Effects of Organic Arsenicals on Enzyme Systems

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Investigations have been carried out by us in the Biochemical Laboratory of the Cardiff City Mental Hospital during 1938, 1939 and early 1940 on the effects of organic trivalent and pentavalent arsenic compounds on proteins and enzymes. The work was primarily undertaken to elucidate the biochemical basis of the trypanocidal and anti-spirochaetal action of a variety of organic arsenic compounds used in the treatment of syphilis. Investigations by Hawking, Hennelly & Quastel (1937) had already shown that administration of the pentavalent arsenical tryparsamide into the body led to the appearance of trivalent arsenic compounds, of high trypanocidal activity, in the spinal fluid. On the other hand, administration of organic trivalent arsenic compounds did not lead to the excretion of active trivalent arsenic compounds in the spinal fluid. Work was therefore undertaken to discover the manner of combination of organic trivalent and pentavalent arsenic compounds with tissue proteins and enzyme systems. It was inevitable that our work led us into problems connected with the mechanism of action of certain war gases (e.g. lewisite) and with the question of reversibility of enzyme systems inactivated by arsenic compounds. Whilst we, ourselves, did not work with actual war gases, our results bore on the problems involved and for this reason publication of our results was not allowed during the war. A report, however, was submitted to the Medical Research Council in November 1940, and an extract containing the main conclusions was published in *Nature* (Gordon & Quastel, 1947) by permission of the Secretary of the Medical Research Council.

The present paper contains the results of investigations made by us on the effects of organic arsenic compounds on enzyme systems during 1938, 1939 and early 1940; they are the results on which the conclusions, already published by Gordon & Quastel (1947), are based.

Much attention has been devoted by workers in chemotherapy to the elucidation of the mechanism of the trypanocidal and anti-spirochaetal activity of arsenic compounds. It is a well established fact, first noted by Ehrlich, that while pentavalent arsenic components are relatively non-toxic, *in vitro*, to protozoa, trivalent arsenic compounds such as substituted phenylarsenoxides and arsenobenzenes are extremely toxic. Moreover, there is good reason to believe that, in the case of the arsenobenzenes, the toxic action is not exerted to any great extent by these compounds as such, but is probably due to the presence, in their aqueous solutions, of highly toxic arsenoxides, rapidly formed by atmospheric oxidation of the —As=As— group. Eagle (1939*a*) has shown that neoarsphenamine is 25–60 times more toxic to trypanosomes under aerobic conditions than it is when strict anaerobic conditions are observed, the change taking place within 2–5 min. of dissolving the compound; there is no difference of this kind shown in the case of mapharside (*m*-amino-*p*-hydroxyphenylarsenoxide). It would appear, therefore, that the arsenoxides are the most highly toxic of the aromatic arsenic compounds, and much experimental evidence has accumulated which has led to the probability that this toxicity is due to the ability of the arsenoxides to react with thiol compounds.

This conclusion was anticipated by Ehrlich (1909) when he suggested that thiol groups could act as chemo-receptors for trivalent arsenic compounds. Later, Voegtlin, Dyer & Leonard (1923) postulated that the toxic action of an arsenoxide to trypanosomes is due to a condensation with thiol groups present in protoplasm, leading to a dysfunction of the system regulating cell respiration. The fact that arsenic compounds are capable of combining with organic compounds containing thiol groups (such as thiolacetic acid or thioglycollic acid) was demonstrated by Barber (1929), who showed that a condensation takes place between phenylarsenoxides and thiol-substituted compounds, giving a derivative (arylthioarsenite or thioglycollate) according to the equation

$$C_6H_5.AsO + 2RSH = C_6H_5.As\begin{matrix} \diagup SR \\ \diagdown SR \end{matrix} + H_2O.$$

Barber also showed that thiol compounds in sufficient concentration will reduce a phenylarsonic acid to a phenylarsenoxide,

$$C_6H_5.AsO_3H_2 + 2RSH \rightarrow C_6H_5.AsO + RSSR + 2H_2O,$$

which then combines with excess of thiol compound to give an arylthioarsenite as above. Gough & King (1930) subsequently deduced, from a scrutiny of the properties and reactions of arylthioarsenites, that these derivatives are not stable, but are slowly hydrolyzed at ordinary temperatures even in neutral solution, with liberation of small amounts of toxic arsenoxides. This supposition supported their view, that when pentavalent phenylarsonic acid derivatives are administered therapeutically in the treatment of protozoal infections, reduction takes place in the tissues to give arsenoxides, which then condense with thiol compounds of the tissues, and are 'stored' in this manner. The highly trypanocidal arsenoxides are finally set free, by hydrolysis, over an extended period of time, and are responsible for the prolonged therapeutic effect of the drug. It would appear,

therefore, that, in neutral solution, an equilibrium can exist between an arsenoxide and a thiol compound, as follows:

$$C_6H_5.AsO + 2RSH \rightleftharpoons C_6H_5.As\begin{matrix} SR \\ SR \end{matrix} + H_2O.$$

If it is true that the toxicity of arsenic compounds to micro-organisms is due to a combination between arsenoxide and thiol groups, causing a breakdown in some essential metabolic process, then it would be reasonable to suppose that, in presence of excess of added thiol compound, the toxicity of the arsenic compound would be greatly reduced, since the arsenic would then be present in the system mainly in the form of arylthioarsenite, which should be non-toxic since it is incapable of further reaction with thiol groups. Such an effect was reported by Eagle (1939*b*) who discovered that, in presence of sufficient excess of thiol compounds such as cysteine, glutathione, etc., the anti-spirochaetal activity of trivalent arsenic compounds is almost completely removed; no effect of this sort was noticed when methionine was used. The large excess of thiol compound necessary to produce the effect was in harmony with the views of Gough & King (1930) upon the hydrolysis of the arylthioarsenites.

In order to obtain more information regarding the capacity of trivalent arsenic compounds to interfere with metabolic processes by virtue of their affinity for thiol groups, we considered it advisable to undertake an investigation of the manner in which arsenic compounds may influence the activities of a variety of enzyme systems. Evidence has been obtained by a number of workers that certain enzymes require, for their activity, the integrity of thiol groups in their structure (see review of earlier work by Hellerman, 1937). Toxicity of trivalent arsenoxides to enzymes may throw light on, or confirm, the thiol nature of such enzymes. The experimental results which are now reported provide evidence confirming the conclusions of earlier workers on the thiol structure of certain enzymes, and indicating for the first time the thiol structure of choline dehydrogenase. At the same time evidence will be given clearly demonstrating the reactivation by thiol compounds of enzyme systems inhibited or poisoned by arsenic compounds.

EXPERIMENTAL METHODS

Urease

Preparation and measurement of activity. A commercial (Dunning) urease tablet (25 mg.) was ground in 5 ml. saline. CO_2 gas was passed through the suspension for a few minutes at room temperature and the mixture was centrifuged. The clear supernatant fluid had high urease activity and 0·2–0·4 ml. of this fluid was used for the enzyme studies.

A manometric technique (Barcroft or Warburg) was exclusively used. The urease preparation was introduced into the main compartment of the vessel of a Warburg or Barcroft manometric apparatus in presence of 0·13 M-sodium acetate buffer, pH 5·0, at 37°. Urea (0·2 ml. of 10% solution) was placed in the side tube of the manometer vessel, and after thermal equilibrium the urea was tipped into the main vessel and readings of CO_2 output were noted at frequent intervals as the urea decomposed. The rate of CO_2 output was a measure of the activity of the urease preparation.

Additions of organic arsenic compounds and other inhibitors. These substances were added to the urease preparation in the main manometer vessel prior to the addition of urea. Thiol compounds, or other substances under investigation, were also added to the urease in the main compartment of the Warburg or Barcroft manometer vessel before addition of the urea. Any divergence from this procedure is noted in the text.

Addition of animal tissues. Experiments were carried out to determine whether the presence of animal tissues would inactivate organic arsenic compounds. Usually, known quantities of the arsenical were incubated with about 1 g. animal tissue (e.g. liver slices, or blood) at 37° for 2 hr. in presence of 0·037 M-$NaHCO_3$ solution in an atmosphere of 7% CO_2 + 93% O_2. At the end of this period 1 ml. of the supernatant was transferred to the acetate-urease mixture present in a Warburg manometric vessel. After thermal equilibrium, urea was tipped in from a side tube and the course of CO_2 evolution was followed. Control experiments in the absence of tissues were always set up.

Hydrolytic enzymes

Cholinesterase. Whole fresh rat brain (1·5 g.) was ground with sand in the presence of saline (8 ml.). The fine suspension was filtered through washed muslin and 0·5 ml. of the filtrate was used for the majority of estimations.

Activity measurements were carried out in either the Barcroft or Warburg manometric apparatus. The enzyme preparation was placed in the main compartment of a manometric vessel in presence of 0·027 M-$NaHCO_3$ solution in an atmosphere of 5% CO_2 + 95% N_2. Acetylcholine chloride (0·2 ml. of a 2% solution in $NaHCO_3$ solution) was placed in a side tube of the apparatus and this was tipped into the main vessel after thermal equilibrium had been set up. The rate of CO_2 evolution was a measure of the activity of the cholinesterase preparation.

Liver esterase. Rat-liver tissue was used as a source of liver esterase either in the form of thin, intact slices of fresh liver or as a suspension made by mincing 1 g. fresh liver in 15 ml. saline, 0·2–0·5 ml. of the suspension being usually employed. Activity was measured as with cholinesterase, with 0·2 ml. tributyrin as substrate. Control experiments without tissue were always set up.

Pancreatic lipase or esterase. Commercial pancreatin was used as a source of the enzyme and 0·5 ml. of a 1 in 100 suspension of pancreatin in saline was usually employed, with 0·2 ml. tributyrin as substrate. Activities were estimated as with cholinesterase.

Invertase. Commercial invertase was used as a source of the enzyme. The preparation (0·1 g.) was ground in 10 ml. water and the suspension was centrifuged. Of the clear centrifugate 1 ml. was usually employed. The general procedure for estimating the effects of organic arsenicals was as follows: In a series of tubes were placed 5 ml. 40% sucrose solution, 3 ml. 0·2 M-sodium acetate buffer solution, pH 5·0, and 1 ml. of either water or an aqueous solution of the organic arsenical. The mixtures were placed in a water bath at 40°, and after 30 min. 1 ml. of the invertase pre-

paration was added to each tube. At known intervals, pairs of tubes were removed from the water bath, and the contents were treated with 2 ml. of 6% trichloroacetic acid and filtered. To 8 ml. of the filtrate 2 ml. N-NaOH were added, and the mixture was transferred to a 1 dm. polarimeter tube and the optical rotation read. The course of inversion of sucrose was followed in this way and estimates were made of the effects of the organic arsenicals on the invertase activity.

Oxidation systems

The activities of these systems were measured either (*a*) under aerobic conditions by estimating the rates of O_2 uptake when tissues, or their extracts, respired in air or O_2 in a Locke 0·03M-sodium phosphate pH 7·4 medium either in the presence or absence of organic arsenicals, or (*b*) under anaerobic conditions by means of the ferricyanide technique of Quastel & Wheatley (1938) whereby the rate of evolution of CO_2 produced from bicarbonate on reduction of ferricyanide, in an atmosphere of 5% CO_2 + 95% N_2, was followed.

Succinic dehydrogenase. Rat liver, or rat kidney, slices were used as sources of this enzyme when intact tissues were investigated. A purified preparation of succinic dehydrogenase made from pig heart by the method of Keilin & Hartree (1940) was also used. In Exp. 6, Table 4, 0·5 ml. of a saline suspension of minced sheep liver (1 g./5 ml. saline) was employed.

Choline dehydrogenase. This enzyme was used either in the form of 1 ml. of a saline suspension of washed, minced rat liver (1 g. liver/5 ml. saline) or as intact liver slices (25 mg. dry weight).

Cytochrome oxidase. A saline suspension (1 ml.) of minced washed rat liver was generally used.

Lactic dehydrogenase. Washed human red blood cells lysed with water (1 part of cells/3 parts water) were used as a source of this enzyme. 1 ml. of the lysed preparation, in presence of cozymase (5 mg.) and neutral NaCN (0·08M), was usually employed.

Oxidations of glucose, lactate, or pyruvate. These oxidation systems were studied with the aid of saline suspensions of minced rat brain (1 g. whole brain/5 ml. saline), 1 ml. of the saline suspension being used.

Catalase. Minced rat liver (1 g.) was shaken with 100 ml. saline and the mixture centrifuged. The clear centrifugate served as a source of catalase, 0·5 ml. of the fluid being used.

RESULTS

Urease

Effects of a variety of organic arsenic compounds in the presence or absence of thiol compounds are shown in Tables 1 and 2 and Figs. 1 and 2.

The results show:

(1) Arsenoxides, in the form of phenylarsenoxide, mapharside(*m*-amino-*p*-hydroxyphenylarsenoxide), *p*-acetamidophenylarsenoxide, *m*-acetamido-*p*-carboxyphenylarsenoxide, and the products of condensation of thioglycollic acid with phenylarsenoxide and the reduced forms of atoxyl and tryparsamide have highly inhibitory effects on urease activity. The effects exceed those due to sodium arsenite at equivalent concentrations.

(2) Stabilarsan (the diglucoside of arsphenamine) has weakly toxic effects on urease, less than those of sodium arsenite at equivalent concentrations.

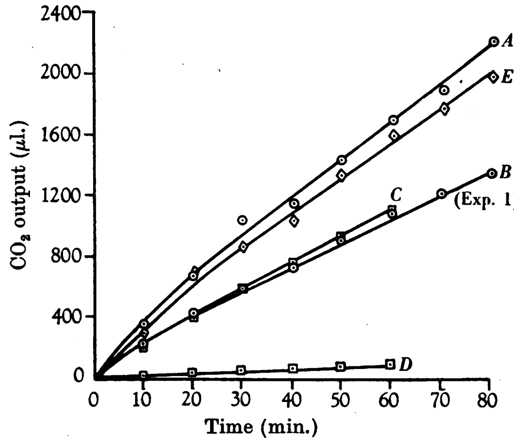

Fig. 1. Effects of arsenic compounds on urease activity at 37° and an acidity of pH 5. Exp. 1, Table 2: *A*, urease control; *B*, urease + 33 µg./ml. mapharside. Exp. 3, Table 2: *C*, urease control; *D*, urease + 67 µg./ml. phenylarsenoxide thioglycollate. Exp. 7, Table 2: *A*, urease control; *E*, urease + 670 µg./ml. tryparsamide.

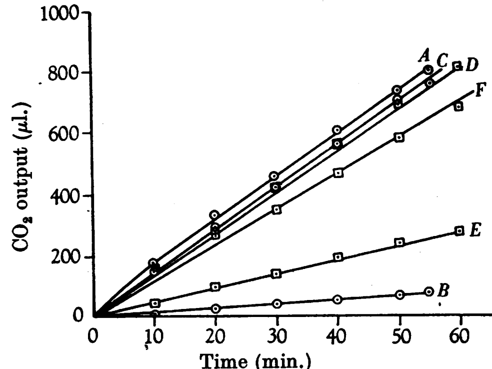

Fig. 2. Effects of arsenic compounds with and without added cysteine on urease activity at 37° and an acidity of pH 5. Exp. 14, Table 2: *A*, urease control; *B*, urease + 67 µg./ml. phenylarsenoxide thioglycollate; *C*, urease + 67 µg./ml. phenylarsenoxide thioglycollate + 2 mg./ml. cysteine. Exp. 15, Table 2: *D*, urease control; *E*, urease + 67 µg./ml. reduced tryparsamide thioglycollate; *F*, urease + 67 µg./ml. reduced tryparsamide thioglycollate + 2 mg./ml. cysteine.

(3) The presence of excess of a thiol compound, such as cysteine or glutathione, eliminates or greatly reduces the toxic effect of arsenoxides or of condensation products of arsenoxides and thioglycollic acid.

(4) Excess of NaCN does not affect the toxicity of an arsenoxide to urease.

Table 1. *Effects of arsenic compounds on urease at* 37° *and an acidity of* pH 5·0

Exp.	Arsenic compound added	Constitution	Conc. (M)	Enzyme activity (CO_2 output, µl./hr.)	Inhibition of enzyme activity (%)
1	Nil		—	1665	—
	Mapharside	AsO; NH_2; OH (benzene ring)	$1{\cdot}6 \times 10^{-4}$	1020	39
	Tryparsamide	AsO_3Na_2; $NH.CH_2.CONH_2$ (benzene ring)	$2{\cdot}1 \times 10^{-3}$	1491	10
2	Nil		—	1120	—
	Phenylarsenoxide thioglycollate	As(S.CH₂.COONa)₂ (benzene ring)	$1{\cdot}8 \times 10^{-4}$	85	92
3	Nil		—	1264	—
	Phenylarsenoxide	AsO (benzene ring)	$4{\cdot}0 \times 10^{-4}$	408	68
4	Nil		—	1308	—
	Sodium arsenite	Na_3AsO_3	$0{\cdot}86 \times 10^{-3}$	876	33
	Sodium arsenite		$1{\cdot}73 \times 10^{-3}$	609	54
5	Nil		—	1245	—
	Reduced atoxyl thioglycollate	As(S.CH₂.COONa)₂; NH_2 (benzene ring)	$2{\cdot}55 \times 10^{-4}$	672	47
6	Nil		—	1152	—
	Stabilarsan	As = As; Glucose-NH, OH; NH-Glucose, OH (two benzene rings)	$0{\cdot}97 \times 10^{-3}$	1008	13
	Stabilarsan		$2{\cdot}4 \times 10^{-3}$	768	34

(5) Toxicity of an arsenoxide to urease does not increase with time, indicating the probable existence of an equilibrium between the arsenoxide and the enzyme.

(6) The presence of tissues such as liver slices, or of whole or lysed blood, diminishes toxicity of arsenoxides to urease. This is due to the fact that the effective concentration of arsenoxide is diminished by combination of the arsenical with the tissue proteins.

(7) The pentavalent arsenic compound, tryparsamide, shows little toxicity to urease.

These conclusions confirm the observations made by previous authors as to the inhibitory effects of

Table 2. *Effects of organic arsenic compounds on urease*

Exp.	Arsenic compound	Conc. of arsenic compound (μg./ml.)	Duration of exp. (min.)	Total CO_2 output (μl.)	Inhibition of enzyme activity (%)
1	Nil	—	80	2220	—
	Mapharside	33	80	1360	39
2	Nil	—	50	633	—
	Mapharside	67	50	160	74·7
	Nil + NaCN (2 mg./ml.)	—	50	627	<1
	Mapharside + NaCN (2 mg./ml.)	67	50	163	74·2
3	Nil	—	60	1120	—
	Phenylarsenoxide thioglycollate	67	60	85	92
4	Nil	—	30	390	—
	Phenylarsenoxide thioglycollate	67	30	20	95
5	Nil	—	40	830	—
	Reduced atoxyl thioglycollate	100	40	449	46
6	Nil	—	40	830	—
	Reduced atoxyl thioglycollate	200	40	363	56
7	Nil	—	80	2220	—
	Tryparsamide	670	80	1990	10
8	Nil	—	30	281	—
	Tryparsamide	1300	30	279	<1
9	Nil	—	15	218	—
	p-Acetamidophenylarsenoxide	33	15	195	10·6
10	Nil	—	15	215	–
	m-Acetamido-*p*-carboxyphenylarsen-oxide	33	15	7	96·7
	Effects of addition of cysteine and glutathione				
11	Nil	—	55	837	—
	Nil + cysteine (2 mg./ml.)	—	55	767	8·4
	Mapharside	67	55	299	64·4
	Mapharside + cysteine (2 mg./ml.)	67	55	761	9·1
12	Nil	—	70	682	—
	Nil + cysteine (2 mg./ml.)	—	70	625	8·4
	Mapharside	67	70	216	68·3
	Mapharside + cysteine (2 mg./ml.)	67	70	591	13·3
13	Nil	—	60	852	—
	Nil + cysteine (2 mg./ml.)	—	60	867	—
	Phenylarsenoxide thioglycollate	67	60	49	94·7
	Phenylarsenoxide thioglycollate + cysteine (2 mg./ml.)	67	60	612	28·2
14	Nil	—	55	809	—
	Phenylarsenoxide thioglycollate	67	55	77	90·5
	Phenylarsenoxide thioglycollate + cysteine (2 mg./ml.)	67	55	761	5·9
15	Nil	—	60	822	—
	Reduced tryparsamide thioglycollate	67	60	278	66·2
	Reduced tryparsamide thioglycollate + cysteine (2 mg./ml.)	67	60	687	16·4
16	Nil	—	45	780	—
	Nil + glutathione (2 mg./ml.)	—	45	741	5
	Mapharside	67	45	185	76·3
	Mapharside + glutathione (2 mg./ml.)	67	45	752	3·6
	Effects of addition of animal (rat) tissues				
17	Nil	—	30	694	—
	Nil + liver slices (330 mg./ml.)	—	30	642	7
	Mapharside	22	30	359	48
	Mapharside + liver slices (330 mg./ml.)	22	30	654	6
18	Nil	—	25	916	—
	Nil + whole blood (0·17 ml./ml.)	—	25	907	1
	Mapharside	33	25	464	49
	Mapharside + whole blood (0·17 ml./ml.)	33	25	821	13
19	Nil	—	30	1070	—
	Nil + lysed blood (0·17 ml./ml.)	—	30	1310	—
	Mapharside	33	30	452	58
	Mapharside + lysed blood (0·17 ml./ml.)	33	30	1390	—

Table 3. *Effects of organic arsenic compounds with and without the addition of cysteine on hydrolytic enzymes*

Exp.	Source of enzyme	Arsenic compound added	Conc. of arsenic compound (μg./ml.)	Duration of exp. (min.)	CO_2 output (μl.)	Inhibition of enzyme activity (%)
		I. Cholinesterase (substrate: acetylcholine 1·3 mg./ml.)				
1	Rat brain suspension (1 ml.)	Nil	—	60	583	—
		Mapharside	500	60	369	36·7
		3-Amino-4-hydroxy-phenylarsonic acid	1300	60	502	14
2	Rat brain suspension (0·5 ml.)	Nil	—	60	373	—
		Mapharside	500	60	209	46
		3-Amino-4-hydroxy-phenylarsonic acid	600	60	347	8
3	Rat brain suspension (0·5 ml.)	Nil	—	100	459	—
		Mapharside	330	100	273	41
		Mapharside + cysteine (10 mg./ml.)	330	100	359	22
4	Rat brain suspension (0·5 ml.)	Nil	—	60	292	—
		Mapharside	330	60	170	41
		Mapharside + cysteine (10 mg./ml.)	330	60	226	22
5	Rat brain suspension (0·5 ml.)	Nil	—	70	501	—
		Nil + cysteine (10 mg./ml.)	—	70	503	—
		II. Liver esterase (substrate: tributyrin 700 μg./ml.)				
6	Rat liver slices (80 mg.)	Nil	—	25	1120	—
		Mapharside	270	25	613	45
7	Rat liver slices (50 mg.)	Nil	—	35	761	—
		Mapharside	270	35	295	61
		Tryparsamide	700	35	543	27
8	Minced liver suspension	Nil	—	20	1250	—
		Tryparsamide	700	20	555	68
9	Minced liver suspension	Nil	—	15	946	—
		Atoxyl	700	15	174	81
10	Minced liver suspension	Nil	—	40	1250	—
		Nil + cysteine (700 μg./ml.)	—	40	934	25·2
		Mapharside	330	40	306	71·2
		Mapharside + cysteine (700 μg./ml.)	330	40	680	45·6
11	Minced liver suspension	Nil	—	35	1070	—
		Nil + cysteine (800 μg./ml.)	—	35	760	29
		Mapharside	100	35	424	60·4
		Mapharside + cysteine (800 μg./ml.)	100	35	635	40·7
		III. Pancreatic lipase (substrate: tributyrin 700 μg./ml.)				
12	Pancreatin suspension	Nil	—	15	633	—
		Mapharside	330	15	628	0·8
13	Pancreatin suspension	Nil	—	20	963	—
		Tryparsamide	700	20	872	9
14	Pancreatin suspension	Nil	—	20	837	—
		Atoxyl	700	20	805	4
		IV. Invertase (substrate: 20% sucrose)			Change of optical rotation (°)	
15	Commercial yeast invertase solution	Nil	—	60	11·23	—
		Mapharside	200	60	10·78	4
16	Commercial yeast invertase solution	Nil	—	90	12·26	—
		Mapharside	200	90	12·11	1
17	Commercial yeast invertase solution	Nil	—	45	2·92	—
		Mapharside	200	45	2·85	2
18	Commercial yeast invertase solution	Nil	—	70	3·82	—
		Mapharside	200	70	3·81	0

substances (e.g. heavy metals, quinones, etc.) which react with thiol groups (Quastel, 1933; Perlzweig, 1932; Hellerman, Perkins & Clarke, 1933; Sumner & Poland, 1933; Smythe, 1936; Weiss, 1937) and support the conclusion that urease depends for its activity on the presence of thiol groups in its structure.

Pentavalent arsenic compounds become appreciably toxic only when reduction to the trivalent arsenoxide occurs, and this toxicity may be eliminated either by combination with thiol compounds, which must be in excess to prevent too great a dissociation of the easily reversible arsenoxide-thiol complex, or by combination with tissue proteins.

Hydrolytic enzymes

Cholinesterase. Typical results showing the effects of arsenicals on cholinesterase are given in Table 3 and Fig. 3. These results demonstrate:

(1) Cholinesterase is inhibited by the trivalent mapharside but not, or only feebly, by the pentavalent 3-amino-4-hydroxyphenylarsonic acid.

(2) The effect of the arsenoxide on the enzyme does not increase with time, showing the existence of an equilibrium between arsenical and enzyme.

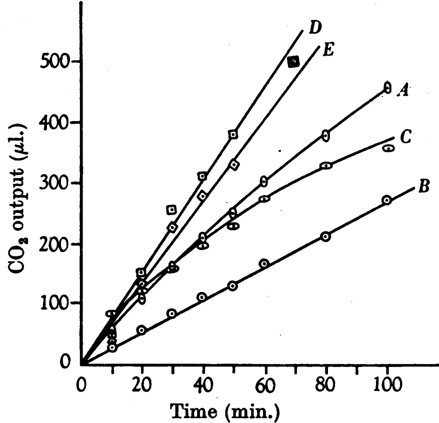

Fig. 3. Effects of arsenic compounds with and without added cysteine on cholinesterase at 37°. Exp. 3, Table 3: *A*, cholinesterase control; *B*, cholinesterase + 330 μg./ml. mapharside; *C*, cholinesterase + 330 μg./ml. mapharside + 10 mg./ml. cysteine. Exp. 5, Table 3: *D*, cholinesterase control; *E*, cholinesterase + 10 mg./ml. cysteine.

(3) The inhibitory action of the arsenoxide is greatly diminished by the presence of excess of cysteine.

The facts support the view that cholinesterase is a thiol enzyme (Nachmansohn & Lederer, 1939).

Liver esterase. Results obtained with this enzyme are shown in Table 3. Liver esterase is exceptional, among the enzymes which have been investigated, in being inhibited by pentavalent as well as by trivalent arsenic compounds. Thus tryparsamide and atoxyl exert toxic effects which are comparable with those due to mapharside. The toxic effect of atoxyl has already been noted by Jowett & Quastel (1935). The inhibitory effects of mapharside are not greatly diminished, or nullified, by the addition of excess of cysteine. A diminution in toxicity does take place, but it is difficult to assess, since cysteine alone exerts inhibitory effects on the activity of the liver esterase preparations we have used.

The facts we have obtained indicate the sensitivity of the enzyme preparation to the presence of arsenic whether this is in the trivalent or pentavalent condition. It would be wise to suspend judgement as to the thiol nature of the enzyme until a purified preparation of liver esterase has been investigated.

Pancreatic esterase. This enzyme preparation, in contrast to that of liver esterase, showed little or no sensitivity to trivalent or pentavalent organic arsenicals, under the conditions of our experiments.

Invertase. This enzyme is not attacked by mapharside at the concentrations tried (see Table 3), and is therefore unlikely to be a thiol enzyme.

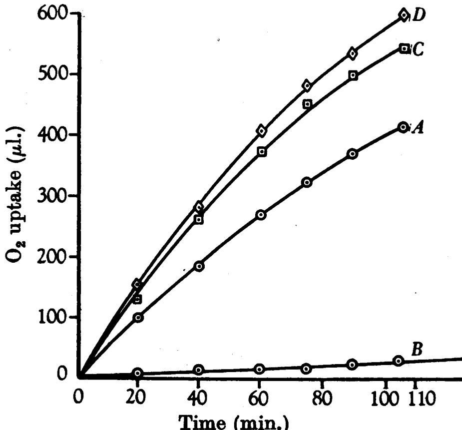

Fig. 4. Effects of mapharside, with and without added glutathione, on succinic dehydrogenase activity. Exp. 4, Table 4: *A*, succinic dehydrogenase control; *B*, succinic dehydrogenase + 2·7 μg./ml. mapharside; *C*, succinic dehydrogenase + 700 μg./ml. glutathione; *D*, succinic dehydrogenase + 2·7 μg./ml. mapharside + 700 μg./ml. glutathione.

Oxidation systems

Succinic dehydrogenase. Results in Table 4 and Fig. 4 show the highly inhibitory effects of mapharside on the activity of succinic dehydrogenase and the lack of toxic action of tryparsamide. The poisoning effect of the trivalent arsenical is nullified by the presence of an excess of glutathione. These

Table 4. *Effects of organic arsenic compounds on oxidizing enzymes in presence and absence of thiol compounds*

Exp.	Source of enzyme	Arsenic compound added	Conc. of arsenic compound (μg./ml.)	Duration of exp. (min.)	O_2 uptake (μl.)	Inhibition of enzyme activity (%)
		I. Succinic dehydrogenase (substrate: sodium succinate M/30)				
1	Rat liver slices (100 mg.)	Nil	—	30	596	—
		Mapharside	70	30	269	58
2	Rat kidney slices (100 mg.)	Nil	—	130	581	—
		Mapharside	133	130	192	67
3	Rat kidney slices (100 mg.)	Nil	—	70	539	—
		Tryparsamide	7000	70	536	0
4	Pig heart extract (0·5 ml.)	Nil	—	105	414	—
		Mapharside	2·7	105	28	93
		Nil + glutathione (700 μg./ml.)	—	105	544	—
		Mapharside + glutathione (700 μg./ml.)	2·7	105	597	0
5	Pig heart extract (0·5 ml.)	Nil	—	60	470	—
		Mapharside	2·7	60	167	64
		Nil + glutathione (700 μg./ml.)	—	60	544	—
		Mapharside + glutathione (700 μg./ml.)	2·7	60	535	0
					Anaerobic CO_2 output (μl.)	
6	Sheep liver extract (0·5 ml.)	Nil	—	30	599	—
		Mapharside	170	30	137	77
7	Rat liver slices (100 mg.)	Nil	—	60	917	—
		Mapharside	133	60	264	71
		II. Choline dehydrogenase (substrate: choline M/30)			O_2 uptake (μl.)	
8	Rat liver suspension (1 ml.)	Nil	—	40	388	—
		Reduced tryparsamide thioglycollate	170	40	212	45
		Reduced tryparsamide thioglycollate	330	40	115	70
9	Rat liver suspension (1 ml.)	Nil	—	60	626	—
		Reduced tryparsamide thioglycollate	230	60	249	60
		Tryparsamide	1300	60	586	6
10	Rat liver suspension (1 ml.)	Nil	—	60	228	—
		Mapharside	200	60	65	71
		Mapharside + cysteine (5 mg./ml.)	200	60	223	0
					Anaerobic CO_2 output (μl.)	
11	Rat liver slices (25 mg. dried tissue)	Nil	—	45	791	—
		Mapharside	130	45	388	50
12	Rat liver suspension (1 ml.)	Nil	—	60	707	—
		Mapharside	130	60	463	34
13	Rat liver slices (25 mg. dried tissue)	Nil	—	60	640	—
		Mapharside	170	60	212	67
		Mapharside + cysteine (5 mg./ml.)	170	60	539	16
		III. Cytochrome oxidase (substrate: *p*-phenylenediamine 3·3 mg./ml.)			O_2 uptake (μl.)	
14	Rat liver suspension (1 ml.)	Nil	—	40	472	—
		Mapharside	133	40	480	0
		Mapharside	330	40	466	1

Table 4 (*cont.*)

Exp.	Source of enzyme	Arsenic compound added	Conc. of arsenic compound (μg./ml.)	Duration of exp. (min.)	Anaerobic CO_2 output (μl.)	Inhibition of enzyme activity (%)
		IV. Lactic dehydrogenase (substrate: sodium lactate 16 mg./ml.)				
15	Lysed human red blood cells (1 blood : 3 water) (1 ml.) + cozymase + NaCN (neutral, M/12)	Nil	—	60	639	—
		Mapharside	330	60	598	6
		V. Glucose oxidation (substrate: glucose 0·01 M)				
18	Rat brain suspension (1 ml.)	Nil	—	150	349	—
		Mapharside	170	150	176	50
19	Rat brain suspension (1 ml.)	Nil	—	180	476	—
		Mapharside	170	180	99	79
		Tryparsamide	1700	180	650	0
20	Rat brain suspension (1 ml.)	Nil	—	120	583	—
		Mapharside	170	120	264	55
		Mapharside + cysteine (3·3 mg./ml.)	170	120	567	3
21	Rat brain suspension (1 ml.)	Nil	—	180	317	—
		3-Acetamido-4-carboxy-phenylarsenoxide	33	180	241	24
		3-Acetamido-4-carboxy-phenylarsenoxide + cysteine (660 μg./ml.)	33	180	319	0
		VI. Pyruvate oxidation (substrate: sodium pyruvate 0·01 M)				
22	Rat brain suspension (1 ml.)	Nil	—	150	360	—
		Mapharside	200	150	95	74
23	Rat brain suspension (1 ml.)	Nil	—	180	634	—
		Nil + glutathione (5 mg./ml.)	—	180	596	6
		Mapharside	200	180	12	98
		Mapharside + glutathione (5 mg./ml.)	200	180	420	33
24	Rat brain slices	Nil	—	110	185	—
		Mapharside	200	110	74	60
		VII. Lactate oxidation (substrate: sodium lactate 0·02 M)				
25	Rat brain suspension (1 ml.)	Nil	—	60	200	—
		Mapharside	133	60	3	98
		Mapharside	67	60	27	86
26	Rat brain suspension (1 ml.)	Nil	—	150	463	—
		Nil + glutathione (330 μg./ml.)	—	150	452	2
		Mapharside	17	150	25	94
		Mapharside + glutathione (330 μg./ml.)	17	150	363	21
		VIII. Catalase (substrate: H_2O_2, 0·03 %)			O_2 output (μl.)	
27	Rat liver extract (0·5 ml.)	Nil	—	40	621	—
		Mapharside	133	40	614	1
		Mapharside	330	40	556	10

facts point to the thiol nature of succinic dehydrogenase, confirming the observations of Hopkins & Morgan (1938), Hopkins, Morgan & Lutwak-Mann (1938) and of Keilin & Hartree (1940). The greater the purity of the enzyme preparation the greater is the toxicity secured by a given concentration of arsenoxide. This is due to the purified preparation containing smaller quantities of tissue proteins which compete with the enzyme for the arsenical.

Choline dehydrogenase. This enzyme (for details of properties and estimation see Mann & Quastel, 1937; Bernheim & Webster, 1937; Mann, Woodward & Quastel, 1938) is highly sensitive to such trivalent organic arsenic compounds as mapharside and reduced tryparsamide thioglycollate. The pentavalent tryparsamide, however, does not inhibit the enzyme. The presence of cysteine, in excess, reverses the inhibitory action of mapharside on choline

Table 5. *Effects of various inhibitors on choline dehydrogenase*

(Substrate: choline 0·033 M)

Exp.	Source of enzyme	Inhibitor	Conc. of inhibitor (M)	Duration of exp. (min.)	O_2 uptake (µl.)	Inhibition of enzyme activity (%)
1	Saline suspension of washed minced rat liver	Nil (choline absent)	—	60	167	—
		Sodium tetrathionate (choline absent)	2×10^{-4}	60	102	—
		Nil	—	60	621	—
		Sodium tetrathionate	2×10^{-4}	60	382	38
2	Saline suspension of washed minced rat liver	Nil (choline absent)	—	60	75	—
		Sodium tetrathionate (choline absent)	6×10^{-4}	60	43	—
		Nil	—	60	549	—
		Sodium tetrathionate	6×10^{-4}	60	80	93
3	Saline suspension of washed minced rat liver	Nil (choline absent)	—	60	89	—
		Alloxan (choline absent)	0·22	60	31	—
		Nil	—	60	508	—
		Alloxan	0·22	60	107	82
4	Saline suspension of washed minced rat liver	Nil	—	60	482	—
		Alloxan	0·022	60	90	82
5	Saline suspension of washed minced rat liver	Nil (choline absent)	—	150	63	—
		Nil	—	150	317	—
		Oxidized glutathione	0·05	150	204	44

dehydrogenase. Results supporting these conclusions are shown in Table 4 and Figs. 8 and 9. These results lead to the view that choline dehydrogenase is a thiol enzyme. This conclusion is supported by the fact that the following compounds also inhibit choline dehydrogenase, viz. sodium tetrathionate, alloxan, and oxidized glutathione. Typical results are shown in Table 5. These substances, as shown by Hopkins & Morgan (1938) and by Keilin & Hartree (1940), have inhibitory effects on succinic dehydrogenase, presumably by oxidation of the thiol groups of the enzyme.

Pyruvic oxidase. The oxidation of pyruvic acid by minced rat brain is very greatly inhibited by mapharside, the inhibition being suppressed by the presence of excess of glutathione (Table 4, and Fig. 6). These facts point to the thiol nature of pyruvic oxidase, already suggested by Peters (1937) and by experiments of Barron (1936) on pyruvate oxidation of gonococci.

Glucose and lactate oxidation. The respiration of minced rat brain in presence of either glucose or sodium lactate is very greatly inhibited by mapharside and by 3-acetamido-4-carboxyphenylarsenoxide, the inhibitions being greatly reduced or eliminated by the presence of excess cysteine. These results are shown in Table 4 and Figs. 5 and 7. The pentavalent tryparsamide has no inhibitory effect on oxygen uptake by brain in presence of glucose under the conditions of our experiments. We have noted that in all experiments on the respiration of brain tissue in presence of glucose or of lactate, there is an accumulation of a ketonic acid, probably pyruvic acid, in the medium when the trivalent organic arsenical is also present. Doubtless the large inhibitory effects of the trivalent organic arsenicals

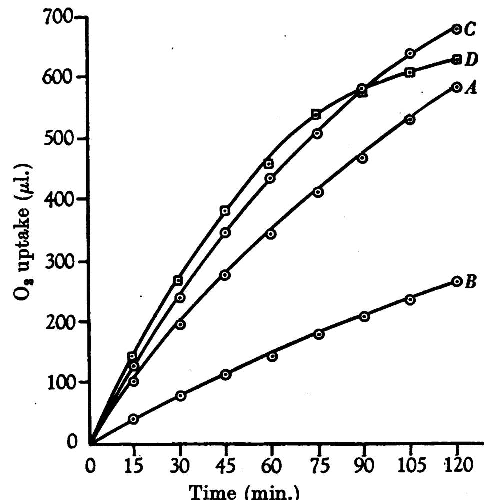

Fig. 5. Effects of mapharside with and without added cysteine, on glucose oxidation at 37°. Exp. 20, Table 4: *A*, glucose oxidation system control; *B*, glucose oxidation system + 170 µg./ml. mapharside; *C*, glucose oxidation system + 3·3 mg./ml. cysteine; *D*, glucose oxidation system + 170 µg./ml. mapharside + 3·3 mg./ml. cysteine.

on brain respiration in presence of glucose or lactate are due partly to the blocking of the respiratory chain at the pyruvic enzyme stage.

Lactic dehydrogenase. Results given in Table 4, Exp. 15, show that lactic dehydrogenase, examined by the anaerobic technique, is unaffected by mapharside and is therefore unlikely to be a thiol enzyme.

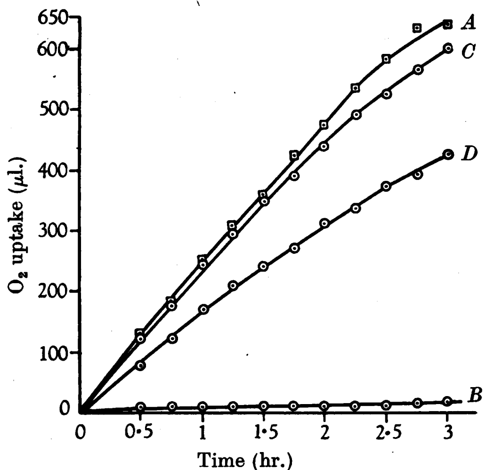

Fig. 6. Effects of mapharside, with and without added glutathione, on pyruvic oxidase activity at 37°. Exp. 23, Table 4: *A*, pyruvic oxidase control; *B*, pyruvic oxidase + 200 µg./ml. mapharside; *C*, pyruvic oxidase + 5 mg./ml. glutathione; *D*, pyruvic oxidase + 200 µg./ml. mapharside + 5 mg./ml. glutathione.

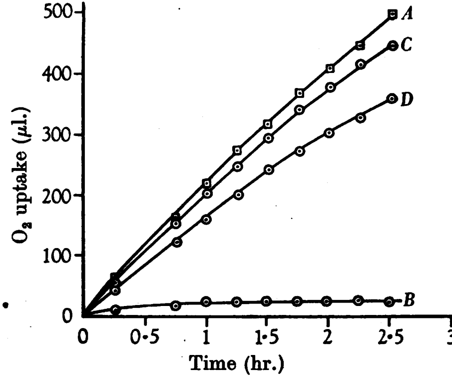

Fig. 7. Effects of mapharside and glutathione on lactic oxidation system at 37°. Exp. 26, Table 4: *A*, lactate oxidation system control; *B*, lactate oxidation system + 17 µg./ml. mapharside; *C*, lactate oxidation system + 330 µg./ml. glutathione; *D*, lactate oxidation system + 17 µg./ml. mapharside + 330 µg./ml. glutathione.

Cytochrome oxidase and catalase. Neither of these enzymes is affected by mapharside under the conditions of our experiments and it is unlikely that they are thiol enzymes.

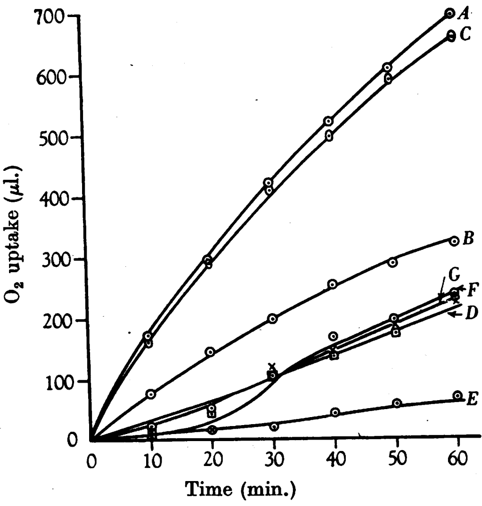

Fig. 8. Effects of arsenic compounds, with and without added cysteine, on choline dehydrogenase activity at 37°. Exp. 9, Table 4: *A*, choline dehydrogenase control; *B*, choline dehydrogenase + 230 µg./ml. reduced tryparsamide thioglycollate; *C*, choline dehydrogenase + 1300 µg./ml. tryparsamide. Exp. 10, Table 4: *D*, choline dehydrogenase control; *E*, choline dehydrogenase + 200 µg./ml. mapharside; *F*, choline dehydrogenase + 200 µg./ml. mapharside + 5 mg./ml. cysteine (cysteine added at same time as mapharside); *G*, choline dehydrogenase + 200 µg./ml. mapharside + 5 mg./ml. cysteine (cysteine added after 2 hr. incubation of enzyme and mapharside).

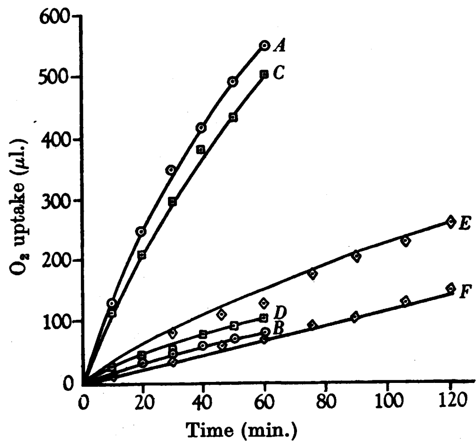

Fig. 9. Effects of thiol inhibitors on choline dehydrogenase activity at 37°. Exp. 2, Table 5: *A*, choline dehydrogenase control; *B*, choline dehydrogenase + 6×10^{-4} M-sodium tetrathionate. Exp. 4, Table 5: *C*, choline dehydrogenase control; *D*, choline dehydrogenase + 0·022 M-alloxan. Exp. 5, Table 5: *E*, choline dehydrogenase control; *F*, choline dehydrogenase + 0·05 M-oxidized glutathione.

Protection of enzymes by their substrates

Experiments were carried out on a few of the enzymes, which were shown to be inhibited by arsenoxides, to determine whether the enzymes could be protected from inactivation by their substrates. The enzymes which were examined in this way were urease, succinic dehydrogenase and choline dehydrogenase. The activities of the enzymes were measured in the manner already described, except that the inhibitor was added before the substrate. Controls were also performed, in which the substrate was added last. Results are shown in Table 6.

arsenoxide. This phenomenon has been confirmed with a variety of organic pentavalent arsenic compounds including tryparsamide in the presence of glutathione or whole blood (Hawking & Quastel, 1936).

It became of interest to see whether the reduction products of tryparsamide and atoxyl, due to incubation with thiol compounds, would inactivate urease.

The arsenical (0·1 g.) was dissolved in 5 ml. water to which 10 mg. cysteine was added. The solution was neutralized, placed in a vacuum tube from which air was withdrawn, and the tube was incubated at 37°. The tube was then opened and various quantities of the solutions were added to urease in a Warburg manometric vessel, from the side tube of which urea was tipped after thermal equilibrium.

Table 6. *Effects of the presence of substrates on inactivation of enzymes by mapharside*

(A) Urease (final concentration of urea 16%)

Final concentration of mapharside (µg./ml.)	Conditions of experiment	CO_2 output (µl.)	Inhibition of enzyme added (%)
0	Urea added last	1749	—
33	Urea added last	960	45
0	Urea added before mapharside	1791	—
33	Urea added before mapharside	1131	37

(B) Succinic dehydrogenase (conc. of substrate M/30)

Source of enzyme	Wt. of wet tissue (mg.)	Conc. of mapharside (µg./ml.)	Conditions of experiment	O_2 uptake (µl.)	Inhibition of activity (%)
Rat kidney slices	100	0	Succinate added last	393	—
	100	33	Succinate added last	225	44
	100	0	Succinate added before mapharside	399	—
	100	33	Succinate added before mapharside	327	19
Pigeon kidney slices	100	0	Succinate added last	334	—
	100	33	Succinate added last	204	39
	100	0	Succinate added before mapharside	322	—
	100	33	Succinate added before mapharside	282	13
(C) Choline dehydrogenase					
Rat liver extract (1 in 4)	1 ml. extract	0	—	368	—
	1 ml. extract	133	Choline added last	194	48
	1 ml. extract	133	Choline added before mapharside	281	32

Urease is obviously not protected by its substrate (see also Quastel, 1932). Succinic dehydrogenase is protected to some extent by succinate when the concentration of mapharside is low, and choline dehydrogenase is also partially protected by its substrate.

Reduction of tryparsamide and atoxyl by thiol compounds and the effects of the reduction products on urease

It is already known (Levaditi, Andrews & Mann, 1928) that atoxyl in presence of glutathione and of tissue extracts becomes strongly trypanocidal, an effect attributed to the reduction of the pentavalent arsenical, by thiol compounds, to the active trivalent arsenoxide.

Typical results are shown in Table 7, which also shows the effects obtained when excess of the thiol compound was added to the urease system. The results show that both atoxyl and tryparsamide may be transformed into highly toxic products by incubation with glutathione, cysteine or thioglycollate. Moreover the toxicities are diminished or eliminated by having an excess of glutathione present with the urease. The facts indicate that urease may be used to detect the formation of trivalent arsenoxide by reduction of pentavalent arsenicals by thiol compounds.

Table 7. *Inhibition of urease activity by pentavalent organic arsenic compounds incubated with thiol compounds*

Exp.	Nature and conditions of incubation mixture	Amount of incubation mixture added to urease (ml.)	Duration of exp. with urease (min.)	CO_2 output (μl.)	Inhibition of urease activity (%)
1	—	Nil	35	612	—
	Atoxyl (20 mg./ml.) + cysteine (2 mg./ml.) incubated 72 hr. at 37°	1	35	44	93
2	—	Nil	40	717	—
	Atoxyl (20 mg./ml.) + cysteine (2 mg./ml.) incubated 72 hr. at 37°	0·5	40	104	86
	Tryparsamide (20 mg./ml.) + cysteine (2 mg./ml.) incubated 72 hr. at 37°	0·5	40	81	89
3	—	Nil	40	471	—
	Tryparsamide (20 mg./ml.) + Na thioglycollate (2 mg./ml.) incubated 19 hr. at 37°	0·1	40	167	65
	Tryparsamide (20 mg./ml.) + cysteine (2 mg./ml.) incubated 19 hr. at 37°. Excess glutathione (10 mg.) finally added on transference to urease	0·1	40	377	20
4	—	Nil	40	640	—
	Tryparsamide (20 mg./ml.) + cysteine (2 mg./ml.) incubated 19 hr. at 37°	0·1	40	167	74
	Tryparsamide (20 mg./ml.) + cysteine (2 mg./ml.) incubated 19 hr. at 37°. Excess glutathione (10 mg.) added on transference to urease	0·1	40	547	7
5	—	Nil	30	457	—
	Tryparsamide (20 mg./ml.) + glutathione (2 mg./ml.) incubated 20 hr. at 37°	0·5	30	271	40
	Tryparsamide (20 mg./ml.) + glutathione (2 mg./ml.) incubated 20 hr. at 37°. Excess glutathione (5 mg.) added on transference to urease	0·5	30	384	16
6	—	Nil	50	323	—
	Tryparsamide (20 mg./ml.) + glutathione (2 mg./ml.) incubated 20 hr. at 37°	0·5	50	195	40

DISCUSSION

Our experimental results justify the view that trivalent organic arsenoxides may be employed for the detection of thiol enzyme systems. Four enzymes, namely urease, succinic dehydrogenase, cholinesterase, and pyruvic oxidase, for which there was prior evidence that thiol groups are important for their activities, are highly sensitive to phenylarsenoxide and its derivatives. Enzymes, however, for which there has been no evidence that thiol groups are essential for their activities, viz. lactic dehydrogenase, cytochrome oxidase, catalase and invertase, are relatively unaffected by the presence of organic arsenoxides.

Pentavalent organic arsenic compounds such as tryparsamide or atoxyl do not affect enzymes (except in the case of liver esterase), but they become active poisons to the thiol enzymes when they are reduced to the trivalent arsenoxides by the presence of thiol compounds.

The organic arsenoxides probably establish an equilibrium with the thiol enzymes, as their toxicities do not increase with time. These toxicities may be diminished or eliminated by the presence of an excess of thiol compounds which convert the arsenoxides into inert condensation products whose dissociations are smaller the greater the excess of free thiol groups.

Choline dehydrogenase is shown, by its high sensitivity to organic arsenoxide, or to the condensation products of these substances with thioglycollic acid, which dissociate freely in absence of excess thiol compounds, to be a thiol enzyme. This is confirmed by showing the enzyme to be sensitive also to the presence of sodium tetrathionate, alloxan or oxidized glutathione, molecules which bring about the oxidation of SH groups. This enzyme, too, probably forms an equilibrium with organic arsenoxide, and the enzyme inactivated by this means may be reactivated by the presence of excess thiol groups, as is also the case with the other thiol enzymes investigated (succinic dehydrogenase, urease, pyruvic oxidase, cholinesterase).

It is obvious that these results have a bearing on the therapeutic properties of organic arsenicals. The reduction of these substances by tissue constituents and the removal of the resultant arsenoxides by

combination with inert proteins, or their transformation into inert condensation products by local high concentrations of SH bodies, are important factors to be considered in the problems of arsenic therapy.

SUMMARY

1. A description is given of experiments carried out during 1938, 1939 and early 1940, details of which have not hitherto been published, on the effects of organic trivalent and pentavalent arsenic compounds on enzyme systems.

2. Urease is highly sensitive to trivalent arsenoxides such as phenylarsenoxide, *m*-amino-*p*-hydroxyphenylarsenoxide (mapharside), *p*-acetamidophenylarsenoxide, and *m*-acetamido-*p*-carboxyphenylarsenoxide. It is also inhibited by the products of condensation of thioglycollate with phenylarsenoxide and with the reduced forms of atoxyl and tryparsamide. The effects are generally greater than those due to sodium arsenite at equivalent concentrations. Urease is also sensitive to stabilarsan, the diglucoside of arsphenamine, but this substance is less toxic than sodium arsenite at equivalent concentrations. The toxicity of arsenoxides may be reduced or eliminated by the presence of excess of thiol compounds such as cysteine or glutathione, or by the presence of proteins which compete with urease for the arsenoxides. An equilibrium probably exists between the arsenoxide and the enzyme. Pentavalent arsenic compounds such as atoxyl or tryparsamide show little toxicity to urease. The results conform with the view that urease is a thiol enzyme.

3. The reaction of cholinesterase with arsenicals also supports the conclusion that this enzyme is a thiol enzyme.

4. Choline dehydrogenase is shown to be a thiol enzyme, since it is inhibited by trivalent arsenoxides, the inhibition being neutralized by the presence of excess of thiol compounds. It is also inhibited by sodium tetrathionate, alloxan, and oxidized glutathione.

5. Pyruvic oxidase is greatly inhibited by mapharside, the inhibition being suppressed by the presence of excess of glutathione. These results point to the thiol nature of the enzyme. The oxidation systems of lactate and of glucose, in brain tissue, are also inhibited by trivalent arsenoxide, the effects being reversed by the presence of excess thiol compounds. Lactic dehydrogenase, however, is unaffected by mapharside and is therefore unlikely to be a thiol enzyme.

6. Cytochrome oxidase, catalase and invertase are relatively unaffected by the presence of organic arsenoxides.

7. Pentavalent organic arsenic compounds do not affect the enzymes investigated, except in the case of liver esterase, but they become active poisons to the thiol enzymes when they are reduced to the trivalent arsenoxides by the presence of thiol compounds.

8. The thiol enzymes apparently form reversible equilibria with organic trivalent arsenoxides.

9. Whereas succinate protects its enzyme from inactivation by a trivalent arsenoxide and choline likewise shows some protection to its enzyme, urea fails to afford any protection to urease.

10. Mapharside is recommended as a useful organic trivalent arsenical for the detection of thiol enzymes.

We are greatly indebted to Dr H. King, F.R.S., for his gift of organic trivalent arsenoxides and their condensation products with thioglycollic acid.

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