Biochemical Pharmacology, 1966, Vol. 15, pp. 1197–1211. Pergamon Press Ltd., Printed in Great Britain.

FACTORS AFFECTING THE INHIBITION OF PHOSPHOFRUCTOKINASE ACTIVITY OF *SCHISTOSOMA MANSONI* BY TRIVALENT ORGANIC ANTIMONIALS*

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(*Received* 27 *December* 1965; *accepted* 24 *February* 1966)

Abstract—As in the case of phosphofructokinases from a number of other tissues and organisms, inhibition of phosphofructokinase (PFK) activity of *Schistosoma mansoni* by high concentrations of ATP is increased by citrate; it is reduced by raising the concentration of fructose 6-phosphate (F6P), and, in varying degrees, by adenosine monophosphate, adenosine diphosphate, cyclic 3′, 5′-adenosine monophosphate, inorganic phosphate, and ammonium and sulfate ions. Evidence is reported indicating that ATP decreases the affinity of schistosome PFK for F6P and that those compounds which reverse the inhibitory effect of high ATP concentrations increase the affinity of the enzyme for F6P. Several differences between the properties of mammalian phosphofructokinases and those of the schistosome enzyme are noted, one of which consists in a 65 to 80 times higher sensitivity of schistosome PFK to the inhibitory effects of trivalent organic antimonials. Inhibition of the activity of this enzyme by antimonials is reversed by increased concentrations of F6P; it is enhanced by high concentrations of ATP and is reduced by the same ions that increase the affinity of schistosome PFK for F6P and reduce the inhibition of enzymatic activity by ATP. It appears that antimonials exert their inhibitory effect on schistosome PFK by a reversible interaction with an F6P site of the enzyme, possibly the substrate site, and a displacement of F6P from this site.

A close association between the reversible chemotherapeutic effects of trivalent organic antimonials in experimental schistosomiasis and reversible changes in the phosphate ester levels of the parasite has been observed, providing additional evidence for the causal relationship between the inhibition of schistosome PFK activity and the antischistosomal action of trivalent organic antimonials *in vivo*.

A PREVIOUS study has demonstrated: (a) that phosphofructokinase activity controls the rate of glycolysis of *Schistosoma mansoni*; (b) that the chemotherapeutic action of trivalent organic antimonials on *S. mansoni in vitro* and *in vivo* is associated with an inhibition of PFK activity of the parasite, demonstrable by an accumulation of the substrate of the enzyme, F6P, and a decrease in the product, FDP, within the parasite; and (c) that inhibition of PFK activity by antimonials can account for the chemotherapeutic effects of these compounds in the treatment of schistosomiasis.[1] Subsequently, applying an identical experimental approach to another helminth belonging

* This investigation was supported by Grants AI-03515 and T1-AI-149 from the National Institutes of Health.

In this paper the following abbreviations are used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; cyclic 3′, 5′-AMP, adenosine 3′,5′-monophosphate; P*i*, inorganic orthophosphate; PFK, phosphofructokinase (EC.2.7.1.11.); F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; FDP, fructose 1,6-diphosphate; TP, triosephosphates (glyceraldehyde 3-phosphate + dihydroxyacetone phosphate.

to the same order of trematodes, *Fasciola hepatica*, Mansour has reported that in this parasite also, as in *S. mansoni*, the rate of the PFK reaction limits the rate of carbohydrate metabolism.[2] In addition, other investigations have indicated the critical role of PFK in the regulation of glycolysis of mammalian tissues and of yeast.[3–7]

The present study deals with a variety of factors affecting schistosome PFK activity and its inhibition by trivalent organic antimonials. Evidence is reported suggesting that this inhibition is brought about by a reversible interaction of antimonials with an F6P substrate site of the enzyme.

MATERIALS AND METHODS

Adult schistosomes (*S. mansoni*) were obtained as in earlier investigations[8, 9] and placed in 75% horse serum. After blotting with filter paper (Whatman No. 50) the worms were homogenized in an all-glass homogenizer at 0° in 0·05 M glyclglycine buffer (pH 7·4) containing 20% glycerol (50 worm pairs or 4·0 to 5·5 mg protein per ml). When kept at 0°, the PFK activity of this homogenate remained stable for at least 3 hrs. Immediately before the assay for enzymatic activity, the homogenate was diluted eightfold in the same medium.

PFK activity was measured spectrophotometrically at room temperature according to the system used by Ling *et al.*[10] involving the enzymatic conversion of the reaction product to triosephosphates and reduction of the latter by NADH. The enzymes used, i.e. aldolase, triosephosphate isomerase, and glycerophosphate dehydrogenase, were commercial preparations (Boehringer) available as suspensions in a concentrated (2 to 2·4 M) ammonium sulfate solution. Since both ammonium and sulfate ions affect not only PFK activity but also the degree of inhibition by antimonials of the schistosome enzyme (see below), it was necessary to remove these two ions prior to the assay. This was done as follows: 0·4 ml of the enzyme suspensions was centrifuged (at 12,800 *g* for 10 min) at 2°–4°, and the residues were suspended in 0·4 ml of 0·05 M glycylglycine buffer (pH 7·4) containing 5×10^{-4} M EDTA. This suspension was then dialyzed at 2°–4° against 150 ml of the buffer for 30 min. Dialysis against fresh buffer was repeated five times, and the dialyzed enzymes were then frozen in aliquots for a single day's use. When diluted, the enzyme solutions contributed less than 3×10^{-7} M ammonium sulfate and about 1×10^{-6} M EDTA to the reaction mixture.

The reaction mixture (total volume 0·8 ml) contained, unless otherwise stated, 0·05 M potassium glycylglycine buffer (pH 8·3), $3{\cdot}75 \times 10^{-3}$ M $MgCl_2$, $1{\cdot}25 \times 10^{-3}$ M ATP, 4×10^{-3} M mercaptoethanol, 0·1% bovine serum albumin, $1{\cdot}4 \times 10^{-4}$ NADH and 0·02 unit* each of dialyzed aldolase, triosephosphate isomerase, and glycerophosphate dehydrogenase (diluted in 0·05 M glycylglycine buffer, pH 8·3). Ten minutes after the addition of 20 μl of the diluted homogenate, a solution of F6P yielding a final concentration of 3×10^{-3} M was added, and the decrease in optical density was recorded at a wavelength of 340 mμ over a period of 4 to 14 min thereafter, during which time the reaction rate was linear. These assay conditions were found to be optimal with regard to the concentrations of substrates and Mg^{++}.

When the effects of other ions or inhibitors were tested, the latter were added after the 10-min preincubation period, but prior to the addition of F6P. Since AMP, in molar concentrations above 3×10^{-6} affected enzymatic activity (see below), a

* A unit is defined as the amount of enzyme that catalyzes the conversions of 1 μmole substrate/min at 25°.

chromatographically purified preparation of NADH (Pabst) was used, which contained less than 0·3% AMP, yielding a final AMP concentration in the reaction mixture of less than 4×10^{-7} M. When the effects of $(NH_4)^+$, $SO_4^=$ and $HPO_4^=$ ions were tested, the chloride and sodium salts were used respectively because NaCl was found to have no effect on the PFK activity of *S. mansoni*.

Prior to its use, 0·2 ml of a crystalline suspension of rabbit muscle PFK (Boehringer) was centrifuged at 14,000 *g* for 10 min. The residue was suspended in 0·2 ml of a solution containing 0·05 M glycylglycine buffer (pH 7·4), 20% glycerol, 4×10^{-3} M mercaptoethanol, 5×10^{-4} M EDTA, and 4×10^{-3} M ATP[11], and dialyzed twice in successsion for 60 min against 50 ml of the same solution. All operations were carried out at temperatures varying between 0° and 4°. The activity of this enzyme was determined as was schistosome PFK, except that the reaction mixture contained 6×10^{-3} M ATP, 2×10^{-3} M F6P, and 5×10^{-3} M $MgCl_2$.

Determination of phosphate ester levels. Schistosomes were homogenized in 3% perchloric acid at 0° (100 pairs/ml). The homogenate was centrifuged at 0° for 10 min at 12,800 *g*, the supernatant neutralized with potassium hydroxide, and centrifuged to remove the potassium perchlorate; aliquots of the neutralized extract were used for analysis. G6P + F6P was determined by incubating 20 to 40 μliters of extract with 0·005 μmole $NADP^+$, 0·06 unit glucose 6-phosphate dehydrogenase, 0·15 unit phosphoglucose isomerase (Boehringer), and 10 μmoles Tris buffer (pH 8·3) at 30° for 20 min in a final volume of 0·2 ml. The resultant NADPH was measured according to the "strong alkali" method of Lowry *et al.*[12] in a Turner fluorometer with primary filter Corning No. 5860 and secondary filters Corning 4308 and 3387. HDP + TP was determined by incubating 20 to 40 μliters of the extract with 0·005 μmole sodium arsenate, 10μmole Tris buffer (pH 8·3), 0·005 μmole NAD^+, 0·01 unit aldolase, 1·0 unit triosephosphate isomerase, and 1·7 units glyceraldehyde 3-phosphate dehydrogenase. The NADH formed was determined fluorometrically in the same manner as NADPH (see above). ATP, ADP, and AMP were determined essentially as described by Lowry *et al.*[13]

RESULTS

Effect of ATP *and* F6P *concentrations on schistosome* PFK *activity*

Although ATP is one of the substrates, it is known that high concentrations of this nucleotide inhibit PFK activity in a large variety of tissues[14–23] including those of schistosomes.[1] At an F6P concentration of 3×10^{-3} M, optimal activity was observed at $1{\cdot}25 \times 10^{-3}$ M ATP. A threefold increase in the concentration of the latter reduced PFK activity significantly (Fig. 1). This inhibition was abolished by raising the F6P concentration (Fig. 2). Furthermore, on lowering the ATP concentration, the concentration of F6P required for half-maximal and maximal activities was reduced also (Fig. 2). Therefore, the affinity of the enzyme for F6P decreased with increasing concentrations of ATP. Because of these relationships, other factors affecting PFK activity of *S. mansoni* were studied at three concentrations of ATP, $2{\cdot}5 \times 10^{-4}$, $1{\cdot}25 \times 10^{-3}$, and $3{\cdot}75 \times 10^{-3}$ M, subsequently referred to as low, optimal, and high, respectively.

Other factors affecting schistosome PFK *activity*

It has been reported that a variety of inorganic ions, such as $(NH_4)^+$, $HPO_4^=$, and of nucleotides, i.e. AMP, ADP, and cyclic 3′, 5′-AMP, stimulate PFK activity in

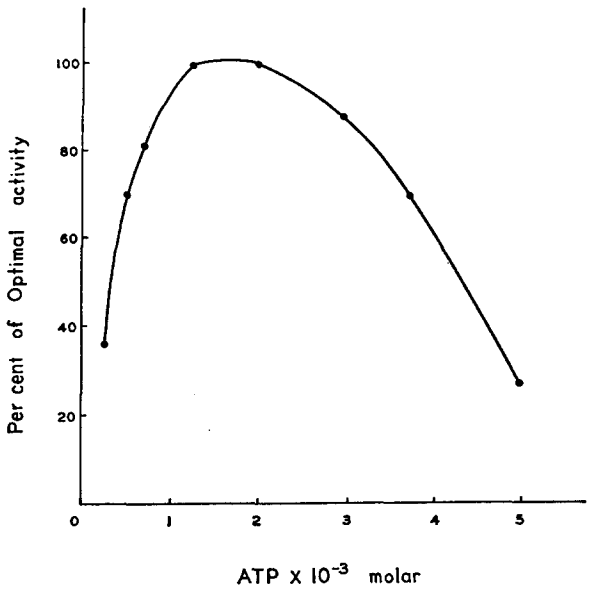

FIG. 1. Effect of ATP concentration on schistosome PFK activity. F6P, 3×10^{-3} M; $MgCl_2$ $3{\cdot}75 \times 10^{-3}$ M

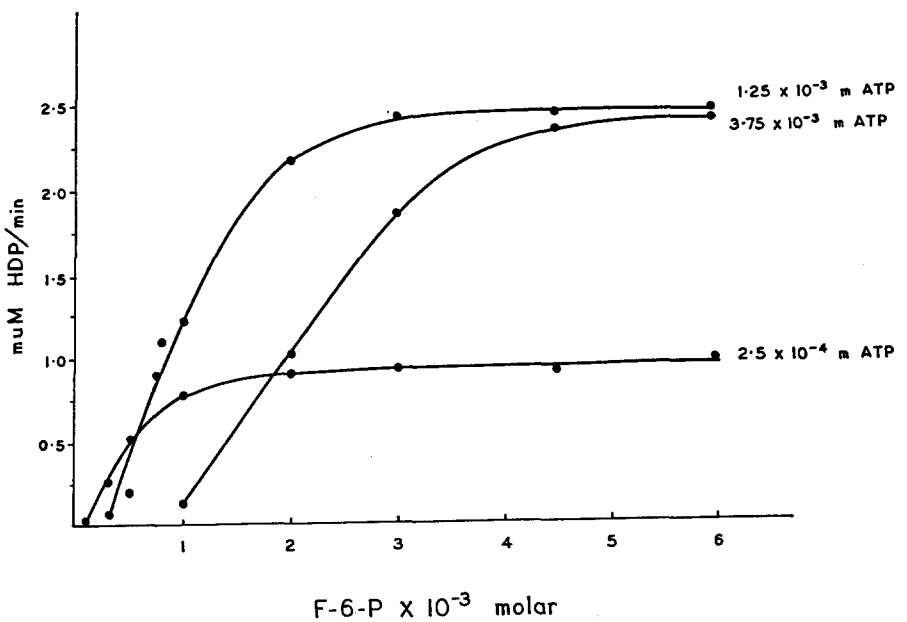

FIG. 2. Effect of the F6P concentration on schistosome PFK activity at low, optimal, and high ATP concentrations

mammalian tissues.[15–17, 20, 22, 24, 25] All these compounds were found to affect schistosome PFK activity under certain conditions; however, differences between these effects on the schistosome enzyme and those on enzymes catalyzing the same reaction in other tissues were noted also.

The activity of schistosome PFK was enhanced significantly by $(NH_4)^+$ at the high, inhibitory concentration of ATP (Table 1). At the low and optimal ATP concentrations, this was far less pronounced. A similar trend was observable with P_i (Table 1).

TABLE 1. EFFECTS OF INORGANIC IONS ON PFK ACTIVITY OF *S. mansoni*

Molar concentration of added ion	$2{\cdot}5 \times 10^{-4}$ M ATP (low)*			$1{\cdot}25 \times 10^{-3}$ M ATP (optimal)			$3{\cdot}75 \times 10^{-3}$ M ATP (high)†		
	$(NH_4)^+$	$HPO_4^=$	$SO_4^=$	$(NH_4)^+$	$HPO_4^=$	$SO_4^=$	$(NH_4)^+$	$HPO_4^=$	$SO_4^=$
3×10^{-3}	10	14	5	20	17	5	56	69	26
1×10^{-3}	10	−3	5	9	20	4	51	70	26
3×10^{-4}	5	5	8	8	6	0	10	39	20
1×10^{-4}		0	3	0	0	0		23	10
3×10^{-5}					0			11	

The figures represent per cent increases in PFK activity over the control rate at a given concentration of ATP. Molar concentration of F6P 3×10^{-3}.

* Control activity about 40% of control activity at $1{\cdot}25 \times 10^{-3}$ M ATP.
† Control activity about 70% of control activity at $1{\cdot}25 \times 10^{-3}$ M ATP.

TABLE 2. EFFECT OF AMP, ADP, AND CYCLIC 3′,5′-AMP ON PFK ACTIVITY OF *S. mansoni*

Molar concentration of added nucleotide	$2{\cdot}5 \times 10^{-4}$ M ATP			$1{\cdot}25 \times 10^{-3}$ M ATP			$3{\cdot}75 \times 10^{-3}$ M ATP		
	AMP	ADP	Cyclic 3′,5′-AMP	AMP	ADP	Cyclic 3′,5′-AMP	AMP	ADP	Cyclic 3′,5′-AMP
1×10^{-2}	−40		−81	+ 7		−54	26	−62	−16
1×10^{-3}	− 7	−70	− 1	+13	−42	+ 3	35	+28	+28
3×10^{-4}	0	−43		+ 9	− 6			+28	
1×10^{-4}	+ 7	−11	+ 1	+ 9	+ 2	0	37	+16	+29
3×10^{-5}	0	− 5		+10	0		23	+12	
1×10^{-5}	0	− 8	+ 7	+ 7	+ 2	− 3	9		+25
1×10^{-6}	+ 7		− 3			+ 5			+ 8

The figures represent per cent change in PFK activity from the control rate at a given concentration of ATP. Molar concentration of F6P 3×10^{-3}.

The effects of $(NH_4)^+$ on schistosome PFK activity differ from those on mammalian phosphofructokinases because the latter are stimulated by $(NH_4)^+$ to the same degree, regardless of the ATP concentration.[17] PFK activity was increased significantly by $SO_4^=$ only at the high ATP concentration (Table 1). Thus it acted as a "reverser," but not as a true enhancer of the parasite enzyme.

When the ATP concentration was low, AMP, in concentrations of 1×10^{-3} M and above, inhibited schistosome PFK activity (Table 2). While AMP stimulated

activity only slightly at the optimal ATP concentration, it reversed in concentrations of 1×10^{-5} M and above, the inhibition produced by the high ATP concentration. At the low ATP concentration, the inhibitory effect of ADP was even greater than that of AMP. In addition, when the ATP concentration was optimal, ADP (1×10^{-3} M and above)—in contrast to AMP—inhibited PFK activity of the parasite (Table 2). On the other hand, both ADP and AMP reversed the inhibition produced by the high ATP concentration (Table 2); AMP was more potent in this respect.

Determination of the concentrations of ATP, ADP, and AMP in the reaction mixtures before and after the assay of PFK activity revealed only small or no changes due to ATPase or myokinase activities. In no instance was the interconversion of the three adenine nucleotides by the schistosome extract large enough to account for the observed changes.

A high concentration of 3′, 5′-AMP (1×10^{-2} M) was inhibitory regardless of whether the ATP concentration was low, optimal, or high; the degree of inhibition was greater, the lower the ATP concentration (Table 2). At the low and optimal ATP concentrations, cyclic 3′,5′-AMP in concentrations of 1×10^{-3} M or less, had no effect on PFK activity (Table 2).‡ At the high ATP concentration, cyclic 3′, 5′-AMP, in concentrations between 1×10^{-3} and 1×10^{-5} M, increased enzymatic activity.

When any of the above compounds produced an increase in PFK activity, combination of two or more of them never resulted in a potentiation but merely proved additive, at the most. This was true also if the F6P concentration was reduced tenfold below its optimal level.

It has been reported that citrate inhibits PFK activity of various tissues.[16, 17, 20, 25] Schistosome PFK activity was inhibited only by relatively high concentrations of citrate (1 to 2×10^{-3} M). Similarly, high concentrations of pyrophosphate proved inhibitory. These effects of citrate and pyrophosphate were reversed by increasing the concentration of Mg^{++}.

Factors affecting the inhibitory activity of trivalent antimonials on PFK *activity of* S. mansoni

The effect of the concentration of potassium antimony tartrate on schistosome PFK activity is reproduced in Fig. 3. On a molar basis, the inhibitory activity of stibophen was the same as that of KSb tartrate, while that of potassium antimony mercaptosuccinate (TWSb) was approximately three times lower.

Since the inhibition of schistosome PFK activity by antimonials is affected greatly by the F6P concentration (see below), the possibility was examined whether the concentration of this substrate in the assay system was reduced by the action of glucose 6-phosphate isomerase present in the worm homogenate. It was found that less than 7% of the added F6P had been converted to G6P at the end of an assay period of 6 min, and less than 14% after 14 min at the termination of the assay. The degree of inhibition of PFK activity by antimonials did not increase over the entire assay period.

‡ Mansour and Mansour[19] have reported that cyclic 3′,5′-AMP produces a three to five-fold increase of schistosome PFK activity. These authors used conditions under which both ATP and F6P concentrations were far above their optima and when enzymatic activity was no longer linear with time. Under these same conditions we failed to detect even a slight activation by cyclic 3′,5′-AMP (final concentration: 1×10^{-5} M); enzymatic activity in the presence of the latter amounted to between − 4 and + 4% of the control activity, which was found to be 5 to 30 times higher than that given by Mansour and Mansour.[19] It varied from 200 to 225 μmoles HDP formed/g worms as contrasted to 6·5 to 42 μmoles reported by these authors.

Therefore, the small decrease in the F6P concentration, produced by the actions of the isomerase and of PFK, did not affect the observed inhibition of PFK activity by antimonials. Furthermore, if enough G6P was added to produce in the reaction mixture a G6P-F6P ratio equal to the equilibrium of the schistosome isomerase,[26] inhibition of PFK activity by a given concentration of KSb tartrate was the same as in the absence of added G6P.

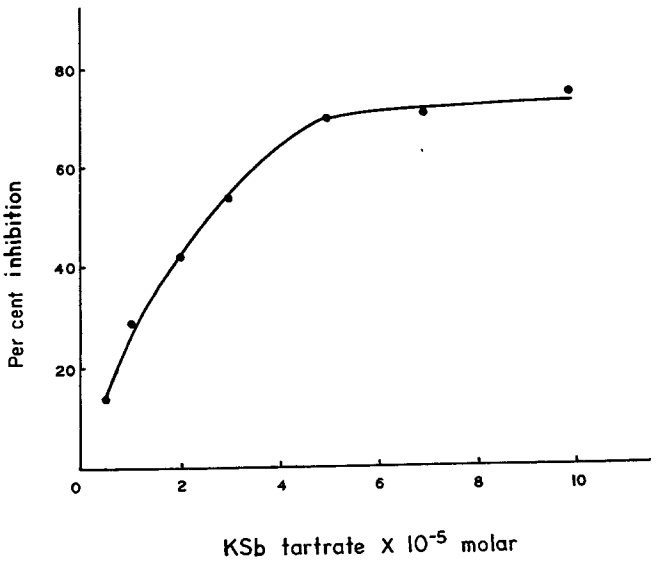

FIG.3. Effect of KSb tartrate on schistosome PFK activity. F6P, 3×10^{-3} M; ATP, $1{\cdot}25 \times 10^{-3}$ M; $MgCl_2$, $3{\cdot}75 \times 10^{-3}$ M.

Although the specific PFK activity of extracts of male schistosomes was four times greater than that of female schistosome extracts—suggesting that a major portion of the enzyme is localized in the muscular tissue[27]—PFK inhibition by a given concentration of an antimonial was essentially the same in extracts of either sex.

When a schistosome extract was incubated in the presence of an inhibitory concentration (1×10^{-4} M) of KSb tartrate at room temperature for 15 min, and when subsequently PFK activity was determined in a 500-fold dilution of this homogenate (resulting in an antimonial concentration of 2×10^{-7} M), no inhibition of enzymatic activity was detectable. Therefore, the inhibitory effect of the antimonial was reversible.

Effect of the ATP *concentration.* Inhibition of PFK activity by KSb tartrate was markedly enhanced when the concentration of ATP was increased. Conversely, at the low ATP concentration ($2{\cdot}5 \times 10^{-4}$ M) a ten times greater concentration of the antimonial was required to inhibit enzymatic activity to an extent of 50 per cent (Table 3).

Effect of the F6P *concentration.* In contrast to ATP, an increase in the concentration of F6P, the other substrate of PFK, reduced the inhibitory potency of the antimonial, regardless of the ATP concentration. The progressive reduction of the inhibitory effect of antimonials on schistosome PFK as a function of the F6P concentration is

illustrated in Fig. 4. The nature and reversibility of PFK inhibition produced by KSb tartrate are demonstrated also by the observation that this inhibition was markedly reduced by the subsequent addition of an excess of F6P (Fig. 5).

It has been reported previously that 80 times greater concentrations of the antimonial are required to inhibit the activity of mammalian brain PFK than those inhibiting

TABLE 3. EFFECT OF ATP CONCENTRATION ON THE INHIBITION OF SCHISTOSOME PFK ACTIVITY BY KSb TARTRATE

Molar concentration of F6P, 3×10^{-3}.

Molar ATP concentration	Molar concentration of KSb tartrate producing 50% inhibition of PFK activity
$2{\cdot}5 \times 10^{-4}$	3×10^{-4}
$1{\cdot}25 \times 10^{-3}$	3×10^{-5}
$3{\cdot}75 \times 10^{-3}$	1×10^{-5}

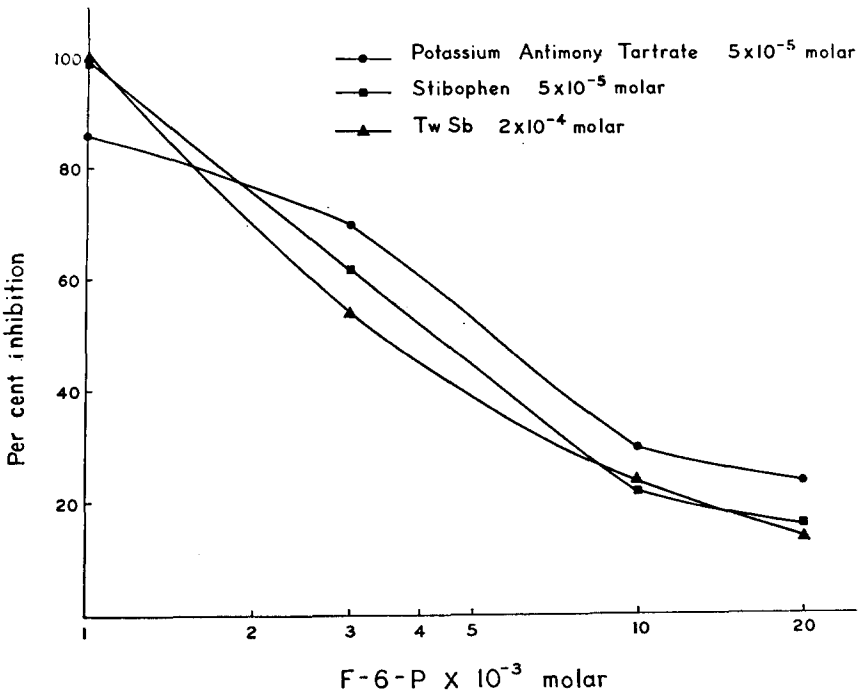

FIG.4. Effect of the F6P concentration on the inhibition of schistosome PFK activity by trivalent organic antimonials.

schistosome PFK activity to the same extent.[28] Similarly, the concentration of KSb tartrate resulting in a 50% inhibition of PFK activity of rabbit muscle at optimal ATP, F6P, and Mg^{++} concentrations was 2×10^{-3} M, or 67 times higher than the antimonial concentration producing the same degree of inhibition of schistosome PFK. While PFK of rabbit muscle had a much lower sensitivity to the antimonial,

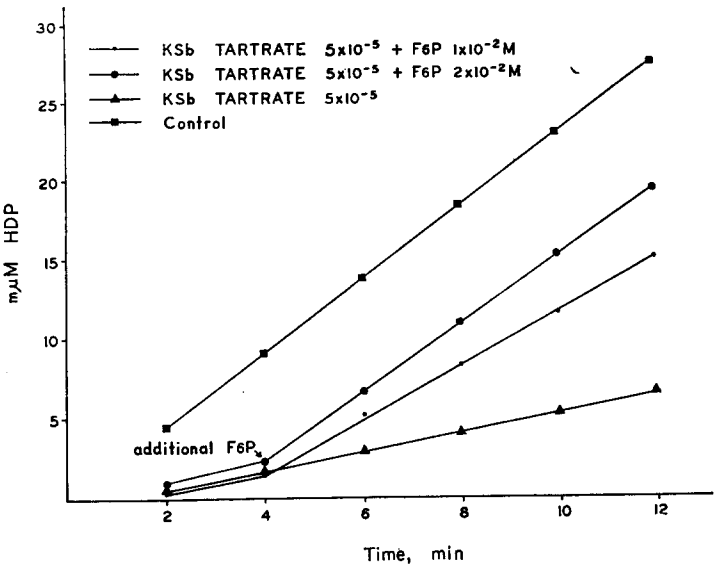

FIG. 5. Reversal by F6P of inhibition of schistosome PFK activity produced by KSb tartrate. ATP $1{\cdot}25 \times 10^{-3}$ M Initial F6P concentration, 3×10^{-3} M. After 4 min (arrow) enough F6P was added to two samples (- · -, - ● -) to raise the final concentration of this phosphate ester to 1×10^{-2} and 2×10^{-2} M respectively.

enzyme inhibition, as in the case of schistosome PFK, was reduced by increased concentrations of F6P.

AMP This proved to be extremely effective in reducing the inhibitory effect of the antimonial on PFK activity (Table 4), when the ATP concentration was optimal or

TABLE 4. EFFECT OF AMP AND CYCLIC 3′,5′-AMP ON THE INHIBITION OF SCHISTOSOME PFK ACTIVITY BY KSb TARTRATE

Figures represent per cent inhibition of PFK activity.

Molar concentration of nucleotide monophosphate	$2{\cdot}5 \times 10^{-4}$ M ATP 3×10^{-4} M KSb tartrate		$1{\cdot}25 \times 10^{-3}$ M ATP 3×10^{-5} M KSb tartrate		$3{\cdot}75 \times 10^{-3}$ M ATP 1×10^{-5} M KSb tartrate	
	AMP	Cyclic 3′,5′-AMP	AMP	Cyclic 3′,5′-AMP	AMP	Cyclic 3′,5′-AMP
	58	58	49	49	65	65
1×10^{-3}	31	36	14	14	9	4
1×10^{-4}	43	35	13	13	37	9
1×10^{-5}	46	36	36	18	67	27
1×10^{-6}		44		38		47
1×10^{-7}		52		50		65

high. The possibility was explored whether this effect might be related to an increased affinity of the enzyme for F6P. In the presence of AMP, half-maximal and maximal activities were observed with 6 times lower concentrations of F6P than in the absence of AMP (Table 5). Thus, AMP markedly increased the affinity of the enzyme for F6P.

The optimal concentration of AMP required to produce this higher affinity was 1×10^{-4} M. Higher concentrations did not change this effect significantly.

Cyclic 3′,5′-AMP. This nucleotide was approximately five times more potent than AMP in reducing the concentration of F6P required for half-maximal and maximal activity (Table 5) and in reversing PFK inhibition by the antimonial (Table 4).

TABLE 5. EFFECT OF $(NH_4)^+$, $HPO_4^=$, $SO_4^=$, AMP AND CYCLIC 3′,5′-AMP ON THE AFFINITY OF SCHISTOSOME PFK FOR F6P.

Added ion	Molar concentration of F6P required for	
	Maximal activity	Half-maximal activity
None	3×10^{-3}	1×10^{-3}
$(NH_4)^+$ (0·01 M)	1×10^{-3}	$4{\cdot}5 \times 10^{-4}$
$HPO_4^=$ (0·01 M)	1×10^{-3}	3×10^{-4}
$SO_4^=$ (0·01 M)	1×10^{-3}	$2{\cdot}5 \times 10^{-4}$
AMP (2×10^{-4} M)	5×10^{-4}	$1{\cdot}5 \times 10^{-4}$
3′,5′-AMP (4×10^{-5} M)	5×10^{-4}	$1{\cdot}5 \times 10^{-4}$

$1{\cdot}25 \times 10^{-3}$M ATP, $3{\cdot}75 \times 10^{-3}$M $MgCl_2$.

ADP. The inhibitory activity of the antimonial at all three ATP concentrations was reduced by ADP, but the latter was less potent in this respect than was AMP.

$(NH_4)^+$, $SO_4^=$, *and* $HPN_4^=$. At the optimal and high ATP concentrations, these three ions markedly reduced the inhibitory activity of the antimonial. At the low ATP concentration, this effect was less pronounced with $SO_4^=$ and $HPO_4^=$ and was barely observable with $(NH_4)^+$ (Table 6). All three ions increased the affinity of the enzyme for F6P (Table 5), although to a lesser extent than did AMP or cyclic 3′,5′-AMP. The inhibitory effect of the antimonial was the same regardless of whether the

TABLE 6. EFFECT OF INORGANIC IONS ON THE INHIBITION OF SCHISTOSOME PFK ACTIVITY BY KSb TARTRATE

Molar concentration of added ion	$2{\cdot}5 \times 10^{-4}$ M ATP 3×10^{-4} M KSb tartrate			$1{\cdot}25 \times 10^{-3}$ M ATP 3×10^{-5} M KSb tartrate			$3{\cdot}75 \times 10^{-3}$ M ATP 1×10^{-5} M KSb tartrate		
	$(NH_4)^+$	$HPO_4^=$	$SO_4^=$	$(NH_4)^+$	$HPO_4^=$	$SO_4^=$	$(NH_4)^+$	$HPO_4^=$	$SO_4^=$
None	60	60	60	50	50	50	68	68	68
1×10^{-2}	46	45	44	15	22	9	15	20	10
3×10^{-3}	50	42	37	29	25	19	38	32	24
1×10^{-3}	50	48	45	28	25	28	59	44	47
1×10^{-4}		48	52	52	38	42	72	67	69

Figures represent per cent inhibition of PFK activity.

optimal concentration of F6P was lower (5×10^{-4} or 1×10^{-3} M) in the presence of cyclic 3′,5′-AMP, $(NH_4)^+$, or $SO_4^=$, or higher (3×10^{-3}) in the absence of these ions. Therefore, inhibition of schistosome PFK by the antimonial was affected not only by the concentration of F6P but also by the affinity of the enzyme for the substrate.

Other ions. In addition to their intrinsic inhibitory effects on PFK activity, pyrophosphate and citrate enhanced the inhibitory action of the antimonial on schistosome PFK activity.

In concentrations varying between $2{\cdot}5 \times 10^{-3}$ and 1×10^{-2} M, Mg^{++} failed to affect the inhibitory activity of the antimonial.

Mammalian phosphofructokinases are readily inactivated by sulfhydryl inhibitors, and their enzymatic activity is increased in the presence of sulfhydryl compounds.[29] Similarly, PFK activity of *S. mansoni* was enhanced by mercaptoethanol. However, this sulfhydryl compound did not reverse or reduce the inhibitory effect of potassium antimony tartrate; in fact, the inhibitory action of a given concentration of the antimonial on schistosome PFK was slightly greater in the presence of mercaptoethanol (final concentration, 4×10^{-3} M). Therefore, it appears that inhibition of schistosome PFK activity by antimonials is not brought about by inactivation of sulfhydryl groups of the enzyme.

PFK *activity of* S. mansoni *under presumed* in vivo *conditions.* In view of the many factors affecting PFK activity and the degree of its inhibition by an antimonial, it is difficult to ascertain the extent of inhibition caused by a given antimonial concentration in the intact worm. An approximation may be obtained by determining this effect at the concentrations of (G6P + F6P), ATP, ADP, AMP, and P_i prevailing in the parasite. At these concentrations (0·25 μmoles G6P + F6P, 1·25 μmole ATP, 0·05 μmole AMP, 0·2 μmole ADP, 1 μmole P_i per ml), PFK activity was inhibited to an extent of 50% by 1×10^{-5} M KSb tartrate. Such a concentration is far below that of antimony found by Khayyal[30] in worms 2 to 4 hrs after the administration to the host of a single chemotherapeutically active dose of an antimonial. Therefore, it is probable that PFK activity of *S. mansoni* is completely inhibited *in vivo* shortly after the administration of such a dose of an antimonial.

Relationship between the "antimonial shift" and PFK *inhibition.* Some observations reported above raise the question whether the reversibility of the inhibitory effect of antimonials on PFK activity of *S. mansoni* can account for the reversibility *in vivo* of the antischistosomal action of a single dose of an antimonial. It is well known that shortly after the administration of such a drug, the worms lose their attachment to the mesenteric veins and are carried to the liver.[31–34] However, unless administration of the antimonial is continued, the worms return to the mesenteric veins. Buttle and Khayyal[35] have found that this "hepatic shift" begins less than 30 min after the intraperitoneal injection of KSb tartrate and is virtually complete after 2 hrs. On the other hand, one to several days thereafter the majority of the schistosomes have returned to the mesenteric veins. As shown in Table 7, these observations were fully confirmed in the present study. When the worms had shifted to the liver, the concentration of the hexosemonophosphate esters was increased in relation to the hexosediphosphate and triosephosphate levels. This was reflected in a very marked increase in the ratio between these two groups of phosphate esters, indicating inhibition of PFK. However, no inhibition of PFK activity was detected in the extracts of the worms when and antimonial, originally present in the intact parasite, was diluted in the final assay system. This demonstrates again the reversibility of the antimonial effect. The concentration and ratios of the phosphate esters returned to their control levels when the worms had moved back to the mesenteric veins (Table 7). Therefore, the "antimonial shift" of schistosomes is associated with an inhibition of the worm's PFK activity, and the

TABLE 7. CONCENTRATIONS OF PHOSPHATE ESTERS IN *Schistosoma mansoni* AFTER THE ADMINISTRATION OF A SINGLE DOSE OF AN ANTIMONIAL TO THEIR HOST

Exp. no.	Antimonial	Hours after injctn. of antimonial	Per cent of worms in: Mesenteric or portal veins*	Per cent of worms in: Liver sinuses*	G6P+F6P (μmoles/g)*	FDP+TP (μmoles/g)*	Ratio G6P+F6P / FDP+TP
1			62	38	0·221	0·078	2·8
	KSb tartrate	2	5	95	0·279	0·041	6·8
	TWSb	2	29	71	0·485	0·045	10·8
	KSb tartrate	48	33	67	0·167	0·062	2·7
	TWSb	48	76	24	0·164	0·078	2·1
2			76	24	0·152	0·088	1·7
	KSb tartrate	2	0	100	0·385	0·082	4·7
	TWSb	2	31	69	0·390	0·071	5·5
	KSb tartrate	72	61	39	0·129	0·081	1·6
	TWSb	72	70	30	0·161	0·089	1·8
3			89	11	0·249	0·249	1·0
	KSb tartrate	0·5	62	38	0·303	0·072	4·2
	TWSb	0·5	74	26	0·156	0·083	1·9
	KSb tartrate	2	0	100	0·250	0·042	6·0
	TWSb	2	46	54	0·300	0·096	3·1
	KSb tartrate	48	87	13	0·087	0·132	0·7
	TWSb	48	98	2	0·226	0·183	1·2

KSb tartrate (25·0 mg/kg) or TWSb (250 mg/kg) was administered in aqueous solutions to mice infected with *S. mansoni*.

* There were no significant differences in the concentrations of phosphate esters of worms removed from the mesenteric and portal veins, or from the liver sinuses of the same animal.

reversal of this inhibition coincides with the recovery of the worms from the effects of antimonials and with their return to the mesenteric veins. A two- to fourfold increase in the ratio (G6P + F6P)/(HDP + TP) was demonstrable also after incubation of the worms for 30 min in 75% horse serum containing concentrations of potassium antimony tartrate as low as 3×10^{-6} M, indicating an inhibition of PFK activity of the worms under these conditions.

DISCUSSION

Since the dissociation constants and optimal concentrations of F6P for schistosome PFK become greater with increasing ATP concentrations, it is evident that ATP, one of the substrates of PFK, reduces the affinity of the enzyme for F6P, the other substrate. This decreased affinity for F6P can explain the inhibition of schistosome PFK activity by ATP, because this inhibition is overcome by raising the F6P concentration. Furthermore, the same nucleotides, i.e. AMP, cyclic 3′,5′-AMP, ADP, and inorganic ions, i.e. $HPO_4^=$, $(NH_4)^+$ and $SO_4^=$, which reverse the inhibitory effect of high ATP concentrations, also increase the affinity of the enzyme for F6P. They enhance PFK activity only if the ATP concentration is high in relation to the concentration of F6P but, if the latter is increased, these ions have no effect.

Schistosome PFK has several properties in common with the phosphofructokinases of other tissues and organisms. For example, it has been demonstrated by Passonneau

and Lowry[15, 17] that progressive increases in the ATP concentrations reduce the affinity of phosphofructokinases from mammalian muscle and brain for F6P and that AMP, ADP, cyclic 3′,5′-AMP, and P_i have the opposite effects. In addition, the phosphofructokinases of *S. mansoni* and of mammalian tissues[16, 17, 20, 25] are inhibited by citrate. However, several differences are to be noted also.

1. $(NH_4)^+$ increases brain PFK activity to the same extent at all levels of ATP.[17] Therefore, this ion acts as a pure enhancer of brain PFK activity. By contrast, $(NH_4)^+$ merely reverses the inhibition of schistosome PFK activity at high ATP levels, but has little or no activating effect on this enzyme at optimal or low ATP concentrations.

2. P_i and AMP markedly enhance brain PFK activity at noninhibitory concentrations of ATP and produce even greater increases at inhibitory ATP levels[15, 17]; on the other hand, both P_i and AMP only reverse the inhibition of schistosome PFK activity at high ATP concentrations but produce no activation at noninhibitory concentrations of ATP.

3. Passonneau and Lowry[16, 17] have found striking potentiations of brain PFK activities by the simultaneous addition of $(NH_4)^+$, P_i, and AMP. This type of activation of schistosome PFK was not observable with such combinations whose effects were, at best, additive. Therefore, schistosome PFK appears to lack at least some of the enhancement sites of the mammalian enzymes.

4. High concentrations of AMP and ADP inhibit schistosome PFK activity; no such effect has been observed with phosphofructokinases from vertebrate tissues, yeast, or bacteria; but ADP, and to a lesser extent AMP, have been found to be inhibitory for some plant phosphofructokinases.[36]

5. The activity of schistosome PFK is inhibited by much higher concentrations of citrate than that of mammalian phosphofructokinases. Since an increase in the Mg^{++} concentration results in a reversal of the inhibition of schistosome PFK activity by citrate, it appears that the effect of citrate on the schistosome enzyme is merely the result of chelation of Mg^{++}. By contrast, the activities of mammalian phosphofructokinases are inhibited by citrate in molarities far below those of Mg^{++},[16, 17, 20] and also by the Mg^{++} salt of citrate.* It has been pointed out that inhibition of mammalian PFK can constitute an important mechanism regulating glycolysis through the tricarboxylic acid cycle.[16, 20, 25] On the other hand, this cycle is of no physiological significance to *S. mansoni* because the parasite depends for its energy supply on glycolysis and does not exhibit a Pasteur effect.

6. The selective toxicity of trivalent organic antimonials is based on their inhibitory action on schistosome PFK activity because the enzymes catalyzing the same reaction in mammalian tissues are much less sensitive to the inhibitory effects of antimonials.

While inhibition of schistosome PFK activity by high ATP concentrations is reversed by P_i and by low concentrations of AMP, ADP, and cyclic 3′,5′-AMP, it has been stated that, in contrast to the latter nucleotide, AMP and ADP do not act as reversers in the case of PFK of the liver fluke *Fasciola hepatica*,[19] another digenetic trematode belonging to the same biological suborder as *S. mansoni*. No information is available on the effect of P_i on PFK of the liver fluke.

The inhibitory effects of high concentrations of ADP, AMP, and cyclic 3′,5′-AMP on schistosome PFK might be due to their ability to combine with an inhibitory site

* O. H. Lowry, personal communication.

for ATP, and thus they might act as structural analogs of the latter. This interpretation is consistent with the observation that the inhibition produced by these nucleotides is more pronounced at low ATP concentrations.

It has been assumed that the chemotherapeutic action of antimonials is brought about by inactivation of sulfhydryl enzymes.[37] Evidence reported previously[28] has suggested that such a mechanism does not apply to the antischistosomal effects of antimonials. Since mercaptoethanol does not reverse the inhibition of PFK activity produced by KSb tartrate, it appears that inactivation of sulfhydryl groups indeed is not involved in this effect.

The inhibition of schistosome PFK activity by trivalent organic antimonials is reduced and eventually reversed, by progressive increases in the F6P concentrations. Therefore, the antimonials may exert their inhibitory effect by an interaction with an F6P site at the enzyme, possibly the substrate site, and a displacement of F6P from this site; conversely, an increase in the concentration of F6P could reverse the inhibition of enzymatic activity by the displacement of the antimonial from an F6P binbing site. Such an interpretation is supported also by the reversal of the inhibitory effect of antimonials by those compounds (AMP, ADP, cyclic 3′,5′-AMP, $(NH_4)^+$, $SO_4^=$, P_i) which increase the affinity of schistosome PFK for F6P, thereby increasing the availability of this substrate for the F6P binding site(s). Furthermore, the marked and progressive increase in the inhibitory potency of KSb tartrate produced by increasing concentrations of ATP can be accounted for by the decreased affinity of the enzyme for F6P. On the other hand, the possibility cannot be ruled out that F6P alters the configuration of schistosome PFK in such a manner as to render it more resistant to the inhibitory actions of antimonials.

Although the F6P levels of schistosomes are far below the concentrations optimal for PFK activity, the ATP, ADP, and P_i levels prevailing in the parasite are sufficiently high to maintain the enzyme in an active state, provided there is no compartmentalization of these phosphate compounds. However, at these low F6P and G6P levels, enzymatic activity is highly susceptible to inhibition by concentrations of KSb tartrate which are considerably lower than those found to prevail after administration of chemotherapeutically active doses of the antimonial.[30] Thus, these relationships can account for an inhibition of PFK activity in schistosomes after the administration of an antimonial to the host.

Several observations reported in this paper indicate that the antimonial readily dissociates from its inhibitory site at the enzyme. Inhibition of PFK activity is reversed by an increase in the F6P concentration and by the subsequent addition of an excess of this substrate. This inhibition is reversed also by mere dilution of an extract of intact schistosomes containing a high concentration of the antimonial.

Shortly after the administration of an antimonial, its chemotherapeutic activity is demonstrable by a shift of the worms from the mesenteric veins towards the liver.[35] This shift is associated with changes in the steady-state concentrations of the phosphate esters in the parasite, i.e. an accumulation of G6P + F6P and a reduction in FDP + TP, indicating that PFK activity of the parasite is inhibited when the drug exerts its antischistosomal action. Conversely, the reversal of this chemotherapeutic effect, one to several days after a single administration of an antimonial, manifests itself in the return of the worms to the mesenteric veins[35] and in the return of the concentrations of the phosphate esters to their initial control levels. This close association of reversible

chemotherapeutic effects and reversible biochemical changes supplies additional evidence for the causal relationship between the inhibition of schistosome PFK activity and the antischistosomal action of trivalent organic antimonials.

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