II. In 3 of the rats, the values higher than this may have been due to the large amount of blood remaining in these organs after death. In one animal, surviving 60 hours, the phosphatase reached a low level of 9.6 units per g of wet kidney. The water content of these organs was found to be 73 to 77% of their initial wet weight.

The reduction in renal phosphatase obtained on chemical analysis was paralleled by that found in histochemical preparations. The disappearance of the enzyme following alloxan injection could readily be detected by the naked eye in differences of color intensity occurring in microscopic sections from kidneys of alloxan treated and of normal rats when tissue sections of both affixed to the same slide were subjected to identical technic for the demonstration of alkaline phosphatase. Sections of normal kidney appeared deep gray or black, while those from treated animals were appreciably paler. The loss of enzyme occurred in the luminal border of proximal convoluted tubules and the ascending limb of the loop of Henle, areas in which the phosphatase is localized normally. Frequently in rats receiving 400 mg per kg of alloxan the phosphatase was not only diminished in the luminal border of the tubular cells but was present in large amounts in the mitochondria within the contiguous cells. Hepler, Simonds and Gurley[11] reported a somewhat similar diffusion into these cells following injury by nephrotoxic drugs. This redistribution of the enzyme was not observed with higher alloxan dosage. In a few instances the phosphatase could not be demonstrated histochemically in the proximal convoluted tubules, having been apparently, completely lost from these areas. In preparations of "Zenker" fixed tissue, with a parallel mounting of pathologic and normal sections on the same slide, which were stained with Heidenhain's iron hematoxylin stain, an augmented intensity of black stain occurred in the mitochondria in the kidneys of the alloxan-treated rats as compared to the normal animal. This finding is further evidence of the chemical change produced in the tubular cells by the alloxan. The mitochondria in the pathologic tissue appeared coarser and occasionally in sections which showed marked granular degeneration these structures also had lost their normal parallel alignment and had an irregular distribution.

Three rats which received 400 mg of alloxan per kg were given insulin on the fifth day, with a restoration of the very high blood sugar to a lower level of 200 mg %. The lowering of the hyperglycemia was not, however, accompanied by any apparent improvement in the renal alkaline phosphatase, as chemically and histochemically there was the same low level as in treated rats not receiving insulin therapy. This finding together with the fact that the diabetogenic dosage is lower than that which produces a reduction in the renal phosphatase leads us to believe that 2 different mechanisms are involved in the damage caused by alloxan in pancreas and kidney. The demonstration by Lehman[12] that alloxan in moderate concentrations inhibits the conversion of the Cori ester to the Robison ester and in high concentration sometimes inhibits the formation of both esters may indicate that the alloxan acts *in vivo* by an interference with certain enzymes which differ quantitatively or qualitatively in various tissues.

Summary. The injection of alloxan in dosage of 300 to 600 mg per kg of rat was followed by a definite decrease in the alkaline phosphatase of the kidney. With the 600 mg dosage this enzyme was reduced 50% or more. The decrease in the renal phosphatase obtained by chemical analysis was roughly paralleled by the reduction demonstrated histochemically.

[11] Hepler, E. E., Simonds, J. P., and Gurley, H., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 221.

[12] Lehman, H., *Biochem. J.*, 1939, **33**, 1241.