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THE GLUCOSE METABOLISM OF *TRYPANOSOMA EVANSI* AND THE ACTION OF TRYPANOCIDES

BY

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It has long been recognized that trypanosomes are dependent on an adequate supply of glucose for the maintenance of their metabolic processes (Yorke, Adams, and Murgatroyd, 1929; Geiger, Kligler, and Comaroff, 1930; von Brand, 1933). Later work has given some indication of the mechanism of the glucose metabolism of various species of the parasite, and the nature of the end-points formed. Reiner and Smythe (1934) showed that the end-point of glucose metabolism in *T. equiperdum* was pyruvic acid, and the same group of workers (Reiner, Smythe, and Pedlow, 1936) showed that *T. lewisi* metabolized glucose more completely to yield formic, acetic and succinic acids, ethyl alcohol and carbon dioxide. Fulton and Stevens (1945) reported that the products of metabolism of *T. rhodesiense* were succinic, pyruvic, lactic, acetic and formic acids, glycerol, ethyl alcohol, and carbon dioxide.

Regarding the respiratory activity of trypanosomes, Christophers and Fulton (1938) showed that oxygen consumption by *T. rhodesiense* was entirely dependent on the presence of glucose, and that 1 molecule of glucose required 1 molecule of oxygen. They also demonstrated the presence of dehydrogenase systems in trypanosome metabolism, and the absence of inhibition by cyanide. Reiner and Smythe (1934) reported that *T. equiperdum* produced very little carbon dioxide in the absence of bicarbonate, and that 1.80 molecules of acid (mostly pyruvic) were produced from the metabolism of 1 molecule of glucose. These workers also showed that trypanosomes could metabolize glycerol as well as glucose, but only under aerobic conditions. More recent work by Searle and Reiner (1940, 1941) has demonstrated the importance of carbon dioxide as an activator of anaerobic glycolysis in trypanosomes.

These investigations show clearly the nature of the end-products of glucose metabolism in various species of trypanosomes, but give little indication of the intermediate processes by which these products are formed. Indirect evidence, such as the ability of glycerol to replace glucose, suggests that the intermediate metabolism of trypanosomes follows the typical phosphorylation course associated with yeast and muscle metabolism. However, only in one instance, a recently published paper by Chen and Geiling (1946), has any direct evidence of glucose phosphorylation been demonstrated. These workers have shown that lysed trypanosomes will transform glucose to fructose-1,6-diphosphate and triose phosphates, and will oxidize phosphoglyceraldehyde to phosphoglyceric acid.

The investigations described in this communication corroborate the findings of other workers regarding the respiratory activity of trypanosomes, and provide further evidence that glycolysis proceeds *via* the typical chain of phosphorylation reactions. Some preliminary indications are given of the points of attack by trypanocidal agents, on which there are no previous reports.

MATERIALS AND METHODS

The organism was a strain of *Trypanosoma evansi* isolated from a camel in the Sudan in 1938, and maintained in mice, in which it produces heavy, acute blood infections. Infected blood was obtained from the exposed hearts of mice killed by placing them in an atmosphere of carbon dioxide.

Respiration experiments.—Oxygen consumption was measured in conventional Warburg constant volume respirometers, using 15 ml. flasks maintained at 38° C. A set of twelve respirometers was run in duplicate, triplicate, or quadruplicate groups, according to the number of variants required. Since normal mouse blood showed only a negligible oxygen uptake, whole blood containing the trypanosomes was usually used, diluted with "isotonic buffer" (0.85 per cent NaCl, 100 ml.; *M*/15 phosphate buffer, *p*H 7.3, 30 ml.) containing a little citrate to prevent clotting. The concentration of trypanosomes was 10 to 100 million per flask. In a few experiments where blood-free trypanosomes were required, the parasites were separated by adding an equal volume of distilled

water to the blood-buffer suspension, which contained 0.05 per cent glucose. The resultant lowering of tonicity lysed the red cells of the blood, but did not disrupt the trypanosomes, which were centrifuged down, washed free from haemoglobin with isotonic buffer-glucose, and finally resuspended in glucose-free isotonic buffer.

Glucose was determined in the incubates from the respirometer flasks by the method of Folin and Wu (1920). Pyruvic acid was determined by the modified method of Lu (1939) described by Umbreit, Burris, and Stauffer (1945), and lactic acid by the method of Barker and Summerson (1941).

Experiments on glucose phosphorylation.—Mouse blood heavily infected with trypanosomes was diluted with isotonic buffer containing a known concentration of glucose. An aliquot of the suspension was deproteinized immediately by adding an equal volume of ice-cold 12 per cent (w/v) trichloroacetic acid. Similar aliquots were incubated at 38° C. for 40–100 min., either alone, or in contact with inhibitors or trypanocides. These were then treated with trichloroacetic acid, chilled in the cold-room, and the precipitated protein removed by centrifuging. The concentration of trypanosomes was 10 to 100 million per aliquot.

The clear supernatants were subjected to a complete analysis for glucose, phosphorylated intermediates, pyruvic and lactic acids. Glucose was determined in dilutions of the supernatants by the method of Folin and Malmros (1929); pyruvic and lactic acids by the methods already referred to. The remaining supernatant was adjusted to *p*H 8.2 and analysed for hexose phosphates, triose phosphates, phosphoglyceric acid and phosphopyruvic acid using the experimental technique described by Umbreit, Burris, and Stauffer (1945). The amounts of the various intermediates were expressed in molecular proportions (μ moles).

The trypanocides investigated were: phenylarsine oxide—trivalent arsenical; tryparsamide—pentavalent arsenical; undecane diamidine—straight chain diamidine; stilbamidine—aromatic diamidine.

Samples of phenylarsine oxide and tryparsamide were kindly supplied by Dr. T. Dewing of the Wellcome Chemical Laboratories, Dartford.

Results

Respiration experiments

Fig. 1 shows the oxygen uptake of an infected blood suspension containing increasing concentrations of glucose. The initial respiratory rate was the same for all concentrations, but the oxygen uptake ceased abruptly as soon as all the glucose had been used up. This experiment showed clearly the dependence of trypanosomes on an adequate supply of glucose. The more gradual falling off of respiratory rate in the presence of excess glucose was at first presumed to be the result of toxic action by accumulated end-products. However, subsequent experiments showed that the *p*H of the

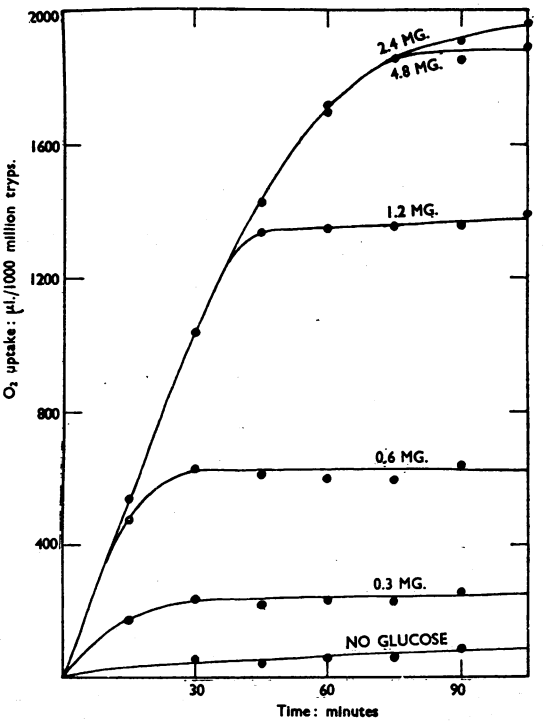

FIG. 1.—Effect of increasing amounts of glucose on the duration of respiration of mouse blood suspension containing *T. evansi*.

medium was not appreciably lowered during the experiment, nor was added pyruvate (the chief end-product) toxic to the trypanosomes. The slowing down of respiratory rate may therefore be caused by exhaustion of available nutritional factors in the parasites or host blood.

The chief end-product of glycolysis by *T. evansi* was pyruvic acid, which accumulated finally in the proportion of 1.75 molecules per molecule of glucose used, one molecule of oxygen being used up in the reaction (Fig. 2).

The rate of oxygen uptake of suspensions of trypanosomes separated from the blood of the host was reduced to 50 per cent of that of the original whole blood suspension. Nearly 75 per cent of the lost respiratory activity was recovered by adding 5 per cent of mouse plasma to the separated parasites (Table I), but addition of lysed mouse red cells had no stimulatory effect. It may be, therefore, that the trypanosome relies on the plasma of the host for many of the nutritional factors required for its metabolism. Individual factors, including pantothenic, nicotinic, adenylic acids, thiamin, riboflavin, pyridoxin, and inositol were

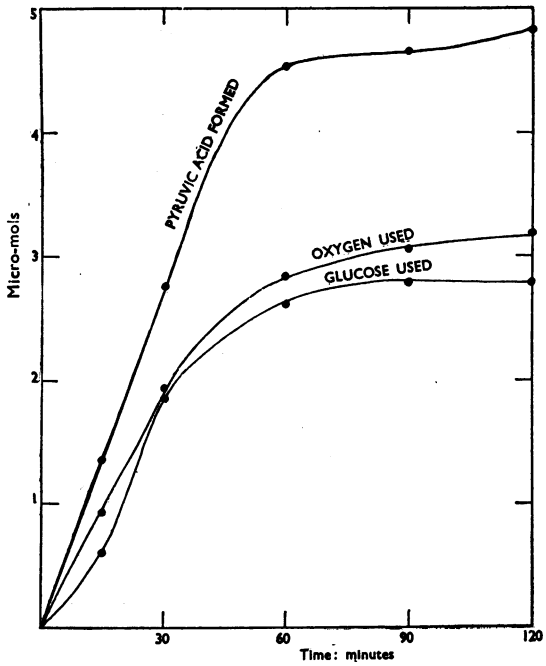

FIG. 2.—The utilization of glucose and oxygen and the formation of pyruvic acid by *T. evansi* (whole blood suspension).

TABLE I

STIMULATORY EFFECT OF MOUSE PLASMA ON THE OXYGEN UPTAKE OF SEPARATED, WASHED TRYPANOSOMES

Washed parasites suspended in isotonic buffer + 0.05 per cent glucose. O_2 uptake compared with that of the original whole blood suspension

Expt.	% plasma added	% of "normal" respiration rate	% recovery
156	none	52.5	—
	5.0	87.8	74.2
157	none	45.2	—
	3.33	79.0	61.5
	1.66	68.5	42.7
	0.66	55.5	18.7
	0.16	55.2	18.4

investigated, but none increased significantly the respiratory rate of separated trypanosomes.

Trypanosome suspensions respired in the presence of glycerol as readily as with glucose. One molecule of oxygen was used up per molecule of glycerol, and one molecule of pyruvic acid was formed. Lactate, fumarate, succinate, glutamate, and aspartate were not metabolized. Pyruvate produced a small additional oxygen uptake, particularly in the presence of glucose. With glycogen, a slow, prolonged oxygen uptake was observed. It was found, however, that glycogen was slowly hydrolysed to glucose by normal mouse blood. The respiration of the trypanosomes in the presence of glycogen therefore resulted from the utilization of this glucose as it was produced, and not from a direct metabolism of the polysaccharide.

TABLE II

EFFECT OF SPECIFIC INHIBITORS ON THE O_2 UPTAKE OF TRYPANOSOMES

Whole blood suspensions containing added glucose

Inhibitor	Concentration		% inhibition
	μg./3 ml.	molar	
Iodoacetic acid ..	50.0	8.98×10^{-5}	92.0
	10.0	1.79×10^{-5}	89.3
	2.0	3.58×10^{-6}	53.3
	0.4	7.71×10^{-7}	4.6
	0.08	1.43×10^{-7}	nil
Sodium fluoride ..	50.0	3.97×10^{-4}	41.5
	10.0	7.94×10^{-5}	13.0
	2.0	1.59×10^{-5}	2.7
	0.4	3.18×10^{-6}	nil
Sodium cyanide ..	50.0	3.4×10^{-4}	10.3
	500.0	3.4×10^{-3}	8.1

Effect of specific inhibitors.—The inhibitory effects of iodoacetic acid, NaCN, and NaF on the oxygen uptake of infected blood suspensions are shown in Table II. Iodoacetic acid and NaF were powerful inhibitors of trypanosome respiration. Cyanide had little effect on the oxygen uptake, but the amount of pyruvic acid formed was reduced to one molecule per molecule of glucose (Fig. 3).

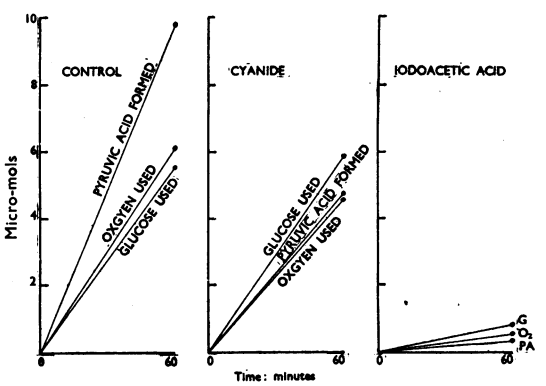

FIG. 3.—Effect of NaCN and iodoacetic acid on the utilization of glucose and oxygen and the formation of pyruvic acid by *T. evansi* (whole blood suspension).

Effect of trypanocides.—The oxygen uptake of trypanosome suspensions was almost completely inhibited by concentrations of 1 in 300,000 of phenylarsine oxide, but was only slightly inhibited by 1 in 30,000 of tryparsamide. Stilbamidine had little inhibitory effect even in concentrations as high as 1 in 6,000. Undecane diamidine, however, produced 50 per cent inhibition of oxygen uptake at 1 in 30,000 concentration, but no further inhibition was obtained on increasing the concentration above this level (Table III).

Phosphorylated intermediate analyses

In these experiments, the standard of activity was taken as the amount of glucose utilized by uninhibited trypanosome suspensions, and the changes in concentration of the intermediate and end-products were expressed as the mean number of molecules used (−) or produced (+) per 100 molecules of glucose consumed by uninhibited, infected blood suspensions.

Glucose phosphorylation by normal and infected blood.—Table IV shows that trypanosome-infected blood used more than 10 times as much glucose as did normal blood ; that glucose-6-phosphate was

TABLE III

EFFECT OF TRYPANOCIDES ON THE O_2 UPTAKE OF TRYPANOSOMES

Whole blood suspensions containing added glucose

Trypanocide	Concentration		% inhibition
	μg./3 ml.	molar	
Phenylarsine oxide (Trivalent As)	10.0	1.98×10^{-5}	81.3
	5.0	9.93×10^{-6}	49.0
	2.0	3.97×10^{-6}	34.0
	0.5	9.93×10^{-7}	14.6
	0.1	1.98×10^{-7}	5.7
Tryparsamide .. (Pentavalent As)	100.0	1.09×10^{-4}	1.70
	10.0	1.09×10^{-5}	4.78
Stilbamidine ..	500.0	6.32×10^{-4}	3.65
	100.0	1.26×10^{-4}	nil
	100.0	1.26×10^{-4}	2.98
	10.0	1.26×10^{-5}	2.08
Undecane diamidine di-HCl	500.0	5.35×10^{-4}	48.5
	100.0	1.07×10^{-4}	51.5
	100.0	1.07×10^{-4}	48.0
	10.0	1.07×10^{-5}	32.4

TABLE IV

EFFECT OF SPECIFIC INHIBITORS AND TRYPANOCIDES ON GLUCOSE PHOSPHORYLATION BY *T. evansi*

Figures denote the mean numbers of μ moles of the substrates formed (+) or utilized (−) per hour per 100 μ moles of glucose consumed by the control suspension (uninhibited trypanosome-blood suspension)

No. of expts.	Inhibitor	Molar conc. of inhibitor	Glucose	ATP	Glucose-1-phosphate	Glucose-6-phosphate	Fructose-6-phosphate	Fruct.-1, 6-diphosphate	Triose phosphates	Phospho-glyceric acid	Phospho-pyruvic acid	Pyruvic acid	Lactic acid
1	(Normal blood)	—	−7	+2.9	+1.2	−0.8	+0.49	+0.34	+0.3	−1.9	+0.18	+4	+13.7
4	(No. inhib.)	—	−100	+6.6	+1.4	−26.7	+0.79	−0.17	+10.7	−4.1	+3.65	+174	−0.21
4	Iodoacetic acid	5.38×10^{-4}	−38	−1.2	+2.2	−13.8	+0.42	+0.38	−2.0	−1.1	+2.55	+12	nil
	(Controls)	—	−100	+6.6	+1.4	−26.7	+0.79	−0.17	+10.7	−4.1	+3.65	+174	−0.21
2	NaCN	4.08×10^{-3}	−173	+9.1	−0.6	−16.8	+0.69	−0.02	+0.5	+0.2	+1.53	+203	+0.7
	(Controls)	—	−100	+5.8	−0.6	−20.3	+1.23	−0.01	−0.1	−2.9	+3.65	+159	−3.8
2	Phenylarsine oxide	5.95×10^{-4}	−24	+10.8	+4.1	−20.9	+0.07	−0.15	+2.8	−20.8		+7	+3.5
	(Controls)	—	−100	+7.4	+3.4	−33.1	+0.36	−0.35	+21.5	−5.4		+189	+3.3
1	Stilbamidine ..	3.79×10^{-4}	−78	−4.1	−10.7	+10.5	+0.96	−0.70	+6.4	−8.9		+202	+8.8
	(Controls)	—	−100	+11.5	+6.8	−1.2	+0.72	−0.70	+21.0	−5.7		+215	+12.3
2	Undecane diamidine	6.4×10^{-4}	−148	+7.2	−0.7	−9.4	+1.36	+0.38	+3.2	−0.7	+5.36	+190	−0.6
	(Controls)	—	−100	+5.8	−0.6	−20.3	+1.23	+0.01	−0.1	−2.9	+3.65	+159	−3.8

very rapidly utilized by infected blood; and that glucose-1-phosphate, triose phosphate, phosphopyruvic acid and particularly pyruvic acid accumulated in parasitized blood incubates. Adenosine triphosphate (ATP) accumulated more rapidly in infected than in normal blood. The mean amount of pyruvic acid formed was 1.74 molecules per molecule of glucose used, which agrees with the results of the respiration experiments. Normal blood produced practically no pyruvic acid, but some lactic acid accumulated.

Effect of specific inhibitors.—In the presence of iodoacetic acid, glucose utilization was strongly inhibited, ATP was consumed, and glucose-6-phosphate disappearance was retarded, while hexose diphosphate accumulated. These observations are in accordance with the accepted view that iodoacetic acid blocks the dehydrogenase reaction which promotes the oxidation of phosphoglyceraldehyde to phosphoglyceric acid. Cyanide actually increased the amount of glucose utilized, but reduced the proportion of pyruvic acid formed. It had little effect on the intermediates of the system.

Effect of trypanocides.—Of the trypanocides investigated, phenylarsine oxide had the most striking effect. In the presence of this compound, very little glucose was utilized, and ATP accumulated in the incubate. This suggests an inhibition of hexokinase, since the ATP released by dephosphorylation was not being re-utilized to phosphorylate more glucose. The intermediates beyond the "block" were rapidly consumed.

Undecane diamidine behaved similarly to cyanide in that it increased the glucose utilization and reduced the proportion of pyruvic acid formed. Stilbamidine inhibited glucose utilization slightly, but increased the proportion of pyruvic acid produced to 2.6 molecules. Its effect on the intermediate metabolism resembled that of iodoacetic acid, though glucose utilization was not inhibited to the same extent.

Discussion

The preliminary observations on *T. evansi* showed that suspensions of the parasites metabolized glucose and glycerol exclusively, the oxygen uptake ceasing abruptly as soon as the added substrate was consumed. The end-point of the metabolism of either substrate was pyruvic acid, and quantitatively the gross reactions taking place conformed fairly closely to the equations

$$\underset{\text{glucose}}{C_6H_{12}O_6} + O_2 \longrightarrow 2\underset{\text{pyruvic acid}}{C_3H_4O_3} + 2H_2O$$

and

$$\underset{\text{glycerol}}{C_3H_8O_3} + O_2 \longrightarrow C_3H_4O_3 + 2H_2O$$

These observations are similar to those reported for *T. equiperdum* (Reiner and Smythe, 1934). No other substrates were utilized by *T. evansi*. Polysaccharides (glycogen) were not broken down directly, and none of the intermediates associated with the subsequent utilization of pyruvic acid (succinate and fumarate), nor amino-acids, stimulated the respiratory activity of the trypanosomes. The metabolic activity of this trypanosome therefore appears to be limited to the conversion of hexoses to pyruvic acid, since glycerol can be regarded as an intermediate of this process.

The next step was to examine the intermediate stages of this reaction. In most living cells, glucose breakdown proceeds *via* the so-called Embden-Meyerhof-Parnas scheme of reactions (Fig. 4) which operates through a chain of phosphorylated hexose intermediates. Previous work on trypanosomes suggested that the metabolism of these organisms followed this scheme. In the present work, analysis of trypanosome suspensions before and after a period of incubation with glucose showed that active changes in the levels of phosphorylated intermediates had taken place. These changes were much greater in parasitized blood than in normal mouse blood. Addition of iodoacetic acid slowed down the rate of utilization of the hexose phosphates as well as the oxygen consumption of the parasites. From these observations it is concluded that the glucose metabolism of the trypanosomes followed the Embden-Meyerhof-Parnas scheme.

The only point at which atmospheric oxygen enters into the above scheme is in the oxidation of phosphoglyceraldehyde to phosphoglyceric acid. Biological oxidations may be accomplished either by transferring oxygen through a chain of oxidation reactions to the compound to be oxidized, or by transferring hydrogen from the compound through a chain of reducing actions to a point where it can be oxidized to water by atmospheric oxygen. Oxidation systems (usually including the cytochrome system) are inhibited by cyanide; dehydrogenase systems by iodoacetic acid. Since the oxygen uptake of trypanosomes was inhibited by iodoacetic acid, but not by cyanide, it is concluded, in agreement with Christophers and Fulton (1938), that only dehydrogenase systems operate in their utilization of oxygen.

In any metabolic system, certain co-factors are necessary for the functioning of the enzyme reactions involved. The factors required by trypanosomes may be obtained from the plasma of the

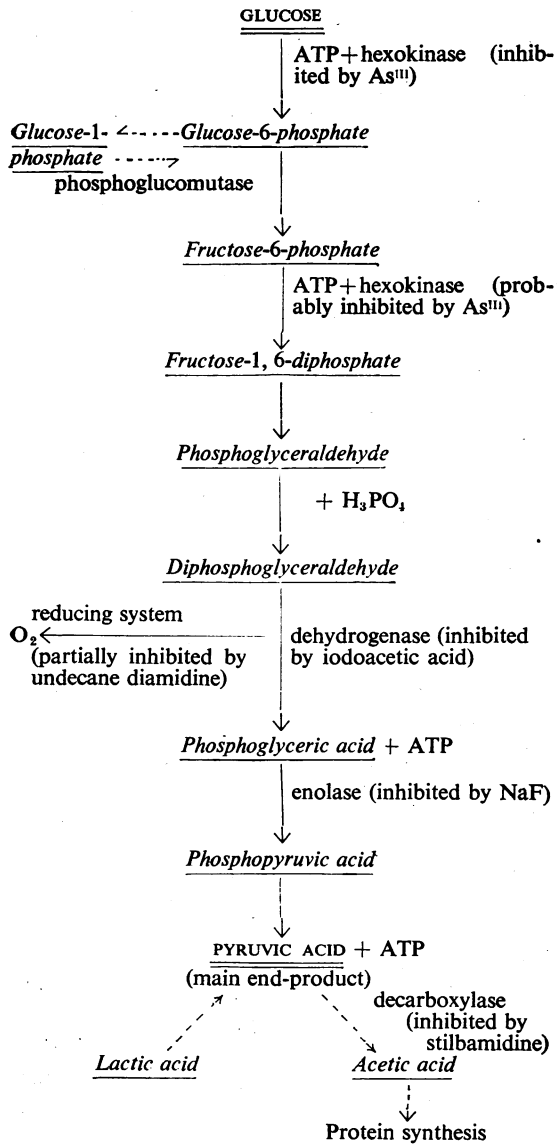

FIG. 4.—Scheme of glucose metabolism of *T. evansi*, with indications of the points of action of trypanocides.

host, since the respiration rate of trypanosomes separated from plasma was reduced by half, and the activity could be restored by adding normal mouse plasma. Other explanations, such as osmotic disturbances and surface phenomena, could account for this loss of metabolic activity, but the fact that only 5 per cent of plasma was required to restore most of the lost respiratory rate would favour the nutritional factors as the most likely explanation.

Although the above equation for the oxidation of glucose to pyruvic acid was fairly well satisfied, it was noticed repeatedly that only 1.75 molecules of pyruvic acid accumulated, instead of the theoretical 2 molecules, and that a little more than one molecule of oxygen was utilized. This suggests that a little of the pyruvate is oxidized by the trypanosomes, and might be the source of the protein which must be synthesized for the multiplication of the parasites. Some indication of the mode of metabolism of the quarter molecule of pyruvic acid was provided by the observation that cyanide decreased the amount of pyruvic acid which accumulated, relative to the amount of glucose used up. Tauber (1938) reported that cyanide was an activator of co-carboxylase, and it is probable, therefore, that the pyruvate undergoes preliminary decarboxylation. The fact that trypanosomes produce a small amount of respiratory CO_2 (Reiner and Smythe, 1934; Christophers and Fulton, 1938) supports this theory, since the reaction ending in pyruvic acid produces no CO_2.

It is now possible to consider at what points in this metabolic scheme the various types of trypanocides exert their action. It is clear from the results that trivalent arsenicals strongly inhibit the hexokinase reaction, and therefore block the utilization of glucose at the first stage, conversion to the 6-phosphate. This observation agrees with the report of Dixon and Needham (1946) on the action of arsenical vesicants on the glucose metabolism of skin. Pentavalent arsenicals showed no inhibition of trypanosome respiration, which is in accordance with the view that these compounds must first be reduced *in vivo* to the trivalent state before becoming active.

The point of action of the diamidines was less obvious than that of arsenic, but it appears that the straight chain compounds (undecane diamidine) differ in action from the aromatic compounds (stilbamidine). The former compound behaved similarly to cyanide in decreasing the amount of pyruvate formed, while stilbamidine had the reverse effect of increasing the pyruvate accumulation. Though stilbamidine caused striking changes in the levels of phosphorylated intermediates, it did not interfere to any extent with the utilization of glucose or oxygen during the 60 to 90 minute period of the manometric experiments. Its trypanocidal action may therefore result from the inhibition of pyruvate metabolism, causing ultimate suppression of growth. These conclusions are in agreement with the obser-

vations of Lourie and Yorke (1937) that this type of compound (synthalin) only showed trypanocidal activity *in vitro* after at least 24 hours' incubation. The point of action of undecane diamidine is not clear, since intermediate glucose metabolism is not greatly influenced by the compound. It did, however, cause a 50 per cent reduction in oxygen uptake, so that its point of action may lie somewhere in the dehydrogenase system (Fig. 4).

Two compounds which do not appear to fit directly into the metabolic scheme of trypanosomes require some consideration. The first one, glucose-1-phosphate, is not an intermediate of glucose but of glycogen phosphorylation. Although it was shown that trypanosomes do not metabolize glycogen directly, an increase in glucose-1-phosphate was observed in some cases in uninhibited incubates and in the presence of inhibitors blocking the normal metabolic chain. In iodoacetic acid inhibition, where the blockage occurs beyond the glucose-6-phosphate stage (Fig. 4), accumulation of glucose-1-phosphate might result from a "feed-back" from the 6-phosphate *via* the reversible phosphoglucomutase reaction. With phenylarsine oxide, however, where the greatest accumulation of glucose-1-phosphate occurred, this "feed-back" could not operate, since the formation of the 6-phosphate itself from glucose was inhibited. The accumulation of the 1-phosphate in this case cannot as yet be explained.

The second compound for consideration is lactic acid, which accumulated in normal blood incubates, but not in incubates containing trypanosomes. Moreover, when glucose metabolism was blocked by iodoacetic acid, lactic acid initially present disappeared during incubation. These observations suggest that indigenous lactic acid can be converted to pyruvic acid by the parasites. Further evidence of this possibility is provided by the observed accumulation of more than the theoretical two molecules of pyruvic acid in the presence of stilbamidine.

Summary

1. Investigations of the oxygen utilization of suspensions of *T. evansi* show that this trypanosome metabolizes glucose mainly to pyruvic acid, though a small quantity of this end-product is probably further utilized by decarboxylation.

2. Analysis of trypanosome suspensions for phosphorylated intermediates in the presence of specific inhibitors shows that the intermediate metabolism of the parasites follows the typical Embden-Meyerhof-Parnas scheme characteristic of yeast and muscle metabolism.

3. At least some of the nutritional factors required in this metabolism may be obtained from the plasma of the host.

4. Trypanosomes appear to be able to utilize the lactic acid in the host blood, probably by conversion to pyruvic acid.

5. Observation of the effects of typical trypanocides suggests that trivalent arsenicals inhibit the hexokinase reaction in trypanosomes; that pentavalent arsenicals are inactive unless reduced *in vivo* to the trivalent state; that straight chain diamidines (undecane diamidine) partially inhibit the dehydrogenase system on which oxygen transport depends in trypanosomes; and that aromatic diamidines (stilbamidine) probably inhibit the decarboxylation of pyruvic acid.

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