6. Gale, G. R., Canad. J. Microbiol., 1966, v12, 73.

7. ———, J. Bact., 1966, v91, 499.

8. Young, C. W., Hodas, S., Fennelly, J. J., Proc. Am. Assn. Cancer Res., 1964, v5, 71.

9. Rosenkranz, H. S., Levy, J. A., Biochim. Biophys. Acta, 1965, v96, 181.

10. Kennedy, B. J., Yarbro, J. W., J.A.M.A., 1966, v195, 1038.

11. Cumulative Index of Materials, Cancer Res., 1965, v25, 1837.

12. Leiter, J., Wodinsky, I., Bourke, A. R., Schneiderman, M. A., ibid., 1960, v20, 539.

13. Reilly, H. C., Falco, E., Myron, S. A., Philips, F. S., Stock, C. C., ibid., 1963, v23, 1731.

14. Lowenthal, J., Nature, 1954, v174, 36.

15. The Pharmacological Basis of Therapeutics, Goodman, L. S., Gilman, A., eds., Macmillan Co., New York, 1965, p330.

16. Goldman, A. S., Yakovac, W. C., Arch. Environ. Health, 1964, v8, 648.

17. Larsson, K. S., Bostrom, H., Ericson, B., Acta Paediat., 1963, v52, 36.

18. Obbink, H. J. K., Dalderup, L. M., Lancet, 1964, v1, 565.

Received May 16, 1966. P.S.E.B.M., 1966, v122.

Effectiveness of THAM in Preventing Cellular Damage Resulting From Oxygen Lack.* (31370)

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Intracellular acidity increases during oxygen lack in a variety of tissues. It has been suggested that this decrease in pH may destroy enzymes and disrupt metabolic function within the cell. The damaging effects of the increased acidity might be lessened if acids were neutralized by a buffer capable of accepting hydrogen ions within the cell. The organic buffer tris-hydroxy methylamino methane (Tris or THAM), is capable of acting as an intracellular buffer(1). THAM buffer had been shown to be more effective in correcting acid-base disturbances, *e.g.*, apneic oxygenation and hypovolemic shock, than carbonate buffers(2,3). Thus the following study was undertaken to determine whether the organic buffer THAM could attenuate the cellular damage associated with oxygen lack. The criteria used to establish the severity of cell damage included: (a) reduction in the activities of selected mitochondrial and microsomal enzymes, (b) labilization of lysosomal enzymes (measured as increased per cent free activity) and (c) degradation of protein (measured by an increased concentration of soluble nitrogen).

* This investigation supported in part by the U.S. Army Research and Development Command, Department of Army, under Research Contract DA-49-193-MD-2371. A major portion of this investigation was performed in the laboratory of Dr. A. T. Miller, Jr., Dept. of Physiology, Univ. of North Carolina, Chapel Hill.

Methods. Male rats of the Sprague-Dawley strain weighing 150-300 g were fed *ad libitum* on Purina Laboratory Chow and water. At the time of death, the animals were decapitated with a guillotine. The left lateral and median lobes of the liver were used for all enzymatic and chemical determinations. The animals were divided into the following groups.

Group A—normal controls.

Group B—liver autolysis. Anoxia was produced by incubation of isolated lobes in a nitrogen atmosphere, at 37°C, moistened with Krebs-Ringer phosphate pH 7.2 for 1 or 12 hours.

Group C—administration of THAM buffer IP followed by liver autolysis. Ten ml of 0.33 M THAM (tris-hydroxy methlyamino methane), pH 7.2, was injected IP. After 18 hours the rat was sacrificed and the liver was allowed to autolyze as in Group B.

Group D—control liver slices for *in vitro* experiments. Liver slices were prepared with a Stadie-Riggs hand microtome. The slices

TABLE I. Effects of Oxygen Lack.

Group	Malic dehydrogenase	Succinic dehydrogenase	Glucose-6-phosphatase	% free acid phosphatase	% free cathepsin	% free soluble N	Tissue buffer capacity
A†	100 ± 32	100 ± 14	100 ± 9	100	100	100	100
B 1 hr	45 ± 21*	71 ± 8*	74 ± 33	80	487*	121	62*
B 12 hr	27 ± 21*	10 ± 9*	7 ± 2*	123‡	337*	210*	62*
D†	100 ± 18	100 ± 12	100 ± 30	100	100	100	100
E 1 hr	58 ± 7*	68 ± 19*	78 ± 29*	169*	319*	131	73*

* $P<0.005$. ‡ $P<0.01$.
† Denotes the respective control groups.

were incubated in 0.3% saline and 0.25 M sucrose or Krebs-Ringer buffer at pH 7.2. The flasks were incubated in a Dubnoff metabolic shaker at 37°C under oxygen for 1 hour.

Group E—liver slices incubated under anoxic conditions. These slices were treated as in Group D except nitrogen was substituted for oxygen.

Group F—control for THAM administered *in vitro*. Liver slices were incubated in 0.33 M THAM, 0.3% NaCl and 0.25 M sucrose, pH 7.2 for 1 hour in oxygen.

Group G—liver slices incubated under anoxic conditions in the presence of THAM. The slices were incubated as in Group F. This was followed by incubation in nitrogen for 1 or 12 hours.

Group H—liver slices incubated in an acid medium with adequate oxygenation. The tissues were treated as Group F except that the pH of the medium was respectively, 6.5, 6.0, or 5.0.

An homogenate was prepared in 0.25 M sucrose and 0.001 M EDTA using 3 strokes of the Potter-Elvehjem teflon pestle. The following enzyme determinations were made: (a) malic dehydrogenase by the methods of Ochoa, Mehler and Kornberg(4); (b) succinic dehydrogenase by the method of Cooperstein, Lazarow and Kurfess(5); (c) glucose-6-phosphatase by the method of Swanson(6), using the method of Chen, Toribara and Warner(7), for determining inorganic phosphorus; (d) free and total acid phosphatase by the method of Gianetto and deDuve(8), total enzymatic activity was released by Triton X-100 (0.1%) with the homogenate; (e) free and total cathepsin by a modification of the methods of Anson(9), Gianetto and deDuve(8). The tyrosine was determined by the Ratnoff and Menzie method(10). Chemical determinations included: (a) acid soluble and total nitrogen by micro-Kjeldahl digestion coupled with Nessler Reagent; (b) titration buffer capacity by deHaan and Field (11); (c) the tissue pH at 37°C with a Beckman tissue electrode.

Results. Preliminary studies showed that 4 methods of producing anoxia, *i.e.*, ligation of the blood vessels, autolysis, pump ventilation with a low oxygen-nitrogen mixture (5:95) and incubation of liver slices in a nitrogen atmosphere, resulted in the same magnitude of increase or decrease of the respective enzymes and chemical determinations tested. Consequently, the liver autolysis studies were selected as being typical in their responses to the cellular damage produced by low oxygen atmospheres. The results are summarized in Table I. Each value represents determinations from 6-10 animals. The control for each group is denoted as 100% ± standard deviation. The experimental results are calculated as a percentage of the respective control group. Where values are computed as % of total concentrations there is no standard deviation given. * denotes $P<0.005$, ‡ denotes $P<0.01$. All others were not significantly different from control value.

These studies showed that oxygen lack caused a significant decrease in the activities of the oxidative enzymes (malic and succinic dehydrogenases) and of the microsomal enzyme (glucose-6-phosphatase). Oxygen lack for a period of 12 hours brought about an increase in the per cent of free acid phosphatase and cathepsin. Per cent of free acid phosphatase changed very little after 1 hour of autolysis. However, when liver slices were

TABLE II. Effects of Acidosis.

Group	Malic dehydrogenase	Succinic dehydrogenase	Glucose-6-phosphatase	% free acid phosphatase	% free cathepsin	% free soluble N
F pH 7.2†	100 ± 44	100 ± 6	100 ± 9	100	100	100
H pH 6.5	82 ± 20	78 ± 3*	94 ± 8	149*	106*	200*
pH 6.0	68 ± 9‡	53 ± 10*	81 ± 26*	185*	163*	209*
pH 5.0	56 ± 4*	43 ± 15*	48 ± 19*	160*	161*	192*

* $P < 0.005$. ‡ $P < 0.01$.
† Denotes the respective control groups.

incubated in a nitrogen atmosphere the per cent of acid phosphatase was increased significantly. After 12 hours of anoxia the free acid phosphatase activity doubled. Total nitrogen concentrations of liver were not altered at 1 hour of oxygen lack, but they were significantly reduced after 12 hours of anoxia. Oxygen lack produced a significant increase in acid soluble nitrogen after 12 hours.

When well oxygenated liver slices were incubated at pH 6.5, 6.0 or 5.0, there was a progressive loss in mitochondrial and microsomal enzyme activity and a progressive rise in per cent of free acid phosphatase and cathepsin (Table II). The acid soluble nitrogen increased to more than twice the control values as the pH decreased from 6.5 to 5.0. These studies indicate that oxygen lack and acidosis cause similar changes in enzyme activities and nitrogen content of the liver.

The prior administration of THAM, either *in vivo* or *in vitro* completely prevented the damaging effects of anoxia on malic and succinic dehydrogenases and glucose-6-phosphatase, observed after 1 hour (Table III). However, 12 hours of anoxia resulted in the usual enzyme loss despite the administration of buffer. The administration of THAM did not affect the mitochondrial and microsomal enzyme activities in control groups. The prior intraperitoneal injection of THAM or addition of THAM to the incubation medium reduced the labilization of acid phosphatase and eliminated completely the labilization of cathepsin even after 12 hours of oxygen lack. Protein degradation was also inhibited by administration of THAM. In all experiments involving the use of THAM, corrections were made for its contribution to acid soluble nitrogen values.

The increase in hydrogen ion concentration in liver during ischemia was demonstrated by the progressive decrease in tissue pH over 12 hours of ischemia from 7.0 to 5.0, while no change occurred in the control animal. The tissue buffer capacity was reduced in all conditions associated with oxygen lack. When THAM was administered, the tissue buffer capacity was reduced, but not to the same magnitude as in those tissues without the presence of THAM.

Discussion. An increase in the hydrogen ion concentration during conditions of oxygen deficiency has been demonstrated in rat liver (12,13). deHaan and Field(11) and Dawes (14) postulated that the accumulation of acid

TABLE III. Effectiveness of THAM Buffer.

Group	Malic dehydrogenase	Succinic dehydrogenase	Glucose-6-phosphatase	% free acid phosphatase	% free cathepsin	% free soluble N	Tissue buffer capacity
A†	100 ± 32	100 ± 14	100 ± 9	100	100	100	—
In vivo							
C 1 hr	106 ± 23	101 ± 15	97 ± 8	98	102	85	—
C 12 hr	69 ± 8‡	14 ± 5*	4 ± 1*	130‡	78	128	—
F†	100 ± 44	100 ± 6	100 ± 9	100	100	100	100
In vitro							
G 1 hr	124 ± 49	103 ± 8	98 ± 33	79	101	89	104
G 12 hr	65 ± 23*	19 ± 10*	1 ± 1*	60*	75	108	80‡

* $P < 0.005$. ‡ $P < 0.01$.
† Denotes the respective control groups.

exhausts the buffer capacity of cells and thus disrupts the enzyme systems. The increased intracellular acidity associated with oxygen lack might directly influence enzymatic reaction and/or cellular structure or it might act on lysosomes, resulting in the liberation of acid hydrolytic enzymes which could attack the remaining subcellular particles and cause disruption of metabolic processes.

In liver, anoxia or hypoxia depresses oxygen consumption(15-17), oxidative enzyme activities(16-21), oxidative phosphorylation and ATP production(20,23-26), and protein synthesis(24), with a concomitant increase in anaerobic glycolysis(27), protein degradation(13,17-19,28,29), and per cent of free acid phosphatase activity(18,22,30,31). It is not certain which of these effects are brought about by oxygen lack *per se* during anoxia, and which are due to the increased acidity resulting from anoxia. The rate of disappearance of ATP and creatine phosphate is probably too rapid to be explained by the increase in tissue acidity. On the other hand, the decline in the functional activities of mitochondrial and microsomal enzymes, and the increase in free activity of lysosomal enzymes and in protein degradation may occur when the rate of acid accumulation is accelerated.

The fact that acid does accumulate in anoxic tissue was demonstrated by the decrease in tissue buffer capacity and tissue pH. The finding of a decreased tissue buffer capacity is in agreement with the report of deHaan and Field(11), and the decreased tissue pH with that of Bradley(13). In the early stages of anoxia the decrease in pH is probably due to lactic acid, but in the later stages the decrease is more likely due to phosphoric acid and organic acids(13).

The decrease in mitochondrial and microsomal enzyme activities after one hour of oxygen lack is in agreement with many reports in the literature.

There was no significant increase in the per cent of free activity of acid phosphatase after 1 or 2 hours of anoxia, but the increase was significant after 6-12 hours. The fact that free acid phosphatase activity did not increase in the early stages of autolysis is not in agreement with other reports(22,30, 31). The rapid increase in free lysosomal hydrolase activity under *in vitro* conditions, *i.e.*, tissue slices incubated in a nitrogen atmosphere, is in agreement with the report of deDuve and Beaufay(31). It would appear that the sliced tissues were more susceptible to the damage caused by anoxia than were tissues that had not been sliced.

It is apparent from Tables I and II that there was a close correlation in the individual enzyme and chemical changes produced by acidosis and anoxia. After one hour's incubation of oxygenated liver slices at pH 6.0, the mitochondrial and microsomal enzyme activities had declined while free acid phosphatase and cathepsin, and acid soluble nitrogen had increased to levels comparable to those observed after one hour anoxia produced by any of the 4 methods used in this study. Since the oxygen supply was normal in the experiments involving incubation of liver slices at the various pH's and since further anoxic effects on enzyme activities can be prevented by buffering with THAM, it seems possible that increased acidity may be largely responsible for the cellular damage observed during oxygen lack. Buffering, by addition of THAM to the incubation medium, prevented the release of acid phosphatase and cathepsin in tissue slices incubated in a nitrogen atmosphere for periods of 1 and 12 hours. However, when the animals received an intraperitoneal injection of THAM 18 hours prior to sacrifice, the liver lobes incubated in nitrogen for 12 hours showed an increase in free acid phosphatase activity, but no change in free cathepsin activity. This suggests two things: (1) administration of larger doses of buffer may be necessary for complete stabilization of the lysosomal membrane, and (2) the mechanism for release of acid phosphatase and cathepsin may be slightly different.

These results indicate that THAM can function as an intracellular hydrogen ion acceptor during anoxia, attenuating the damage produced by oxygen lack to enzymes and perhaps other proteins. Once the buffering capacity of the THAM is exhausted, the acidity of the cells increases and cellular organelles may be damaged. These results also suggest that the damage to mitochondrial and microsomal

enzymes and protein structure in the early stages of anoxia is not due to lysosomal activity, but rather acidity.

Summary and conclusions. The effects of oxygen lack on rat liver were studied in order to: (a) compare the results of a variety of methods of producing oxygen lack; (b) evaluate the role of increased intracellular acidity in production of anoxic cell damage; (c) determine if buffering with an intracellular hydrogen ion acceptor reduces the damaging effects of anoxia on cellular metabolism.

The criteria used to establish the severity of cellular damage included: (a) reduction in the activities of 2 mitochondrial enzymes (succinic dehydrogenase and malic dehydrogenase) and of one microsomal enzyme (glucose-6-phosphatase); (b) the labilization of 2 lysosomal enzymes (acid phosphatase and cathepsin); (c) the degradation of protein.

Oxygen lack of equivalent duration produced by ischemia *in vivo,* autolysis *in vitro,* pump ventilation with a 5% oxygen mixture, and incubation of liver slices in a nitrogen atmosphere caused approximately the same degree of cellular damage: (a) oxygen lack resulted in an early and progressive decrease in the activity of the 2 mitochondrial enzymes and glucose-6-phosphatase activity; (b) the total activities of the 2 acid hydrolases were not affected even by prolonged periods of anoxia, but the free activities of these enzymes were increased by prolonged periods of anoxia; (c) anoxia was associated with a progressive decrease in the titratable buffer capacity and pH of the liver. These studies indicate that acidity is one of the major destructive factors affecting cell metabolism during oxygen lack.

Similar enzymatic and chemical changes were observed in liver slices incubated in an oxygenated medium at low pH's and in a nitrogen atmosphere in a medium buffered to pH 7.4.

THAM buffering largely prevented the early effect of anoxia on the mitochondrial, microsomal and lysosomal enzyme and on protein, but was not effective after long periods of anoxia. Thus it appears the early effect of anoxia on mitochondrial and microsomal enzymes and on protein is due largely to increased acidity.

I wish to thank Dr. Aaron P. Sanders for valuable assistance.

1. Holmdahl, M. H., Nahas, G. G., Am. J. Physiol., 1962, v202, 1011.

2. Mittelman, A., Dos, S. J., Barker, H. G., Nahas, G. G., ibid., 1962, v202, 334.

3. Manger, W. M., Nahas, G. G., Hassan, D., Habif, D. V., Papper, E. M., Ann. Surg., 1962, v156, 503.

4. Ochoa, S., Mehler, A. H., Kornberg, A., J. Biol. Chem., 1948, v174, 979.

5. Cooperstein, S. J., Lazarow, A., Kurfess, N. J., ibid., 1950, v186, 129.

6. Swanson, M. A., ibid., 1950, v184, 647.

7. Chen, P. S., Toribara, T. Y., Warner, H., Analyt. Chem., 1956, v28, 1756.

8. Gianetto, R., deDuve, C., Biochem. J., 1955, v59, 433.

9. Anson, M. L., J. Gen. Physiol., 1937, v20, 565.

10. Ratnoff, O. D., Menzie, C., J. Lab. Clin. Med., 1951, v37, 316.

11. deHaan, R. L., Field, J., Am. J. Physiol., 1959, v197, 449.

12. Severinghaus, E. L., Koehler, A. E., Bradley, H. C., J. Biol. Chem., 1923, v57, 163.

13. Bradley, H. C., Physiol. Rev., 1938, v18, 173.

14. Dawes, G. S., Selective Vulnerability of Brain in Hypoxaemia, F. A. Davis, Co., Phila., Pa., 1963, pp37-40.

15. Mengebier, W. L., Am. J. Physiol., 1954, v177, 231.

16. Wilhelmi, A. E., Engel, M. G., Long, C. N. H., ibid., 1946, v147, 181.

17. Wilhelmi, A. E., Russel, J. A., Engel, F. L., Long, C. N. H., ibid., 1945, v144, 669.

18. Berenbom, M., Chang, P. I., Betz, H. E., Stowell, R. E., Cancer Res., 1955, v15, 1.

19. Klein, P. D., Thompson, J. F., Am. J. Physiol., 1956, v187, 259.

20. Gallagher, C. H., Judah, J. D., Rees, K. R., J. Path. Bact., 1956, v72, 247.

21. Moore, K. E., Brody, T. M., Am. J. Physiol., 1960, v198, 677.

22. Van Lanker, J. L., Holtzer, R. L., J. Biol. Chem., 1959, v234, 2359.

23. Strickland, E. H., Ackerman, E., Anthony, A., J. Appl. Physiol., 1962, v17, 535.

24. Sanders, A. P., Hale, D. M., Miller, A. T., Jr., Am. J. Physiol., 1965, v209, 443.

25. Duspiva, D., Noltenius, H., Beit. Path. Anat. u. Allg. Path., 1958, v118, 52.

26. Dawkins, M. J. R., Judah, J. D., Rees, K. R., J. Path. Bact., 1959, v77, 257.

27. Laser, H., Biochem. J., 1937, v31, 1971.
28. Bradley, H. C., J. Biol. Chem., 1922, v52, 467.
29. Luck, J. M., Eudin, J., Nimmo, C. C., ibid., 1939, v131, 201.
30. Franklin, T. J., Biochem. J., 1962, v82, 118.
31. deDuve, C., Beaufay, H., Biochem. J., 1959, v73, 610.

Received May 16, 1966. P.S.E.B.M., 1966, v122.

Antigen Induced Histamine Release from Platelets of Rabbits Producing Homologous PCA Antibody. (31371)

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Humans, guinea pigs, rats and mice produce antibodies which are capable of sensitizing homologous species for anaphylaxis. The human antibody (reagin) is capable of sensitizing human leukocytes so that on the addition of antigen histamine is released(1). There is no requirement for added serum or plasma factors, such as complement, for histamine release. The corresponding guinea pig and rat antibodies are able to sensitize their respective mast cells and, here also, it has been demonstrated that complement is not needed(2).

Recently Zvaifler and Becker(3) described an antibody in rabbits appearing as early as 6 days after immunization which sensitizes rabbits for passive cutaneous anaphylaxis (PCA). The antibody was not a γG or γM immunoglobulin and had no detectable precipitating or complement fixing properties. Onoue *et al*(4) described a similar rabbit skin sensitizing antibody and ascribed it to 7SγA immunoglobulin.

All the previous studies of *in vitro* release of histamine from rabbit platelets involved rabbit γG antibodies and the presence of fresh plasma was found to be obligatory. In this study it will be shown that sensitized rabbits whose plasma contains rabbit PCA antibody also have platelets which even after thorough washing are capable of releasing histamine on the addition of antigen in the absence of added plasma.

Materials and methods. Preparation of dinitrophenylated proteins. Bovine gamma globulin (BGG) and bovine serum albumin (BSA) was dinitrophenylated by reacting with 2,4 dinitrobenzene sulfonate as described by Eisen(5). The conjugated antigens contained approximately 35 moles of DNP per mole of BGG and 15 moles of DNP per mole of BSA. As work progressed it was found that the DNP-BSA conjugate became contaminated and was capable of releasing histamine from platelets of unimmunized rabbits presumably because of its endotoxin content. Subsequent DNP-BSA material was filtered and stored at -20°C in small quantities.

Immunization of rabbits. One ml of the DNP-BGG in Freund's complete adjuvant (1.4 mg AgN/ml) was injected in 0.25 ml volumes into each foot pad. Beginning on the fifth day after immunization the animals were bled daily from the marginal ear vein. The blood was collected into ice cold polypropylene tubes which contained sufficient sodium heparin to yield a final concentration of 7 units of heparin per ml of whole blood. In most instances the blood was used for histamine release as soon as possible after collection. In those experiments where release of histamine from whole blood was compared with that from washed platelets, the whole blood was stored in an ice bath until used for release. To obtain washed platelets, a known volume of whole blood was centrifuged at 3000 rpm for 15 minutes, the plasma removed and the platelets washed 3 times with 3 to 5 times their volume of ice cold Tyrode's solution. After washing, the sediment was suspended up to the original volume of whole blood and used for histamine release. The suspension, although it contained platelets, leukocytes and red cells, is referred to as "washed platelets"