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THE RELATIONSHIP BETWEEN INHIBITION OF PHOSPHOFRUCTOKINASE ACTIVITY AND THE MODE OF ACTION OF TRIVALENT ORGANIC ANTIMONIALS ON *SCHISTOSOMA MANSONI*

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The addition of purified mammalian phosphofructokinase to homogenates of *Schistosoma mansoni* increased the rate of lactic acid production from glucose and reversed the inhibition of glycolysis produced by low concentrations of trivalent organic antimonials. Neither mammalian phosphofructokinase nor trivalent antimonials affected the rate of lactic acid production from fructose-1 : 6-diphosphate (HDP) by schistosome homogenates. Accordingly, in the schistosome, the rate of glycolysis of glucose is determined by the activity of phosphofructokinase.

The aldolase of *S. mansoni* has a high requirement for HDP; relatively slight reductions in the concentration of this substrate below the optimum resulted in a sharp decline of aldolase activity. Therefore, decreased formation of HDP, due to inhibition of schistosome phosphofructokinase activity by antimonials, reduced the activity of aldolase and resulted in an inhibition of glycolysis of schistosome homogenates.

Kinetic data revealed differences in the nature of the phosphofructokinase of *S. mansoni* and that of the enzyme catalysing the same reaction in the host. Exposure of schistosomes to low concentrations of potassium antimonyl tartrate or administration of subcurative doses of stibophen to the host resulted in an accumulation of the substrate (fructose-6-phosphate), and a reduction of the product (HDP) of the phosphofructokinase reaction, indicating that the activity of this enzyme was inhibited by antimonials in the intact parasite. It is concluded that inhibition of phosphofructokinase activity can account for the mechanism of the chemotherapeutic action of trivalent organic antimonials in schistosomiasis.

Despite their relatively high toxicity and other undesirable properties, trivalent organic antimonials are still considered to be the most effective available chemotherapeutic agents in the treatment of schistosomiasis. Information about the mechanism of the schistosomicidal action of these drugs might reveal opportunities for the development of compounds equally effective against the parasite, but less damaging to the host. In an attempt to explore this problem it was observed that trivalent antimonials produce a decrease in the utilization of carbohydrate by *Schistosoma mansoni* (Bueding, 1950). Subsequent studies revealed that organic antimonials selectively inhibit the phosphorylation of fructose-6-phosphate by adenosine triphosphate, catalysed by the enzyme phosphofructokinase (Mansour and Bueding, 1954). The present paper is concerned with the relationship between this biochemical effect of trivalent antimonials and their mode of action in schistosomiasis. The reaction sequences which are discussed are shown in Fig. 1.

METHODS

In this paper the following abbreviations are used: F-6-P, Fructose-6-phosphate; HDP, fructose-1 : 6-diphosphate; G-6-P, glucose-6-phosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide.

The preparation of schistosome homogenates and measurement of their rate of glycolysis were carried out as in a previous study (Mansour and Bueding, 1954). Phosphofructokinase of rabbit muscle was prepared according to Ling, Byrne, and Lardy (1955), aldolase according to Taylor, Green, and Cori (1948).

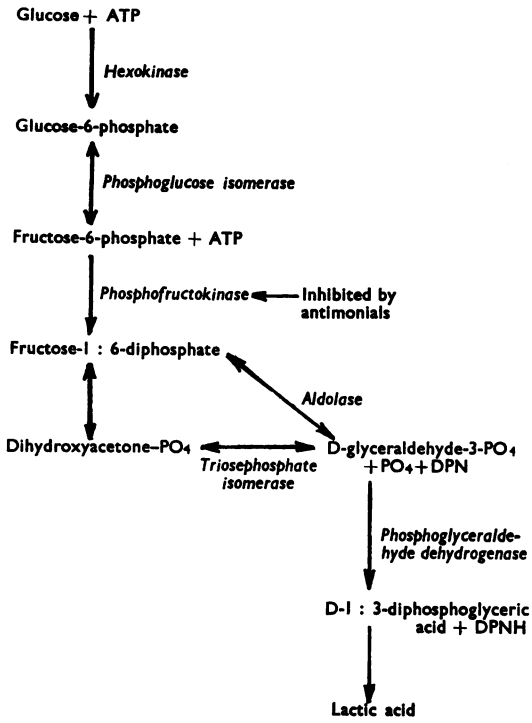

FIG. 1.—Glycolytic reactions and enzymes of *Schistosoma mansoni*.

and glyceraldehyde phosphate dehydrogenase according to Cori, Slein, and Cori (1948). Glucose-6-phosphate dehydrogenase from yeast was prepared by a procedure identical to that used in a previous study (Bueding and MacKinnon, 1955a). An extract containing schistosome phosphofructokinase was obtained as follows: adult schistosomes were cut with a pair of small scissors in potassium glycylglycine buffer (*p*H 7.5; 0.01 M; 50 worm pairs/ml.) for 2 min. After stirring for another 2 min. the mixture was centrifuged at 3,500 *g* and the supernatant was used as the source of the enzyme. All operations were carried out between 0 and 4° C.

Enzymic activities are expressed in arbitrary units. One unit is defined as the amount of enzyme which turns over 1 μM of substrate in 1 min. at 25° C. Rates of phosphofructokinase activity were determined spectrophotometrically according to Ling *et al.* (1955) (*System b*). In the presence of excesses of aldolase and of glyceraldehyde-3-phosphate dehydrogenase, the formation of HDP, catalysed by phosphofructokinase, was followed by the conversion of HDP to glyceraldehyde-3-phosphate and the oxidation of the latter by DPN. Reduction of DPN was measured in a Beckman spectrophotometer at 340 mμ. It was possible to use this procedure because schistosome homogenates and extracts did not contain any measurable 2-glycerophosphate dehydrogenase activity.

The concentration of hexose phosphates in the worm was determined in the following manner: the parasites were homogenized in 0.2 N-HCl (1 ml./100 worm pairs) in an all-glass homogenizer. The homogenate was centrifuged for 20 min. at 34,000 *g* in a Spinco preparative ultracentrifuge. The supernatant was neutralized with 0.16 volumes of 2 N-$KHCO_3$ and then centrifuged for 20 min. at 34,000 *g*. Aliquots of the second supernatant (S2) were assayed for HDP, F-6-P, and G-6-P as described below.

Determination of HDP.—Glyceraldehyde phosphate dehydrogenase (0.8 units) was added to a Beckman spectrophotometer cell (capacity, 1 ml.; light path, 10 mm.) containing an aliquot of S2, potassium glycylglycine buffer (*p*H 8.3; 5×10^{-2} M), $MgCl_2$ (5×10^{-3} M), potassium arsenate (6×10^{-3} M), L-cysteine (1×10^{-3} M), and neutralized DPN (3.4×10^{-4} M), to give a total volume of 0.8 ml. No reduction of DPN was observed at a wavelength of 340 mμ. Therefore, no triose phosphate was detectable in S2. Aldolase (0.5 units) was then added and the subsequent reduction of DPN was equivalent to the molar amount of HDP present in S2. Reduction of DPN was completed within 15 min.

Determination of F-6-P and G-6-P.—0.05 units of glucose phosphate dehydrogenase was added to a spectrophotometer cell containing an aliquot of S2, potassium glycylglycine buffer (*p*H: 8.3; 5×10^{-2} M), $MgCl_2$ (1.25×10^{-2} M), and TPN (1.5×10^{-4} M). The final volume was 0.8 ml. The G-6-P present in S2 was completely oxidized in 15 min. with a corresponding reduction of TPN. The enzyme preparation was contaminated with a small amount of phosphohexose isomerase, and therefore 20 to 30% of the F-6-P in S2 was converted to G-6-P. This percentage was determined by assaying a standard solution of F-6-P along with each unknown. After 15 min., an excess of rabbit muscle phosphohexose isomerase was added and the subsequent reduction of TPN was proportional to the F-6-P remaining in S2. The total F-6-P in S2 was calculated from the amount of TPN reduced after addition of phosphohexose isomerase, plus % of the standard F-6-P solution which was converted to G-6-P in the first 15 min. The amount of G-6-P present in S2 was calculated by subtracting the value found for F-6-P from the total hexosephosphates. Calculations of the amounts of reduced DPN and TPN were based on their molar extinction coefficients of 6.3×10^3 at the wavelength of 340 mμ.

In each of the experiments described below worms were used which originated from mice infected on the same day by the same group of cercariae; in these worms variations in the respective concentrations of the three phosphate esters did not exceed 15%. Much greater differences were encountered among schistosomes removed from animals infected with different batches of cercariae.

Aldolase and glyceraldehyde phosphate dehydrogenase activities of schistosomes were measured spectrophotometrically for a period of 5 min. with

predetermined optimal concentrations of the various constituents of the reaction mixture. These are given below; for aldolase: potassium glycylglycine buffer: *p*H 8.3, 5×10^{-2} M; MgHDP: 5×10^{-3} M; potassium arsenate: 6×10^{-3} M; DPN: 5×10^{-4} M; glyceraldehyde phosphate dehydrogenase: 0.8 units/ml.; for glyceraldehyde phosphate dehydrogenase: potassium glycylglycine buffer: *p*H 8.7; 5×10^{-2} M; L-cysteine: 1×10^{-3} M; DPN 1.4×10^{-3} M; potassium arsenate: 6×10^{-3} M; MgHDP: 5×10^{-3} M; aldolase: 0.5 units per ml.

Lactic dehydrogenase activity of *S. mansoni* was determined according to a procedure described previously (Mansour and Bueding, 1953).

The commercially available magnesium salt of HDP was purified by dissolving 100 mg. of this material in 1 ml. of ice-cold water and adding 30 mg. of charcoal (Nuchar). After stirring the mixture for 3 min. it was filtered and the colourless filtrate was used.

RESULTS

Addition of purified mammalian phosphofructokinase resulted in an increase in the rate of glycolysis of schistosome homogenates when glucose was used as the substrate (Table I). These observations indicated that the rate of the reaction catalysed by phosphofructokinase determined the rate of lactic acid production from glucose by cell-free extracts of the worms. Other evidence recorded in Table I suggests that inhibition of

TABLE I

LACTIC ACID PRODUCTION BY HOMOGENATES OF *SCHISTOSOMA MANSONI*

Substrates: Glucose+ATP. Phosphofructokinase (PFK) from rabbit muscle (0.5 to 0.6 units/ml.). Activities are expressed as μ moles of lactic acid produced/mg. protein in 30 min. (37° C.).

Additions	Activity			
	1	2	3	4
None	1·4	1·1	0·5	1·2
Stibophen (1×10^{-4} M)	0·6	0·2	0·2	0·4
PFK	2·1	1·4	1·8	2·0
Stibophen+PFK	2·3	1·5	1·9	1·9
Potassium antimonyl tartrate (5×10^{-5} M)				0·4
Potassium antimonyl tartrate +PFK				1·7

phosphofructokinase activity by trivalent antimonials can account for the inhibition of glycolysis. For example, the inhibitory effects of stibophen and of potassium antimonyl tartrate on lactic acid production from glucose were abolished by the addition of phosphofructokinase of rabbit muscle. Neither phosphofructokinase nor the antimonials altered the rate of lactic acid production when fructose-1:6-diphosphate instead of glucose and ATP was used as substrate (Table II).

TABLE II

LACTIC ACID PRODUCTION BY HOMOGENATES OF *SCHISTOSOMA MANSONI*

Substrate: Hexose diphosphate. Activities and PFK as in Table I.

Additions	Activity			
	1	2	3	4
None	1·1	1·7	1·9	1·9
PFK	1·1	1·7	2·0	1·9
Stibophen (1×10^{-4} M)	1·1		2·1	1·7
Potassium antimonyl tartrate (7×10^{-3} M)			1·9	1·7

The experiments described above suggested that the phosphofructokinase reaction might be the rate-limiting process in the glycolysis of schistosomes; that is, the conversion of F-6-P to HDP might proceed at a slower rate than any other step in the series of reactions concerned with the production of lactic acid from glucose. Accordingly, the reaction rates of five glycolytic enzymes of schistosomes were measured under optimal conditions, which did not differ substantially from the standard conditions used in the earlier experiments. The activities of the enzymes, expressed as μ moles of substrate utilized/mg. of protein/min. at 25° C., were as follows: Phosphohexose isomerase, 0.42; phosphofructokinase, 0.42; aldolase, 0.17; phosphoglyceraldehyde dehydrogenase, 1.58; and lactic dehydrogenase, 0.36. The rate of the reaction catalysed by the phosphofructokinase of the parasite was considerably more rapid than the next step in glycolysis, namely the formation of two molecules of triosephosphate from one molecule of HDP catalysed by aldolase. Therefore, the rate of the phosphofructokinase reaction was not limiting the rate of glycolysis of schistosomes. The possibility was then explored that the concentration of HDP, the product of the phosphofructokinase reaction, might affect the rate of glycolysis of the parasite. Fig. 2 shows the effect of the concentration of HDP on the activity of aldolase. Optimal activity was observed at a relatively high substrate concentration (5×10^{-3} M). If the concentration of the substrate was reduced below a certain critical level (1×10^{-3} M) there was a very sharp decline in the activity of aldolase.

This low activity was not caused by instability of the enzyme in the presence of sub-optimal concentrations of substrate. In several experiments aldolase activity was measured initially with HDP at a concentration of 3×10^{-4} M for 5 min.; when subsequently the substrate concentration in the reaction mixture was raised to the optimum (5×10^{-3} M), the enzymic activity was as high as in the

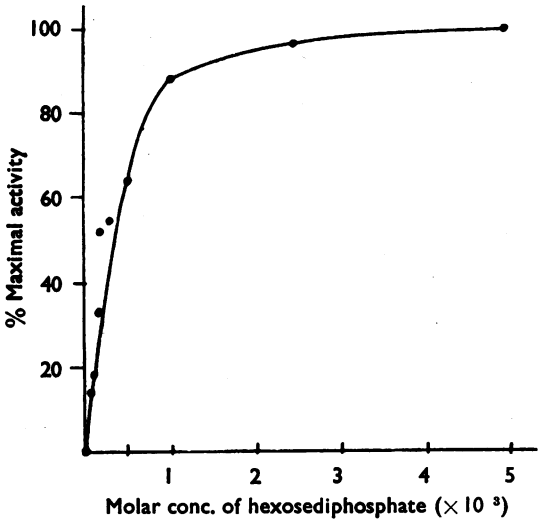

FIG. 2.—Relation of substrate concentration to the activity of aldolase of *Schistosoma mansoni*.

control assay in which aldolase activity was determined at once at the optimal substrate concentration without prior exposure of the enzyme to a lower concentration of HDP. Since the concentration of HDP in schistosomes never exceeded 3×10^{-4} M (see below), a slight decrease below this concentration, due to inhibition of phosphofructokinase activity by antimonials, would markedly reduce the activity of aldolase, resulting in an inhibition of glycolysis. Conversely, addition of mammalian phosphofructokinase to homogenates of schistosomes would increase the rate of HDP formation, giving rise to a significant increase in aldolase activity, thereby increasing the rate of lactic acid production. Furthermore, inhibition of HDP formation by antimonials should be abolished by the addition of an excess of mammalian phosphofructokinase to schistosome homogenates. These are the changes that were found experimentally (Table I).

The observations reported above were made with cell-free preparations of the worms; the problem arose whether similar mechanisms operate in the intact parasite. It was found in earlier studies that utilization of glucose and production of lactic acid by intact schistosomes are reduced by antimonials in concentrations similar to those which inhibited the activity of the parasite's phosphofructokinase (Bueding, 1950). If antimonials were inhibiting this enzyme in intact worms, they would produce an increase in the concentration of the substrate and a decrease in the concentration of the product of the phosphofructokinase reaction. Accordingly, schistosomes were incubated in dilute horse serum (3 vols. serum + 1 vol. water) containing potassium antimonyl tartrate in a concentration (5×10^{-5} M) which markedly inhibits the activity of schistosome phosphofructokinase and the rate of glycolysis of worm extracts. Exposure to the same concentration of the antimonial reduced the survival of the worms *in vitro* from 30 days to approximately 8 hr. The concentrations of hexosemonophosphates and of HDP were determined as soon as a definite reduction in motility became evident;

TABLE III

EFFECT OF POTASSIUM ANTIMONYL TARTRATE ON THE CONCENTRATIONS OF HEXOSEPHOSPHATES IN SCHISTOSOMES

The period of incubation in 75% horse serum at 37° C. was 1 hr. in experiments 1 to 5 and 2 hr. in experiment 6.

Expt.	Molar Conc. of Antimonial	F-6-P		G-6-P		HDP	
		Molar Conc. $\times 10^{-4}$	% Change	Molar Conc. $\times 10^{-4}$	% Change	Molar Conc. $\times 10^{-4}$	% Change
1	—	3·2		2·4		1·1	
	5×10^{-5}	6·1	+91	6·3	+163	0·58	−47
	1×10^{-4}	7·3	+128	7·7	+220	0·42	−62
2	—	1·0		1·2		3·5	
	5×10^{-5}	1·9	+90	2·2	+83	2·2	−37
3	—	—	—	—	—	1·5	—
	5×10^{-5}	—	—	—	—	0·5	−67
	1×10^{-4}	—	—	—	—	0	−100
4	—	0·8		0·6		2·7	
	1×10^{-4}	2·8	+250	2·1	+150	1·3	−52
5	—	1·2		1·1		2·4	
	1×10^{-4}	3·4	+182	2·8	+154	1·4	−42
6	—	2·9		2·4		1·7	
	5×10^{-5}	6·7	+136	5·5	+120	0·9	−47

TABLE IV

EFFECT OF SUBCURATIVE DOSES OF STIBOPHEN ON THE CONCENTRATIONS OF HEXOSEPHOSPHATES IN SCHISTOSOMES

30 mg./kg. of stibophen (0.12% in saline) were administered intraperitoneally to mice infected with *S. mansoni* twice daily at intervals of 8 hr. Infected mice receiving equal volumes of saline served as controls. The worms were removed 1 hr. after the last dose.

Expt.	Total Dose of Stibophen in mg./kg.	F-6-P		G-6-P		HDP	
		Molar Conc. $\times 10^{-4}$	% Change	Molar Conc. $\times 10^{-4}$	% Change	Molar Conc. $\times 10^{-4}$	% Change
1	Control	2·1		1·8		3·0	
	150	3·2	+52	2·7	+50	1·6	−47
2	Control	1·5		1·2		2·6	
	90	2·3	+53	1·8	+50	1·5	−42
3	Control	1·8		1·5		2·0	
	90	2·7	+50	2·1	+40	1·2	−40
4	Control	0·6		0·6		1·3	
	150	2·2	+256	1·9	+217	0·75	−42
5	Control	2·9		2·6		1·4	
	90	4·1	+41	3·3	+27	0·8	−43
	150	5·7	+97	5·1	+96	0·55	−61
	210	6·1	+110	5·6	+116	0·1	−93

TABLE V

KINETICS OF THE PHOSPHOFRUCTOKINASES OF RABBIT MUSCLE AND OF *SCHISTOSOMA MANSONI*

All concentrations are expressed on the basis of molarity. K_m is the Michaelis–Menten dissociation constant.

	F-6-P		ATP		Mg^{++}		*p*H
	K_m	Optimal Conc.	K_m	Optimal Conc.	K_m	Optimal Conc.	Optimum
Rabbit muscle enzyme ..	1.5×10^{-4}	7.5×10^{-4}	9×10^{-5}	2×10^{-3}	1.25×10^{-3}	5×10^{-3}	8·6
Schistosome enzyme ..	4×10^{-4}	1.5×10^{-3}	3×10^{-4}	3.75×10^{-3}	9×10^{-4}	3.75×10^{-3}	8·3

this consistently occurred following incubation with the antimonial for a period of 1 to 2 hr. In these worms the concentration of HDP was reduced while F-6-P and G-6-P accumulated (Table III). These two esters are in equilibrium through the action of phosphoglucose isomerase, and the changes indicated an inhibition of phosphofructokinase activity during the relatively brief exposure of the parasites to potassium antimonyl tartrate. A similar reduction in the concentration of HDP and an increase in that of F-6-P was observed in worms obtained from mice which had received subcurative doses of stibophen (Table IV). The dosage regime used produced a shift in the distribution of the worms from the mesenteric towards the portal veins; therefore, a slight but demonstrable chemotherapeutic effect had occurred (Schubert, 1948; Standen, 1953).

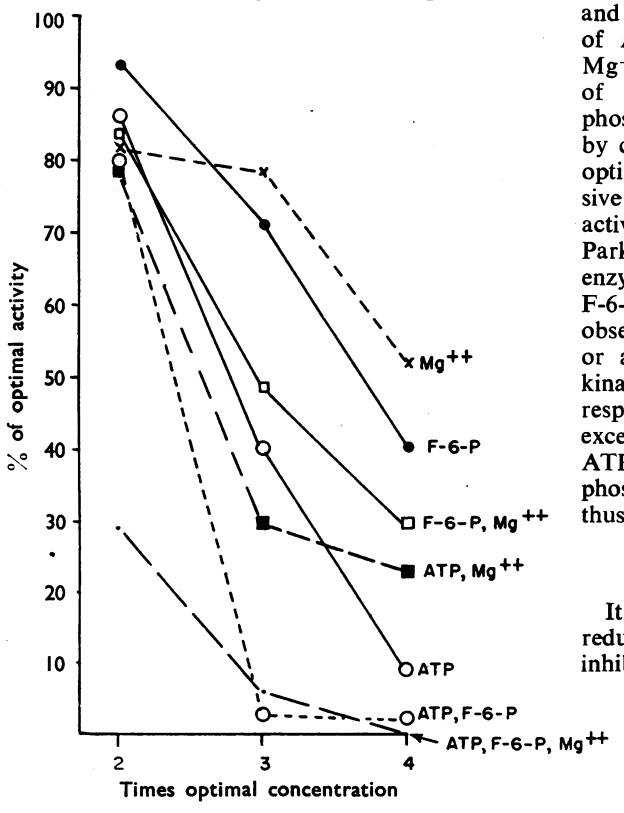

FIG. 3.—Effect of ATP, F-6-P and Mg^{++} in excess of the optimal concentrations on the activity of phosphofructokinase of *S mansoni*.

Because of the critical role of phosphofructokinase in the glycolysis of schistosomes, an attempt was made to determine whether and in what manner this enzyme differs from the one catalysing the same reaction in the host. Measurements of reaction kinetics revealed differences between these two enzymes with regard to their affinities for F-6-P, for ATP and, to a lesser degree, for Mg^{++}, as well as to the effect of *p*H on their activity (Table V). Recently Lardy and Parks (1956) have shown that concentrations of ATP in excess of the molar equivalent of Mg^{++} reduce the activity of phosphofructokinase of rabbit muscle. Similarly, the activity of phosphofructokinase of schistosomes was inhibited by concentrations of ATP which were above the optimal (Fig. 3). On the other hand, while excessive concentrations of Mg^{++} had no effect on the activity of the mammalian enzyme (Lardy and Parks, 1956), they did inhibit the activity of the enzyme of the parasite. The same was true for F-6-P. Inhibition of enzymic activity was observed also when the concentrations of any two or all three constituents of the phosphofructokinase reaction mixture were raised above their respective optima (Fig. 3). It would appear that excessively high intracellular concentrations of ATP, of F-6-P and of Mg^{++} critically reduce the phosphofructokinase activity of schistosomes, thus decreasing the rate of glycolysis of the worms.

DISCUSSION

It was found that trivalent organic antimonials reduce the rate of glycolysis of schistosomes by an inhibition of the activity of phosphofructokinase; this inhibition brings about a decreased formation and thus a lower concentration of HDP which in turn results in a decrease in the activity of aldolase.

Because of the role of phosphofructokinase in determining the rate of glycolysis of *S. mansoni*, it would seem of interest to

point out that the phosphofructokinase of rabbit muscle is much less sensitive than that of schistosomes to the inhibitory effects of antimonials (Mansour and Bueding, 1954). Accordingly, the toxicity of antimonials for the host cannot be ascribed to an inhibition of phosphofructokinase activity. In addition, differences in affinities of the phosphofructokinase of the host and of the parasite for their substrates and in the effect of *p*H on their activities were observed. Finally, in contrast to the rabbit enzyme, excessive concentrations of Mg^{++} markedly inhibited the activity of the worm enzyme. Therefore, it is evident that the enzymes catalysing the same reactions in the parasite and its host are not identical. Because of such differences, opportunities are available to develop compounds which would reduce the activity of the schistosome enzyme without inhibiting the activity of the host enzyme. Such and other differences in the nature of homologous glycolytic enzymes of schistosomes and of mammals have been demonstrated also with hexokinase (Bueding and MacKinnon, 1955a), with lactic dehydrogenase (Mansour and Bueding, 1953 ; Mansour, Bueding and Stavitsky, 1954 ; Henion, Mansour and Bueding, 1955) and with phosphoglucose isomerase (Bueding and MacKinnon, 1955b).

The selective action of trivalent antimonials on phosphofructokinase of *S. mansoni* raises the question about the relationship between this drug-enzyme interaction and the chemotherapeutic effect of antimonials in schistosomiasis. While a multitude of enzymes are affected by drugs, it has been demonstrated only in relatively few instances that such effects are responsible for the pharmacological or chemotherapeutic action of a particular drug. In the opinion of the authors this in no way invalidates the drug-enzyme theory first formulated by Clark (1933), but is ascribed to many experimental difficulties and to frequent neglect in correlating the effects of drugs on isolated biological systems with their action on the intact organism. In a recent review on this subject, Hunter and Lowry (1956) have directed attention to certain requirements which must be met before rigorous proof that a drug acts by inhibiting a particular enzyme can be accepted. In the following, these criteria will be applied to the inhibitory effect of antimonials on phosphofructokinase of schistosomes.

(1) The enzyme concerned should be inhibited in the intact cell. There is evidence for the inhibition of phosphofructokinase by antimonials in the intact worms *in vitro* and *in vivo*: exposure of schistosomes to low concentrations of potassium antimonyl tartrate or administration of subcurative doses of stibophen to the host resulted in an accumulation of F-6-P and in a reduction in the concentration of HDP in the worms ; therefore, under these conditions the substrate of the enzyme was increased and its product was decreased, indicating that the activity of phosphofructokinase within the intact schistosomes was inhibited.

(2) The inhibition of the enzyme should quantitatively explain the effects of the drug. The observations reported in this paper support the conclusion that inhibition of phosphofructokinase is responsible for the reduction in the rate of glycolysis of *S. mansoni*. Previous studies have demonstrated that glycolysis supplies the major, if not exclusive, source of energy for the schistosomes (Bueding, 1950 ; Bueding and Peters, 1951) ; therefore, it is quite conceivable that inhibition of glycolysis accounts for the death of the worms.

(3) Enzyme inhibition must occur with an amount of drug not greater than that necessary to produce the drug action. Survival of the parasites *in vitro* is reduced from 30 days to approximately 8 hr. by exposure to potassium antimonyl tartrate or to stibophen in concentrations (5×10^{-5} M and 1×10^{-4} M respectively) which produced an inhibition of schistosome phosphofructokinase activity to an extent of 50%. Therefore, there is good agreement between the concentration producing an inhibition of the enzyme and that which exerts a schistosomicidal effect.

(4) Other cell constituents must not bind or inactivate a substantial fraction of the drug. Given concentrations of an antimonial inhibited phosphofructokinase activity of crude schistosome homogenates to the same degree as that of purified preparations of this enzyme ; therefore it appears that there is no significant binding of antimonials by other constituents of schistosome cells.

On the basis of these considerations, it is concluded that inhibition of phosphofructokinase activity can account for the schistosomicidal effect of trivalent organic antimonials. While the possibility cannot be excluded that these drugs may interfere also with other, as yet unknown, mechanisms essential for the survival of the parasite, the present study has revealed a vulnerable point in the metabolism of schistosomes which is susceptible to inhibition by a group of chemotherapeutic agents.

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