cosal activity *is not* destroyed appreciably at any temperature up to 100°C.

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Action of Amino Acids on Bacterial Dehydrogenases and Glycolysis. (18852)

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In a previous communication(1) it was shown that in a system containing washed resting *Micrococcus pyogenes* var. *aureus* and glucose the reduction of triphenyltetrazolium was greatly accelerated by nitrogenous substances. Effects of amino acids on biological systems have been variously reported. Amino acids were found to increase markedly the motility, fertilizing power and agglutination ability of starfish sperm(2), greatly to extend the functional life span of sea urchin spermatozoa(3) and under anaerobic conditions to induce motility in these spermatozoa which are otherwise quickly immobilized by lack of oxygen(4). In these studies no amino acid utilization was reported to occur.

The effect of amino acids observed under our conditions was subjected to quantitative analysis to characterize the underlying mechanism.

Materials and methods. Micrococcus pyogenes var. *aureus* strain 1A was carried on extract agar slants kept at 4°C. The cells washed from a slant which had been incubated at 37°C for 20 hours were used as source of inoculum. A casein hydrolyzate medium containing added glucose (0.5%), tryptophan (20 μg/ml), thiamin (1 μg/ml), and nicotinamide (1 μg/ml) was inoculated with 250,000 cells/ml, and when growth had reached the middle of the log phase, the cells were collected and washed twice with 0.066 M phosphate buffer (pH 7.0). The amount of cells used in the reaction systems was determined by comparing the turbidity of the systems with a previously calibrated curve relating turbidimetric readings (filter 56) and the dry weight of cells. The glucose content of the samples removed from the reaction systems at the start and at the end of the experiment was determined by the method of Folin and Wu(5). Under the conditions of the experiment triphenyltetrazolium, formazan and amino acids did not interfere with these determinations. Lactate was determined by the method of Barker and Summerson(6). The copper-lime treatment removed glucose, tetrazolium, histidine and alanine so that these substances did not interfere with the determinations. The red formazan formed by the reduction of triphenyltetrazolium chloride was measured by the use of the method described by Kun and Abood(7). Acetone (7 ml) was added to a 4 ml sample, the mixture shaken, centrifuged, and the supernate read on the Klett-Summerson colorimeter (filter 42). The quantity of formazan was determined by reference to a standard curve relating known concentrations of tetrazolium which had been reduced with a few crystals of sodium hydro-

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4. Tyler, A. and Rothschild, Lord, PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 52.

5. Folin, O. and Wu, H., *J. Biol. Chem.*, 1920, v41, 367.

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7. Kun, E. and Abood, L. G., *Science*, 1949, v109, 144.

TABLE I. Effect of Various Compounds on the Reduction of Tetrazolium by *Micrococcus Pyogenes* var. *Aureus*.

Systems (1A)*	Colorimetric reading†	Systems (1B)*	Colorimetric reading†
No addition	11	Glycine	165
Glycine	90	Pyruvate	21
Ethylamine	65	α-oxo-glutarate	20
Sarcosine	50	Acetate	22
Betaine	53	Benzoic acid	18
Choline	24	Salicylate	24
PABA	33		
Ammonium chloride	35	Cells + glycine + TTZ	0

* (1A and 1B). Each system consisted of 6 ml volume. Each ml contained 30 μg cells. 500 μg glucose, 100 μg tetrazolium dissolved in 0.067 M phosphate buffer (pH 7.05). Concentration of substances added in Systems (1A) was 0.0025 M; in (1B) 0.005 M.

† Colorimetric readings (filter 54) indicate increment in red color due to formazan formation after incubation of 120 min at 37°C. Readings corrected for zero hr readings.

TABLE II. Effect of Amino Acids and Tetrazolium on Glycolysis by *Micrococcus Pyogenes* var. *Aureus*.

Systems*	μMol of glucose utilized	μMol of lactate formed	Molar ratio glucose/lactate	μMol of formazan formed
1. Cells + glucose	.55	.17	3.2	—
2. " " " + histidine†	.55	.27	2	—
3. " " " + TTZ	.13	.11	1.2	.005
4. " " " + histidine + TTZ	.33	.16	2	.11
5. " " "	.90	1.13	.78	—
6. " " " + histidine	.90	1.35	.66	—
7. " " " + TTZ	.90	1.07	.84	.18
8. " " " + histidine + TTZ	.90	1.13	.78	.18
9. " " histidine + TTZ	—	—	—	0
10. Heat killed cells + glucose + histidine + TTZ	0	0	--	0

* Each ml of the 30 ml system contained 40 μg (Systems 1-4) and 80 μg (Systems 5-10) cells, 2.77 μMols glucose, 0.32 μMols histidine, and 0.29 μMols of TTZ, as indicated, which were dissolved in 30 ml of 0.066 M phosphate buffer (pH 7.05). At start of experiment 15 ml were removed and inactivated by heating in boiling water, the remaining 15 ml were incubated for 3 hr at 37°C in 50 ml centrifuge tubes, then similarly inactivated. The pH measured in Beckman pH meter remained constant. Formazan was determined by the method of Kun and Abood(7).

† Methionine and alanine gave similar results. Chemical analysis for histidine showed that, if any, < 1 μg/ml was utilized in the 3 hr experiment. Microbiological determination for methionine showed that, if any, < 1 μg/ml methionine was metabolized.

sulfite and the colorimetric readings of their acetone supernate. A more sensitive method used by us consisted in reading the red color due to formazan formation directly in the reaction system. This method permitted us to follow on a comparative basis the effect of various factors on the progress of the reduction of tetrazolium in a constant volume. It could be used when the amount of cells (10 to 40 μg/ml) used in the experiment was small and remained in a homogeneous suspension throughout the experiment. Histidine was determined by the modification of the method of MacPherson(8) as described by Straughn and Sevag(9). This method permitted the determination of 1 μg of histidine, but did not allow differentiation between histidine, histamine and the keto derivative of histidine. Methionine was determined microbiologically in usual salt-glucose medium by means of an *E. coli* mutant kindly furnished by Dr. J. S. Gots of this department. This method permitted the detection of 1 μg of the amino acid. Anaerobic experiments were performed in Thunberg tubes which fitted into the Klett-Summerson colorimeter. The tubes attached to a manifold were evacuated to a residual

8. MacPherson, H. T., *Biochem. J.*, 1942, v36, 59.

9. Straughn, W. R. and Sevag, M. G., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 360.

pressure of 10-15 mm Hg by means of a high vacuum pump, equilibrated in the water bath at 37°C for 5 minutes, after which the content of the side arm was mixed and colorimetric readings made periodically.

The results of the single experiments presented are representative of a series of experiments the results of which were closely similar. All determinations were carried out in triplicate.

Results. Amino acids while unable to serve as substrates for the reduction of tetrazolium by resting cells of *Micrococcus pyogenes* var. *aureus* are the most effective of the compounds tested (Table I) in accelerating the reduction when added to the reaction system in combination with glucose.

Due to a greater reaction rate, the effect of the amino acid on the amount of lactic acid formed is masked when a larger amount of cells is used† (Table II, compare systems 1 and 2 and 5 and 6). This is indicated by the fact that the accelerative effect of amino acids on the reduction of tetrazolium in such systems can only be shown during a very early phase of reaction (compare systems 3 and 4 with 7 and 8, Table II). With smaller concentrations of cells the presence of the amino acid can be shown to cause 50% more lactate formation (in other experiments not reported here as high as 300% more lactate production was observed) and to cause the reduction of 20 times more tetrazolium than in the absence of the amino acid (Table II, systems 1 and 2, 3 and 4).

The rate of reduction and the amount of formazan formed may appear to be proportional to the concentration of the amino acid added (Fig. 1); the relationship, however, is not a stoichiometrical one. The quantity of amino acid utilized, if any, must be less than 1 μg/ml (0.0064 μm histidine) and since the

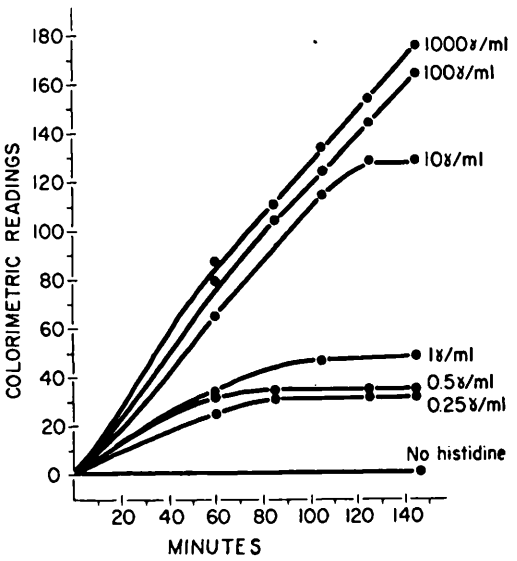

FIG. 1. Reaction systems in Klett tubes consisting of 8 ml volume. Each ml contained 40 μg cells, 500 μg glucose, 100 μg of tetrazolium, histidine.HCl as indicated dissolved in 0.067 M phosphate buffer (pH 7.30) and incubated at 37°. The colorimetric readings (filter 54) represent increment in red formazan formation and are corrected for the small zero hr readings.

quantity of tetrazolium reduced is in the order of 40 μg/ml (0.11 μm) (Table II, systems 3 and 4) the action of the amino acid might be catalytic in nature. No disappearance of histidine could be demonstrated. The method was shown to permit the detection of the disappearance of 1 μg of histidine from a system of 6 ml containing 10 μg of histidine. Also in microbiological assay using a methionine-less *E. coli* mutant, we failed to demonstrate any disappearance of methionine from the medium during the reaction. Control experiments showed that this method would detect the removal of as little as 1 μg of methionine per ml. The fact that oxidation products of the amino acids, the keto acids, and the ammonium ion have only a slight effect (Table I) and the variety of nitrogenous substances (*e.g.*, sulfathiazole, ethylamine, etc.) causing some degree of acceleration of the reduction of tetrazolium, tends to eliminate utilization as a mechanism for the observed effects of amino acids.

Tetrazolium chloride acting as an unnatural hydrogen acceptor inhibits glucose metabol-

† Since in Warburg set-up satisfactory measurements of gas exchange require at least from 500 to 1,000 μg cells/ml, its use for the study of the reported effect of amino acids could not be adapted. In contrast, the use of tetrazolium in place of oxygen as hydrogen acceptor permitted the measurement of dehydrogenase activity of as little as 20 to 40 μg cells/ml.

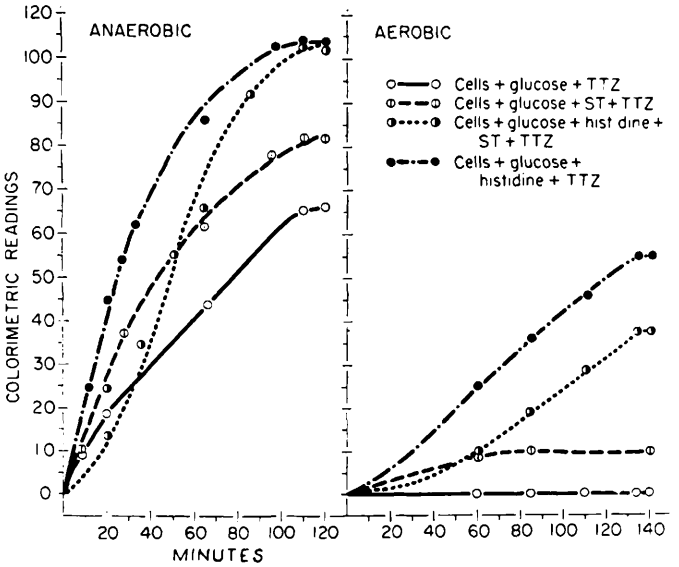

FIG. 2. Anaerobic systems in Thunberg tubes consisted of 6 ml volume. Each ml contained 20 μg of cells, 500 μg sulfathiazole (where added) in the main compartment, and 500 μg glucose, 100 μg tetrazolium, 100 μg histidine.HCl (where added) in the side arm dissolved in 0.067 M phosphate buffer (pH 7.30). The tubes were evacuated to a residual pressure of from 10-15 mm Hg, equilibrated for 5 min at 37°, after which the contents of the side arm were mixed and periodical readings taken in the colorimeter using filter 54. Aerobic systems in 50 ml centrifuge tubes consisted of 6 ml volume. Each ml contained 40 μg of cells, and was otherwise identical with the systems used in the anaerobic experiments. To insure good aerobic conditions the mixtures were periodically pipetted into Klett tubes, at which times the color increment was read on the colorimeter. No formazan was formed aerobically or anaerobically in systems containing only cells, histidine and tetrazolium.

ism (Table II, systems 1 and 3); this inhibition is not apparent when larger concentrations of cells are used. The specific effect of the amino acids is partially to relieve the inhibition of the oxidative enzymes involved in the metabolism of glucose (Table II, systems 3 and 4).

Inhibition of the reduction of tetrazolium. Comparison of parallel aerobic and anaerobic systems show that oxygen is probably competing with tetrazolium as a hydrogen acceptor indicated by the inhibition of the reduction of tetrazolium by resting cells in the presence of glucose (Fig. 2). Sulfathiazole slightly accelerates the reduction of tetrazolium when glucose alone is a substrate. The acceleration by amino acids of the reduction of tetrazolium aerobically or anaerobically is however inhibited by sulfathiazole, showing that the drug interferes with the effect of the amino acids on dehydrogenases.‡

Discussion. Flavoprotein enzymes have been reported to be the site of reduction of tetrazolium(10-12). In view of the striking effect of the amino acid on the reduction of tetrazolium, and lactic acid production from glucose, it would seem reasonable to assume that this action of amino acids is related to their interaction with flavoproteins. This view is corroborated by the partial relief by amino acids of the inhibition of glucose consumption by tetrazolium. Similarly, the gradual decrease in the sulfathiazole inhibition of the amino acid accelerated reduction of tetrazolium can be explained by a gradual displacement by amino acids of the drug from its site of action on flavoprotein. The inhib-

‡ The inhibitory effect of α-oxo-glutaric acid reported previously(1) was due to inadequate neutralization of compound and consequential lowering of pH of medium.

10. Bielig, H. J., Kausche, G. I. and Haardick, H., *Z. Naturforsch.*, 1949, v46, 80.

11. Kuhn, R., personal communication.

12. Brodie, A. F. and Gots, J. S., *Science*, 1951, v114, 40.

itory action of sulfonamides on flavoprotein has been abundantly reported(13).

The results of the parallel aerobic and anaerobic experiments therefore show that oxygen, tetrazolium and amino acids, and tetrazolium, sulfathiazole and pyruvate compete for the same enzyme site.§

In the absence of evidence showing utilization of the amino acids, the observed effects of these substances appear to be contingent upon the inhibition of reactions competing for the available hydrogen. As to the nature of these competing reactions it is of interest to note that Meyerhof and Schulz(14) found that only upon addition of certain nitrogenous substances (certain amino acids, amines or ammonium sulfate) to fermentation mixtures did yeast completely convert hexoses to CO_2, because under these conditions assimilation of sugar was restricted. In the absence of added nitrogenous substances only 75 to 80% of the theoretical yield of CO_2 was obtained.

Summary. Amino acids, aerobically and anaerobically, cause an acceleration of the reduction of tetrazolium by *Micrococcus pyogenes* var. *aureus* in the presence of glucose, and can be shown to shift glucose metabolism towards increased lactate production. This effect of amino acids appears to be related to the inhibition of normally occurring reactions resulting in an increased availability of hydrogen for the reduction of tetrazolium or pyruvate.

Oxygen inhibits the reduction of tetrazolium in the absence of added amino acids, while sulfathiazole inhibits the amino acid accelerated reduction of tetrazolium. Since flavoprotein is reported to mediate tetrazolium reduction and to be susceptible to inhibition by sulfathiazole, the accelerative effect of amino acids on the reduction of tetrazolium could be related to an interaction of the amino acids with the flavoprotein enzyme. Competition for this enzyme site between tetrazolium, oxygen and pyruvate, modified by the presence of amino acids and sulfathiazole is suggested as an explanation consistent with the observed accelerations and inhibitions of the reduction of tetrazolium.

13. Sevag, M. G., Gots, J. S., and Steers, E., *The Enzymes,* Vol. I, New York, Academic Press, 1950, pp115-186.

§ The alternative postulate that the amino acid acts by lowering the oxidation-reduction potential of the medium to a level more favorable for tetrazolium reduction does not appear to find support in the fact that all the amino acids and other nitrogenous substances tested are active in low concentrations and under strictly aerobic and anaerobic conditions under which O/R potentials would widely vary.

14. Meyerhof, O. and Schulz, W., *Biochem. Z.,* 1936, v287, 206.

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Protein Depletion Enhances Pancreatic Damage Caused by Ethionine. (18853)

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Destruction of pancreatic tissue by the administration of ethionine in the rat has been recently reported by Farber and Popper(1) and by Goldberg, Chaikoff, and Dodge(2). In an attempt to reproduce the results of these investigators pancreatic damage was found to be slight and seen only irregularly in rats on a complete diet. It is the purpose of this communication to describe a procedure by which pancreatic damage can be regularly produced by simply putting the rats on a protein depletion regime prior to the administration of the drug.

1. Farber, E. and Popper, H., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 838.

2. Goldberg, R. C., Chaikoff, I. L., and Dodge, A. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 869.