Biochemical Pharmacology, Vol. 16 pp. 1421–1428. Pergamon Press Ltd. 1967. Printed in Great Britain

THE INACTIVATION OF *SCHISTOSOMA MANSONI* LACTATE DEHYDROGENASE BY 4-IODOACETAMIDOSALICYLIC ACID

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(*Received* 5 *January* 1967; *accepted* 16 *March* 1967)

Abstract—4-iodoacetamidosalicyclic acid inactivates *S. mansoni* and rabbit skeletal muscle lactate dehydrogenases, but has little or no effect on the enzyme from ox heart, mouse liver or human serum. The results support previous reports that the first stage in inactivation is the reversible formation of an enzyme-inhibitor complex. The compound is toxic to intact schistosomes in culture, but no changes were found in their lactate dehydrogenase activity, although glycolysis was inhibited. These effects, and the toxicity in mice, are probably due to the high non-specific reactivity of the alkylating group.

SINCE *S. mansoni* is largely dependent on glycolysis for obtaining its energy,[1] an agent which inhibits one of its glycolytic enzymes without inhibiting the corresponding enzyme in the mammalian host should be a potential means of chemotherapy against this parasite. Such a selective inhibition of schistosomal phosphofructokinase (E.C. 2.7.1.11.) has been shown to occur with antimonials.[2] For selective inhibition to occur, specific differences must exist between the mammalian and schistosomal forms of the enzyme. It was thought that lactate dehydrogenase (LDH; L-lactate: NAD oxidoreductase, E.C. 1.1.1.27) would be a suitable enzyme for study in this respect. It is known to exist in different forms in various species and tissues,[3, 4] and differences between *S. mansoni* and mammalian LDH have been reported.[5, 6]

It has been shown by Baker *et al.*[7] that 4-iodoacetamidosalicylate (IAS) and similar compounds will inactivate rabbit skeletal muscle LDH but not the ox heart enzyme. It was suggested that the salicylate residue binds to the enzyme, causing some reversible inhibition; if a suitable group on the enzyme is then adjacent to the iodoacetamido group, alkylation and inactivation follow. This last effect is highly specific because it depends on the detailed structure of the enzyme near the salicylate binding site.

Some compounds of this type were therefore tested for their ability to inactivate selectively *S. mansoni* LDH. Their effects on intact schistosomes in culture and in mice, and their toxicities, were examined with a view to assessing their chemotherapeutic potential.

MATERIALS AND METHODS

Experimental compounds. The following compounds were synthesized: IAS,[8] 4-chloroacetamidosalicylic acid,[9] 4-(*N*-iodoacetyl) glycylamidosalicylic acid[8] and α-iodoacetanilide.[8] Melting points and elementary analyses agreed with those previously reported and those calculated, respectively. Iodoacetic acid, iodoacetamide,

salicylic acid and 4-aminosalicylic acid were obtained from the British Drug Houses Ltd.

Aromatic acids were dissolved by adding a minimal quantity of 0·1 N NaOH solution to a suspension in water, and then bringing the pH to 7·4 with dilute HCl. Other compounds were dissolved in water and the pH brought to 7·4. α-Iodoacetanilide was dissolved in 10% (v/v) dimethyl formamide in water, corresponding controls also being run in 10% dimethyl formamide. Glass distilled water was used for all experiments except those involving *in vitro* cultivation of schistosomes, when deionised water was used.

S. Mansoni. The strain used was originally obtained from the Liverpool School of Tropical Medicine, and has subsequently been maintained in this laboratory. Mice (infected at least 7 weeks previously with about 110 cercariae each) were killed by cervical dislocation and the schistosomes dissected from the mesenteric and portal veins. The paired worms were not separated, as no differences were found between LDH from male and female worms.

Lactate dehydrogenase preparation. The ox-heart and rabbit skeletal muscle enzymes were purchased from the Koch-light Laboratories, Colnbrook, Bucks. LDH from other sources was prepared as follows:

S. mansoni were washed with water and then homogenised in about 1 ml water on an M.S.E. ultrasonic disintegrator. The sample was cooled in an ice-salt mixture to avoid inactivation of the enzyme. The preparation was sufficiently homogeneous for use without centrifuging.

Mouse liver (0·5 g) was homogenised in 50 ml water at 0° in a glass homogeniser. The supernatant was used after centrifuging for 1 hr at 4° and 2000 *g*.

Human serum was prepared in the usual way from blood obtained from a finger-prick, care being taken to avoid haemolysis.

An equal volume of 64% (w/v) sucrose solution was added to all the enzyme preparations, which were kept at 0° until used on the same day. The sucrose improved the stability of the *S. mansoni* LDH;[5] the results were corrected for the slight remaining instability.

Lactate dehydrogenase assay. The reduction of pyruvate to lactate was coupled with the oxidation of $NADH_2$ to NAD, measured by the fall of absorption at 340 mμ. on a Hilger and Watts 'Uvispek' at room temperature. The reaction mixture (volume 3 ml) contained 0·13 mM $NADH_2$, 0·9 mM sodium pyruvate, 0·05 M phosphate buffer (pH 7·4) and sufficient enzyme to give a rate of change of optical density of about 0·05/min in a 1-cm cell.

The optical density was usually measured at 1 min intervals for 6 min, and the rate of change found by fitting a regression line to the points, this method being quicker and more accurate than graphical plotting of the results. One unit of enzyme activity was taken as that amount which oxidised 1 μmole of $NADH_2$ per minute.

Effects of compounds on LDH

IAS and related compounds both reversibly inhibit and irreversibly inactivate LDH. The inhibition is virtually instantaneous while the inactivation by alkylation is time-dependent. The latter was measured by incubating the enzyme at 25° and pH 7·4 with the compound under test (0–35 mM) and $NADH_2$ (1·95 mM). At intervals 0·2 ml aliquots were diluted to 3·0 ml in buffer containing pyruvate (0·9 mM) and

assayed immediately. The reversible inhibition, being constant in all samples, was eliminated by expressing activities as a percentage of that in the first aliquot removed.

Reversible inhibition was measured by adding the enzyme to the other components of the assay mixture, including the compound under test (0–2·5 mM), and assaying immediately. Under these conditions no more than 2 per cent of the enzyme should have been inactivated by alkylation in the 6–min assay period; no such inactivation was in fact detected. This was also true for the assay of the diluted aliquots described in the preceding paragraph.

Investigations on intact S. mansoni

Schistosomes were carefully removed from mice, being discarded if there was any evidence of damage under low–power microscopy. They were then cultured at 37° in 1:1 horse serum; Tyrode's buffer containing the compound under test. LDH assay was carried out on individual worm pairs as described above, after confirming that they were still alive and washing them thoroughly in water to remove the compound. Some measurements of carbohydrate metabolism were made on schistosomes which had been cultured for 16 hr with IAS. Four viable worm pairs were placed in a flask containing 2·5 ml of Tyrode's buffer with glucose (0·4 mg/ml) and the test compound, but without serum (which gave a high lactate blank). After incubation for 1 hr at 37° with gentle agitation, the glucose[10] and lactate[11] concentrations were measured. The worms were digested in 30% KOH at 100°, and glycogen precipitated with ethanol, washed and assayed.[12]

RESULTS

Effects of IAS on heart and skeletal muscle LDH

The actions of IAS on ox heart and rabbit skeletal muscle LDH were determined for comparison with Baker's results.[7] The compound inhibited both enzymes, but a plot of v_0/v (reaction velocity without and with inhibitor respectively) against IAS concentration gave an upward curvature (Fig. 1). It is clear however, that the heart enzyme was less sensitive to IAS, as found by Baker.[7] The curvature of the lines in

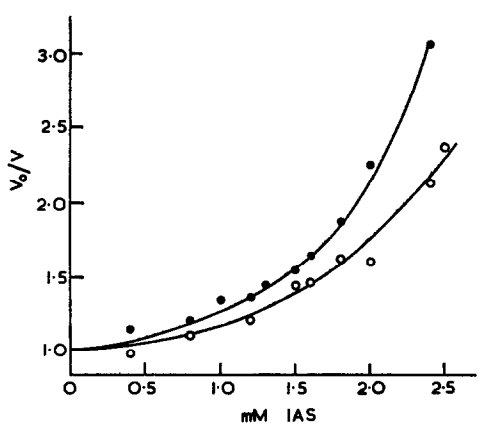

FIG. 1. Inhibition of rabbit skeletal muscle (●) and ox heart LDH (○) by IAS. v_0/v is plotted against IAS concn., where v and v_0 are the reaction velocities with and without IAS.

Fig. 1 is unlikely to be due to inactivation by alkylation occurring at high IAS concentrations, for the extent of curvature was not time-dependent and salicylate gave similar curves (though approximately 10 × higher concentrations were required to give the same amount of inhibition).

Fig. 2 shows the inactivation of the two enzymes by IAS. Heart LDH was apparently insensitive at the concentrations used. The rate of inactivation of skeletal

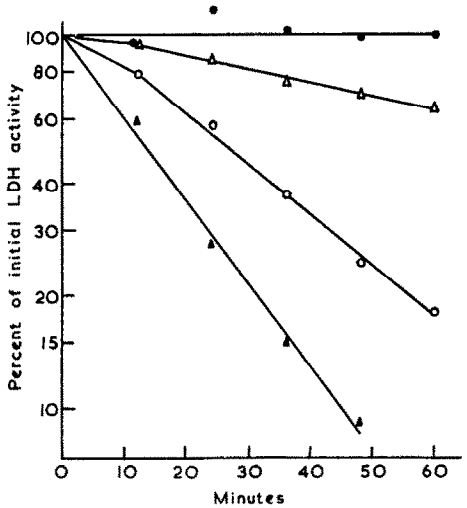

FIG. 2. Inactivation of rabbit skeletal muscle and ox heart LDH by IAS at 25°. Enzyme activities are plotted logarithmically. Key: Ox heart LDH, IAS at 35 mM (●). Rabbit skeletal muscle LDH, IAS at 1·75 mM (△), 17·5 mM. (○), 35 mM (▲).

muscle LDH was not proportional to IAS concentration, increasing only 3·21 and 6·03 times as the IAS level rose from 1·77 to 17·7 and 35·3 mM. These results are in agreement with those previously reported.[7]

Inactivation of S. mansoni *LDH by IAS*

LDH from *S. mansoni*, mouse liver and human serum was incubated with IAS. The schistosome enzyme was inactivated much more rapidly than the others (Fig. 3); human serum LDH was virtually unaffected. The rate of inactivation of *S. mansoni* LDH slowed as the experiment progressed; this could indicate heterogeneity of the enzyme.

The compounds related to IAS shown in Table 1 were tested for inactivation of *S. mansoni* LDH. Replacement of the iodine by a chlorine or lengthening of the chain by insertion of a glycyl residue reduced the activity. The presence of both the salicylate and the alkylating groups was necessary for activity. In particular, iodoacetamide had little effect in spite of its alkylating properties, and salicylate produced no significant inactivation although it reversibly inhibited the enzyme.

Effects of IAS on intact S. mansoni

IAS at 1 mM killed schistosomes *in vitro* in 24 hr, 0·1 mM reduced their activity, and 0·01 mM was ineffective. 1 mM salicylate had no effect in 4 days, but iodoacetamide was toxic at 0·1 mM.

Table 2 shows the changes in certain parameters of glucose metabolism in schistosomes incubated for 16 hr in media containing IAS at concentrations producing a reduction of activity but not death. Both glucose uptake and lactate production were depressed, being almost completely abolished at 0·4 mM IAS. The glycogen content of control worms incubated without IAS was much lower than that of normal

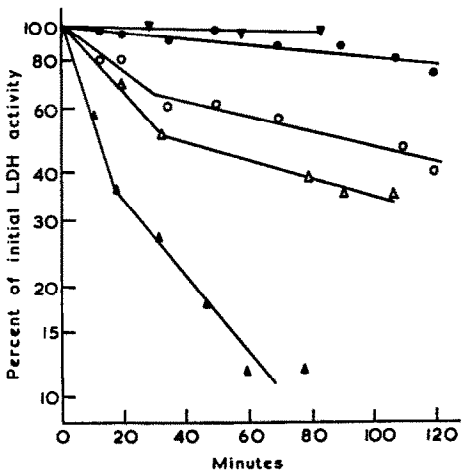

FIG. 3. Inactivation of *S. mansoni*, mouse liver and human serum LDH by IAS. Enzyme activities are plotted logarithmically. Key: At 25°, IAS at 7·5 mM; human serum LDH (▼), mouse liver LDH (●), *S. mansoni* LDH (○). *S. mansoni* LDH at 25°, IAS at 15 mM (△). *S. mansoni* LDH at 37°, IAS at 20 mM (▲).

TABLE 1. INACTIVATION OF *S. mansoni* LACTATE DEHYDROGENASE BY 4-IODOACETAMIDOSALICYLIC ACID AND RELATED COMPOUNDS

Compound	% Inactivation in 30 min
4-iodoacetamidosalicylic acid	39·4 ± 4·3
4-(N-iodoacetyl) glycylamidosalicylic acid	21·7 ± 3·7
4-chloroacetamidosalicylic acid	1·9 ± 3·7
α-iodoacetanilide	4·7 ± 3.4
Iodoacetamide	0·7 ± 3·8
Iodoacetic acid	2·5 ± 3·5
4-aminosalicylic acid	−1·7 ± 3·7
Salicylic acid	0·1 ± 3·4

The table shows the per cent loss of activity of *S. mansoni* LDH when incubated at 25° for 30 min with the compounds at 7·5 mM (iodoacetanilide 5 mM), allowing for reversible inhibition (see text) and loss of activity of the enzyme in the absence of compound (4·4 ± 2·8 per cent). Each result is the mean ± S.E. of 6 observations.

schistosomes, suggesting that depletion of glycogen occurred during incubation. This depletion was partially prevented by IAS. It would therefore appear that suppression of glucose metabolism was due to inhibition of glycolysis rather than to any effect on glucose absorption.

No significant changes in LDH activity were detected. This part of the experiment was repeated several times with varying incubation times and IAS concentrations, but in no case was any significant fall in LDH activity found before the death of the worms.

TABLE 2. EFFECTS OF 4-IODOACETAMIDOSALICYLIC ACID ON CARBOHYDRATE METABOLISM IN *S. mansoni*

IAS Conc. (mM)	Glucose Uptake (μmole/ worm pair/hr)	Lactate Production (μmole/ worm pair/hr)	Glycogen Content (μmole/ worm pair)	LDH Activity (milliunits/ worm pair)
0	0·346 ±0·007	0·117 ±0·014	0·049 ±0·003	70·1 ± 4·1
0·2	0·099* ±0·093	0·065* ±0·013	0·065 ±0·001	71·5 ± 5·7
0·4	0·027† ±0·009	0·001† ±0·003	0·102† ±0·011	71·5 ± 7·2
Normal worms	—	—	0·132† ±0·011	62·8 ± 4·6

Schistomes were incubated for 16 hr in medium containing IAS. LDH was assayed in some worms, while others were incubated for 1 hr in fresh medium to measure glucose uptake and lactate production; their glycogen content was then found. Normal worms were freshly removed from mice. See text for further details.

Results are the mean ± S.E.M. of 4 observations, each using four worm pairs, except for LDH activities which are the mean of six results using individual worm pairs. Significance of differences from controls (no IAS), by *t*-test: *, $P = < 0{\cdot}05$; †, $P = < 0{\cdot}001$; otherwise $P = > 0{\cdot}05$.

Bueding[1] found the glucose uptake and lactate production of *S. mansoni* to be about 195 and 210 μg/mg dry wt./hr respectively, corresponding to 0·17 and 0·36 μmole/worm pair/hr. Our values for glucose uptake were rather higher, although of the same order of magnitude, but we did not observe the almost quantitative conversion to lactate that he found. This difference may be connected with the 16-hr pre-incubation used in the present experiments. However, the close correlation between inhibition of glucose utilisation, reduction of lactate output, and death of the worms strongly supports Bueding's conclusion[1] that glycolysis is of great importance in schistosomal metabolism.

Toxicity of IAS in mice

The 7-day LD_{50} was determined on mice weighing 19–21 g, using groups of 10 animals at each of 5 dose levels differing by a ratio of 1·4. The results were as follows:
Oral LD_{50}; 365 mg/kg body wt. (95 per cent limits, 329–405).
I.P. LD_{50}; 77·5 mg/kg body wt. (95 per cent limits, 68·5–87·7).

In both groups over 95 per cent of the deaths occurred within 2 days of administration of IAS. At necropsy of mice surviving for 7 days, or killed when moribund (in a separate experiment) the kidneys were usually (16/19 mice) found to be enlarged and pale; histological examination showed that severe tubular necrosis had occurred.

Intraperitoneal injection of 400 or 800 mg IAS/kg body wt. caused a marked reduction of activity, irritability, and respiration; death occurred within 1 hr, apparently due to respiratory failure. These effects could be the results of CNS depression. Equivalent doses (on a molar basis) of iodoacetamide, iodoacetate and 4-chloroacetamidosalicylate had closely similar effects, but salicylate and 4-aminosalicylate were without effect at 800 mg/kg body wt. The toxicity of IAS did not appear to be reduced by substitution of chlorine for iodine.

Effects of IAS on schistosomal infection in mice

The compound was given orally at 150 mg/kg on each of 5 days, to 5 mice infected 7 weeks previously with about 110 cercariae. Two mice died; in the remaining three, no effects on the mesenteric-hepatic distribution of the schistosomes or on the maturity of the eggs in the intestine were found.

DISCUSSION

Our results concerning the action of IAS on rabbit skeletal muscle and ox heart LDH are in general agreement with those of Baker *et al.*[7] Both enzymes are reversibly inhibited by IAS, but progressive inactivation is observed only with the former. The lack of effect on *S. mansoni* LDH of alkylating agents not containing a salicylate residue is strong evidence in favour of Baker's hypothesis[7] that inactivation of the enzyme by alkylation follows the formation of an enzyme—IAS complex. The formation of this complex is presumably responsible for the reversible inhibition. However, the curvature of the plots of v_0/v against inhibitor concentration (Fig. 1) is not consistent with the hypothesis that IAS is bound to the active site of LDH in competition with pyruvate.[7] The steady-state kinetic equations for the binding of an inhibitor to the active site in LDH (or in the LDH-$NADH_2$ or LDH-NAD complexes) should give linear plots for $1/v$ against inhibitor concentration.[13] Whatever the cause of the reversible inhibition, it is clear that a subsequent alkylation reaction would cause irreversible inactivation by preventing the inhibitor from dissociating from the enzyme, in accordance with Baker's hypothesis.[7]

S. mansoni LDH resembles the rabbit skeletal muscle enzyme in being inactivated by IAS, whereas mouse liver, human serum and ox heart LDH are affected slightly or not at all. We have confirmed the observations of Conde-del Pino *et al.*[14] that on electrophoresis *S. mansoni* LDH remains close to the origin, thus resembling the skeletal muscle rather than the heart isoenzymes.[3] In this respect there is a correlation between inactivation by IAS and electrophoretic mobility, but in the small number of results presented here the variation in sensitivity to IAS could equally well be due to species differences.[4]

The death of schistosomes *in vitro* on treatment with IAS is associated with a reduction in glycolysis but not with inhibition of LDH. The compound is toxic to mice, and has no effect on *S. mansoni* infection when administered at the maximal tolerated dose. These results are to be expected in view of the reactivity of the alkylating group in IAS. Other enzymes are probably more susceptible to non-specific alkylation than LDH, particularly those in which a thiol group is closely involved in their activity; it will be noted that iodoacetamide is as toxic as IAS to both mice and schistosomes, though it has little effect on LDH. We have found that IAS reacts readily with both

tissue homogenates and thiol compounds, the reaction being complete in 5 min at 37°. On administration to mice, therefore, it is unlikely that significant free IAS levels are attained in the blood.

Acknowledgements—We should like to thank the members of this department, particularly Dr. J. N. Green, for their advice and help. Synthesis of experimental compounds was carried out by members of the Chemistry Department under Dr. S. S. Szinai. The experiments on living animals were performed by Mrs. P. R. M. Jackson and by members of the Pharmacology Department under Mr. C. H. Cashin.

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