Brit. J. Pharmacol. (1948), 3, 1.

THE GLUCOSE METABOLISM OF *PLASMODIUM GALLINACEUM*, AND THE ACTION OF ANTIMALARIAL AGENTS

BY

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(Received May 1, 1947)

Studies on the metabolism of the erythrocytic forms of plasmodia were initiated by Christophers and Fulton (1938), who showed that monkey red cells infected with *P. knowlesi* consumed oxygen independently of glucose, though added glucose was utilized; the oxygen uptake of the parasitized cells was completely inhibited by cyanide. Maier and Coggeshall (1941) showed that parasitized red cells were dependent to some extent on glucose, and that mannose, fructose, and glycerol would replace glucose as a metabolite. During the war years, a lot of research on the metabolism of the malaria parasite has been done by various groups of workers in America. This work has been comprehensively reviewed in *Federation Proceedings* (vol. 5, No. 3). It was reported that, under aerobic conditions, red cells infected with malaria parasites could metabolize lactate, pyruvate, succinate, fumarate, and amino-acids, besides glucose, and that glucose was converted to lactic acid by phosphorylation, as in muscle (Evans, Ceithaml, Speck, and Moulder, 1945; Bovarnick, Lindsay, and Hellerman, 1946a, b; McKee, Ormsbee, Aufinsen, Geiman, and Ball, 1946). Speck and Evans (1945a) showed that cell-free extracts of plasmodia would phosphorylate glucose similarly to yeast extracts.

Regarding the action of antimalarials, Silverman, Ceithaml, Taliaferro, and Evans (1944) reported that quinine and mepacrine (atebrin) inhibited the respiration of plasmodia, though the inhibition was delayed until the third or fourth hour of incubation with the drug. Speck and Evans (1945b) reported that quinine and mepacrine inhibited the phosphorylation of glucose by cell-free extracts of plasmodia and of normal red cells, and that the degree of inhibition corresponded to the amount of hexokinase activity present. Investigating isolated enzyme systems, these workers showed that quinine and mepacrine inhibited yeast hexokinase, but not 3-phosphoglyceraldehyde dehydrogenase. Lactic dehydrogenase from plasmodium extracts and from ox-heart was inhibited more by mepacrine than by quinine. More recent work by Bovarnick, Lindsay, and Hellerman (1946b) showed that separated plasmodia, thoroughly exhausted of substrates by washing and preliminary incubation, were more sensitive to antimalarial drugs. The recovery of respiration after addition of glucose to the exhausted parasites was strongly inhibited by mepacrine, but no inhibition was observed with other substrates. Inhibition of glycolysis by mepacrine was antagonized by adenylic acid or adenosine triphosphate.

The present paper, reporting preliminary work on the metabolism of plasmodia, confirms generally the above reports on the glucose metabolism of malaria parasites, and presents fresh evidence of glycolysis by phosphorylation and on the mode of action of quinine and mepacrine.

Materials and Methods

Blood from chicks 2–4 weeks old, heavily infected (70–95 per cent of cells parasitized) with *P. gallinaceum*, was used as the source of parasite material. Since, in initial experiments, whole blood suspensions showed no response to added glucose, washed, parasitized red cells were used in most of the experiments described. The chicks were killed by placing in an atmosphere of CO_2, and blood drawn from the exposed heart was suspended in isotonic buffer (0.85 per cent NaCl, 100 ml.; $M/15$ phosphate buffer, pH 7.3, 30 ml.) containing a little citrate to prevent coagulation. The suspension was centrifuged and the clear supernatant discarded; the red cell layer was shaken with more isotonic buffer, re-centrifuged, and the red cells finally suspended in sufficient isotonic buffer to produce the required volume of suspension. This treatment removed all but a trace of indigenous glucose from the red cells.

Respiration experiments.—Oxygen consumption was measured in conventional Warburg respirometers, using 15 ml. flasks, maintained at 38° C. Determinations were carried out with a set of twelve respirometers, in duplicate, triplicate, or quadruplicate groups, according to the number of variants required. The amount of oxygen consumed was expressed in microlitres (μl.) per 1,000 million red blood cells, the percentage of cells parasitized being 70–95.

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.—Aliquots of a suspension of washed, parasitized red cells were incubated at 41° C. with a known amount of glucose, in contact with various inhibitors and antimalarial agents. A control aliquot was deproteinized immediately by adding an equal volume of ice-cold 12 per cent (w/v) trichloroacetic acid. The test aliquots were incubated for 4–5 hours, during which they were oxygenated, either by continuous bubbling, or at frequent intervals. At the end of the incubation period, the test aliquots were deproteinized, chilled in the cold-room, and the precipitated protein removed by centrifuging.

The clear supernatants were subjected to a complete analysis for glucose, phosphorylated intermediates, and pyruvic and lactic acids. Glucose was determined in dilutions of the supernatants by the method of Folin and Malmros (1929), and 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, and phosphoglyceric acid, using the experimental technique described by Umbreit, Burris, and Stauffer (1945). The amounts of the various intermediates were expressed as gram-molecules.

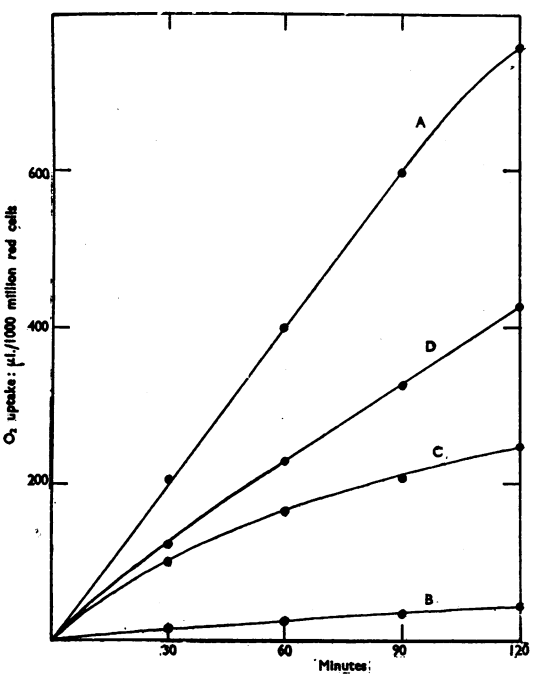

FIG. 1.—Oxygen uptake of normal and parasitized chick blood (single experiment). A.—Whole blood suspension (parasitized). B.—Whole blood suspension (normal). C.—Washed, parasitized red cells. D.—Washed, parasitized red cells + glucose (0.5 mg./ml.).

RESULTS

Respiration experiments

Though normal chick red cells showed a substantial oxygen uptake (compared with non-nucleated mammalian red cells), the respiration rate of heavily parasitized (96 per cent) chick red cells was about 15 times as rapid (Fig. 1). The respiratory rate of infected red cells (whole blood suspensions) increased proportionately with the number of cells parasitized (Table I). The oxygen uptake of infected whole blood suspensions was not increased, but actually depressed by adding glucose. If, however, the parasitized red cells were washed free from indigenous glucose, the oxygen uptake was reduced to 37 per cent (mean of 9 determinations; range 27.8–50.8 per cent) of that of the whole blood suspension (Fig. 1). The respiration of glucose-free infected cells was stimulated by added glucose, the recovery being graded

TABLE I

THE RELATION BETWEEN OXYGEN UPTAKE AND THE NUMBER OF RED CELLS PARASITIZED WITH *P. gallinaceum*

Whole blood suspensions; no added substrate

% cells parasitized	No. of respirometers	O_2 uptake (μl./hr./1000 million red cells)
normal	6	65.4
,,	6	48.4
,,	6	27.5
,,	5	36.2
,,	5	25.5
,,	2	19.5
,,	2	17.1
		Mean 34.2
30	5	77.8
30	5	89.0
44	3	146.0
80	5	250.0
85	5	296.0
88	2	235.0
96	6	314.0

TABLE II

EFFECT OF GRADED CONCENTRATIONS OF GLUCOSE ON THE OXYGEN UPTAKE OF WASHED, PARASITIZED RED CELLS

Glucose added (mg./3 ml.)	% of O_2 uptake of whole blood suspension	% recovery of lost respiration
0.05	43.7	6.2
0.1	38.0	nil
0.5	57.2	28.7
0.5	56.5	35.5
0.5	52.1	26.3
		Mean 30.2
1.0	60.7	42.0
1.0	65.4	36.5
1.0	65.4	42.4
1.0	66.3	51.5
		Mean 43.1
2.0	81.7	71.8
2.0	73.0	45.1
		Mean 58.5
5.0	84.0	75.4
10.0	80.4	70.0

up to 5 mg./3 ml., when a maximum recovery of 75 per cent of the lost respiration rate was attained (Table II).

The quantity of oxygen consumed by plasmodia was not accounted for by the amount of glucose used; for example, when infected red cells were washed, the oxygen consumption was still about 40 per cent of that of the whole blood suspension, though the amount of available glucose was very small. Thus the oxygen consumption per molecule of glucose used was apparently increased (Table III). Again, since additional glucose did not increase the oxygen uptake of infected whole blood suspensions, the oxygen consumption and the amount of lactic acid formed per molecule of glucose appeared to decrease with increasing concentration of glucose (Table III). No pyruvic acid was formed. Table IV shows the course of quantitative utilization of glucose added to washed, parasitized red cells, and the amount of *extra* oxygen consumed. Neither lactic nor pyruvic acids accumulated in this instance.

TABLE III

QUANTITATIVE UTILIZATION OF GLUCOSE BY PLASMODIA

(a) Effect of graded concentrations on whole blood suspension.
(b) Effect of washing the infected red cells.

Preparation	Conc. of glucose g./100 ml.	Mols O_2 used per mol glucose consumed	mols lactic acid formed per mol glucose consumed
(a)	*added*		
Whole blood suspension	nil*	5.3	4.0
	0.01*	3.1	2.5
	0.05*	2.1	1.56
	0.10*	1.9	1.95
(b)	*present*		
Whole blood suspension	0.002	4.04	—
Washed cell suspension	0.00005	26.0	—

* + 0.02 g./100 ml. indigenous glucose.

TABLE IV

QUANTITATIVE UTILIZATION OF GLUCOSE BY WASHED, PARASITIZED RED CELLS

Time after adding glucose (min.)	μ-mols glucose used	μ-mols extra O_2 used	Mols O_2 used per mol glucose consumed
30	1.15	0.69	0.60
60	1.70	1.32	0.78
90	2.73	2.68	0.98
120	2.76	3.08	1.12

Substrates metabolized by plasmodium.—The oxygen uptake of washed, parasitized red cells was stimulated by glycerol, pyruvate, and lactate to the same extent as by glucose, but was only slightly stimulated by succinate and fumarate. Glutamate, aspartate, and tyrosine were inactive (Table V).

TABLE V

EFFECT OF ADDED SUBSTRATES ON THE OXYGEN UPTAKE OF WASHED, PARASITIZED RED CELLS

Substrate added	Concentration (mg./3 ml.)	% stimulation of oxygen uptake
Glucose	1.0	75.6
Tyrosine	1.0	nil
Glucose	2.0	155
Pyruvate	2.0	161
Lactate	2.0	179
Succinate	2.0	13.5
Fumarate	2.0	12.9
Glucose	2.0	174
Glycerol	2.0	207
Glutamate ..	2.0	nil
Aspartate	2.0	nil

Effect of specific inhibitors.—Cyanide strongly inhibited the oxidation of glucose, lactate, and pyruvate by washed, parasitized red cells. Iodoacetic acid, in low concentration (1 in 30,000),

strongly inhibited glucose, but not pyruvate, oxidation; at higher concentration (1 in 6,000), the specificity of inhibition disappeared, and both substrates were equally inhibited (Table VI).

TABLE VI

EFFECT OF SPECIFIC INHIBITORS ON THE OXYGEN UPTAKE OF WASHED, PARASITIZED RED CELLS ALONE, AND WITH ADDED SUBSTRATES

Inhibitor	Conc. (mg./3 ml.)	Percentage inhibition of O_2 uptake			
		No added substrate	Glucose	Pyruvic acid	Lactic acid
NaCN ..	0.5	80.4	87.5	93.7	87.7
Iodoacetic acid	0.5	36.0	42.0	86.0	74.6
	0.5		76.0	83.6	
	0.1		63.5	10.1	

Effect of antimalarial agents.—Quinine and mepacrine did not markedly inhibit the oxygen uptake of plasmodia either when respiring alone (washed, parasitized red cells), or in the presence of active substrates (Table VII). At fairly high concentration (1 in 6,000), there was little differentiation between the substrates, but at lower concentration (1 in 60,000), quinine showed a slightly greater inhibition of glucose and lactate oxidation, and mepacrine of lactate oxidation (Table VII).

TABLE VII

EFFECT OF QUININE AND MEPACRINE ON THE OXYGEN UPTAKE OF WASHED, PARASITIZED RED CELLS ALONE, AND WITH ADDED SUBSTRATES

Anti-malarial agent	Conc. (mg./3 ml.)	Percentage inhibition of O_2 uptake after 2 hours			
		No added substrate	Glucose	Pyruvic acid	Lactic acid
Quinine	0.5	22.4	25.7	29.1	35.7
	0.05	6.0	17.3	2.3	18.9
Mepacrine	0.5	11.9	34.4	25.6	34.5
	0.05	9.0	2.0	5.8	14.6

At still lower concentration (1 in 300,000), the inhibitory effects, particularly of mepacrine, did not appear until the drugs had been incubated with the parasites for 2–6 hours (Table VIII).

Glucose phosphorylation

Changes in glucose, phosphorylated intermediates, and lactic and pyruvic acids during incubation of infected red cell suspensions were expressed as molecules per 100 molecules of glucose used by uninhibited, parasitized red cells. Table IX shows the comparison between the glucose metabolism of uninfected chick red cells and parasitized red cells. Parasitized cells used about four times as much glucose as normal red cells, and, with parasitized cells, a large amount of lactic acid accumulated (0.5 to 1.5 molecule per molecule of glucose used). Glucose-6-phosphate, fructose-6-phosphate and fructose-1, 6-diphosphate were metabolized more rapidly in parasitized cells than in normal cells, but triose phosphate and pyruvic acid accumulated to a greater extent than in uninfected red cells. These observations showed that active glucose phosphorylation was proceeding more rapidly in infected red cell incubates than in normal cell incubates.

TABLE VIII

INHIBITION OF OXYGEN UPTAKE OF WASHED, PARASITIZED RED CELLS BY LOW CONCENTRATIONS OF QUININE AND MEPACRINE

Anti-malarial agent	Substrate	Percentage inhibition after incubation for		
		2 hr.	4 hr.	6 hr.
Quinine (0.01 mg./3 ml.)	none	nil	5.4	16.8
	glucose	9.4	15.7	20.0
Mepacrine (0.01 mg./3 ml.)	none	nil	nil	3.1
	glucose	nil	6.8	9.8

Effect of specific inhibitors.—Iodoacetic acid completely inhibited the utilization of glucose and accumulation of lactic acid, and, in the accumulation of hexose and triose phosphates, showed the typical effects of phosphoglyceraldehyde dehydrogenase blockage. Cyanide inhibited the utilization of glucose by 40 per cent, but the amount of lactic acid formed was greater than in the controls. Cyanide stimulated the utilization of phosphorylated intermediates and of pyruvic acid, as shown by a decrease during the incubation period.

Effect of antimalarial agents.—Quinine inhibited glucose utilization more powerfully than mepacrine did. The most marked effect of mepacrine was the large accumulation of adenosine triphosphate (ATP), indicating inhibition of the enzyme hexokinase by which glucose is initially phosphorylated. Quinine also inhibited hexokinase, but to a smaller extent than mepacrine. Both drugs caused accumulation of glucose-6-phosphate, but not to the same extent as iodoacetic acid. In the presence of quinine, pyruvic acid accumulated; mepacrine reduced the accumulation of lactic acid more than quinine did.

TABLE IX

EFFECT OF SPECIFIC INHIBITORS AND ANTIMALARIALS ON GLUCOSE PHOSPHORYLATION BY WASHED CHICK RED CELLS INFECTED WITH *P. gallinaceum*

Figures denote the mean numbers of μ mols of the substrates formed (+) or used (−) per hour per 100 μ mols of glucose consumed by the control suspension

No. of expts.	Inhibitor	Molar conc. of inhibitor	Glucose	ATP	Glucose-1-phosphate	Glucose-6-phosphate	Fructose-6-phosphate	Fructose-1, 6-diphosphate	Triose phosphates	Phosphoglyceric acid	Phosphopyruvic acid	Pyruvic acid	Lactic acid
1	(Normal blood)	—	−23	+1.66	+1.16	+1.19	+0.24	+0.65	+0.66	nil	nil	+1.51	nil
4	(No inhibitor)	—	−100	+0.79	+6.99	−3.81	−0.05	−0.01	+4.47	−1.29	nil	+5.19	+115
3	Iodoacetic acid	1.08×10^{-3}	+4	−0.42	+7.90	+10.22	+0.70	+0.23	+4.67	+1.00	+1.07	+2.01	+6
	(Controls)	—	−100	+1.05	+9.32	−0.17	+0.19	+0.15	+5.96	−1.10	nil	+6.04	+143
2	NaCN	4.08×10^{-3}	−60	−0.93	+4.26	−4.13	−0.07	+0.61	+0.53	−1.56	+5.84	−0.42	+90
	(Controls)	—	−100	+0.66	−0.92	−9.80	−0.30	−0.25	+0.40	−1.10	nil	+5.02	+69
3	Quinine	5.05×10^{-4}	−42	+2.39	+5.97	+1.14	−0.18	+0.41	+1.87	−0.28		+5.37	+94
	(Controls)	—	−100	+0.61	+9.93	−3.47	−0.11	−0.02	+5.69	−1.60		+4.44	+118
3	Mepacrine	3.94×10^{-4}	−79	+6.46	−0.91	+2.26	−0.17	+0.47	+3.48	−0.30	nil	+1.02	+57
	(Controls)	—	−100	+0.61	+9.93	−3.47	−0.11	−0.02	+5.69	−1.60	nil	+4.44	+118

Discussion

Although the malaria parasite utilizes glucose, its metabolism of this substrate is comparatively slow; thus, in whole blood suspensions, sufficient indigenous glucose is present to last the parasites for several hours, and added glucose produces no stimulation of respiratory activity. That glucose is a necessary substrate for the full activity of plasmodia is, however, shown by the fact that infected red cells lose 60 per cent of their respiratory activity when washed free from glucose, and that most of this lost activity can be restored by adding glucose to the washed cell suspension.

The respiration rate of washed, parasitized red cells can be stimulated by substrates other than glucose. As observed by previous workers (Speck, Moulder and Evans, 1946), lactic and pyruvic acids are as active as, or slightly more active than, glucose in this respect (see, however, the next paragraph). Succinic and fumaric acids show a small but definite stimulatory effect. From these observations it is apparent that the carbohydrate metabolism of plasmodia proceeds to some end-point beyond lactic and pyruvic acids. The stimulation by succinate and fumarate suggests that pyruvic acid is metabolized *via* the Krebs citric acid cycle. Using parasites freed from red cells, Speck, Moulder, and Evans (1946) obtained much greater stimulation with succinate.

Analysis of parasitized red cell incubates for phosphorylated intermediates confirmed the view of previous workers that the initial stages of glucose metabolism by plasmodia follow the so-called Embden-Meyerhof-Parnas system characteristic of yeast and muscle metabolism. Since considerable quantities of lactic acid accumulated in these incubates, it seems that the conversion of glucose to pyruvic acid is more rapid than the subsequent utilization of pyruvate, the excess pyruvate being reduced to lactate. McKee *et al.* (1946) reported that lactate was produced six times as rapidly as it was utilized.

The amount of oxygen utilized by plasmodia bears no constant relation to the amount of glucose used. In parasitized red cell incubates respiring in the presence of glucose, the amount of oxygen consumed is at first less than one molecule per molecule of glucose used, but gradually increases to more than one molecule during the incubation period. This gradual increase in oxygen consumption probably indicates the "starting up" of additional oxidation systems as the metabolism of the initial quantity of glucose reaches the various stages. The fact that it takes several hours to build

up to the full rate of oxygen consumption emphasizes the sluggishness of carbohydrate metabolism in plasmodia.

Oxygen enters into the metabolic system of plasmodia at two points at least; firstly in the oxidation of glucose to pyruvic acid, and secondly in the subsequent oxidation of the pyruvate. Since the first oxidation (which takes place at the conversion of phosphoglyceraldehyde to phosphoglyceric acid) is inhibited by iodoacetic acid, it is concluded that it takes place through a chain of reducing reactions catalysed by dehydrogenase systems. However, the final connection with atmospheric oxygen is probably made through the cytochrome oxidase system, since glucose oxidation is as powerfully inhibited by cyanide as pyruvate oxidation. Furthermore, in the presence of cyanide, glucose and the phosphorylated intermediates are rapidly converted to lactic acid, which is an anaerobic reaction requiring no oxygen. Pyruvate oxidation is not appreciably inhibited by iodoacetic acid, but is strongly inhibited by cyanide, and is therefore entirely dependent on the cytochrome oxidase system.

Considerable quantities of glucose-1-phosphate (an intermediate of glycogen, but not of glucose, metabolism) accumulated in parasitized red cell incubates. This accumulation probably resulted from a "feed-back" from glucose-6-phosphate through the reversible phosphoglucomutase reaction. This view is strengthened by the observation that accumulation of glucose-1-phosphate was increased in the presence of iodoacetic acid, where the later stages of normal glucose phosphorylation were blocked.

Parasitized red cells from which all traces of indigenous glucose have been removed by repeated washing still show an appreciable oxygen uptake, which is still proceeding after 6 hours' incubation. It is generally believed that plasmodia can metabolize substrates other than carbohydrate, but, in view of the slowness of glucose metabolism, the possibility cannot be ignored that sufficient quantities of the glucose intermediate compounds might remain in the parasites or red cells after washing to account for the "residual" respiration observed. The oxygen uptake of washed, parasitized red cells was not stimulated by amino-acids (glutamate, aspartate or tyrosine) nor could any change in non-protein or ammonia nitrogen be detected in the incubates. This is not, however, in agreement with the results of previous workers (Moulder and Evans, 1946), who found both that amino-acids were utilized, and that non-protein and ammonia nitrogen were produced by plasmodia.

Having obtained some indication of the course of carbohydrate metabolism in plasmodium, we may consider at what points in this system quinine and mepacrine exert inhibitory actions. In agreement with the observations of previous workers (Silverman *et al.*, 1944) it was noted that neither drug, in low concentration, inhibited the oxygen uptake of parasitized red cells until it had been in contact with the cells for 2–6 hours; even then, the inhibition was not very pronounced.

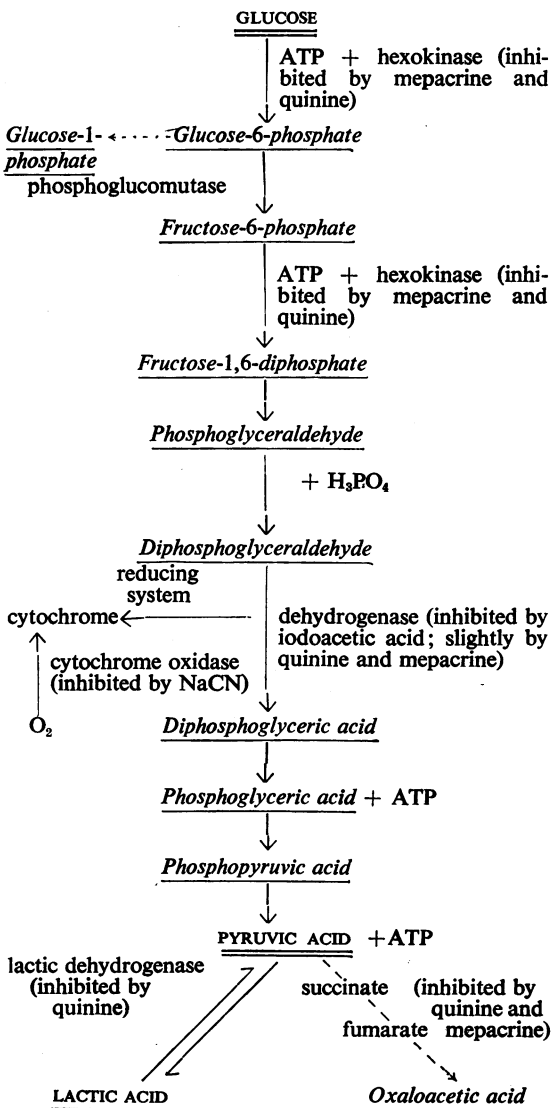

FIG. 2.—Scheme of glucose metabolism of *P. gallinaceum*, with indications of the points of action of quinine and mepacrine.

Bovarnick *et al.* (1946b) reported that mepacrine inhibition of plasmodia could be reversed by ATP, and Speck and Evans (1945b) showed that both quinine and mepacrine inhibited yeast hexokinase. In the present investigation, the analyses showed that, while quinine inhibited glucose utilization to a greater degree than mepacrine, the latter compound showed a more pronounced inhibition of hexokinase. In the presence of mepacrine, the accumulation of glucose-1-phosphate was inhibited, presumably because of the formation of glucose-6-phosphate was reduced. Both drugs showed some iodoacetate-like inhibitory effects, but these were not well marked. Quinine caused lactate and pyruvate to accumulate, and therefore inhibited lactate as well as pyruvate oxidation. These possible points of action are indicated in Fig. 2.

From the above observations, it is clear that quinine and mepacrine exert inhibitory activity at several points in the metabolic scheme of plasmodia. With an organism possessing a comparatively complicated metabolism, it is necessary that a therapeutic agent should have this capacity for multiple attack in order to be effective against the organism. For instance, trivalent arsenical compounds, which inhibit only one type of enzyme, the so-called -SH enzymes, are active against certain trypanosomes which possess a simpler type of metabolism, but are inactive against plasmodia, in which alternative metabolic paths probably exist, or a greater variety of substrates can be utilized. The present investigation has given indications of the points of action of antimalarial drugs in one metabolic system — carbohydrate metabolism. Further studies are necessary to find what proportion of the total inhibitory activity takes place at the different points, and, indeed, whether the greater part of the inhibition does take place against the carbohydrate metabolism, or against other metabolic functions.

SUMMARY

1. Investigations on the oxygen uptake of *Plasmodium gallinaceum* show that washed, parasitized chick red cells can oxidize glucose, glycerol, lactate, and pyruvate, but not glutamate, aspartate, or tyrosine. Stimulation of oxygen uptake by succinate and fumarate indicates that pyruvate metabolism proceeds *via* the Krebs citric acid cycle.

2. Observation of the changes in phosphorylated intermediates in washed, parasitized red cell incubates confirms the view that glucose metabolism in plasmodia proceeds *via* the Embden-Meyerhof-Parnas system characteristic of yeast and muscle metabolism.

3. The possibility of metabolic systems in plasmodia other than carbohydrate is discussed.

4. Quinine and mepacrine exert inhibitory activity at several points in the glucose metabolism of plasmodia. Quinine inhibits hexokinase and phosphoglyceraldehyde dehydrogenase moderately, and possibly lactic dehydrogenase and pyruvate oxidation. Mepacrine inhibits hexokinase strongly, phosphoglyceraldehyde dehydrogenase moderately, and probably pyruvate oxidation.

The author is indebted to Mr. L. G. Goodwin (Pharmacology Dept.) for supplying chicks infected with *P. gallinaceum*, and to Messrs. P. A. Hankin and R. W. Neville for valuable technical assistance.

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