cysteine (as ethyl ester); other experiments gave similar results; the inactivation with the —S—S compound and reactivation with —SH was definite.

DISCUSSION

Systematic investigations upon —SH groups in an enzyme appear to have been first made upon urease by Hellerman, Perkins & Clark (1933) (see Hellerman, 1937).

Some facts as to the action of maleate upon brain tissue have already been published by Weil-Malherbe (1938), who quoted earlier literature. He found that 20 mM-maleic acid inhibited the respiration of brain slices in bicarbonate-glucose-Ringer by 10–50%; the inhibition was comparatively small in presence of pyruvate, and it thus appears that the pyruvate oxidase system is even less sensitive in the slice than in the *brei*. Our facts are consistent with the idea that an —SH group is essential for the activity of this system, and that this group is so activated as to be specially sensitive to maleate. It is still necessary to qualify this by pointing out that the evidence is indirect, because the enzyme concerned has not yet been obtained in pure form; the evidence has been much strengthened by the dithiol theory of Stocken & Thompson (1940–41) (for a brief account see Peters, Stocken & Thompson, 1945). (Recently, Barron & Singer (1945), see also Waters & Stock (1945), have also concluded that the pyruvate oxidase system is specially sensitive to —SH reagents; independently Bacq (1942) has found the —SH fraction in proteins abolished by some vesicants.)

SUMMARY

1. Arising from earlier work on chemical warfare agents, and from a research upon antidotes, the sensitivity of the pyruvate oxidase system from brain to some —SH reagents was investigated.

2. The pyruvate oxidase system and the pyruvate dehydrogenase component were much more sensitive to sodium maleate than succinodehydrogenase. Pyruvate dehydrogenase was inactivated by cystine ester and reactivated by cysteine ester. Both these effects are explained by the presence of an essential —SH group in the enzyme concerned.

We are grateful to Dr L. A. Stocken for the preparations of the esters of cystine and cysteine (as hydrochloride).

REFERENCES

Bacq, Z. M. (1942). *Enzymologia*, **10**, 48.
Banga, I., Ochoa, S. & Peters, R. A. (1939*a*). *Biochem. J.* **33**, 1109.
Banga, I., Ochoa, S. & Peters, R. A. (1939*b*). *Biochem. J.* **33**, 1980.
Barron, E. S. G. & Singer, T. P. (1945). *J. biol. Chem.* **157**, 221.
Dickens, F. (1933). *Biochem. J.* **27**, 1141.
Dixon, M. (1937). *Nature, Lond.*, **140**, 806.
Hellerman, L. (1937). *Physiol. Rev.* **17**, 454.
Hellerman, L., Perkins, M. E. & Clark, W. M. (1933). *Proc. Nat. Acad. Sci., Wash.*, **19**, 855.
Hopkins, F. G. & Dixon, M. (1922). *J. biol. Chem.* **54**, 526.
Hopkins, F. G. & Morgan, E. J. (1938). *Biochem. J.* **32**, 611.
Lipmann, F. (1937). *Skand. Arch. Physiol.* **76**, 255.
Long, C. (1938). *Biochem. J.* **32**, 1711.
Long, C. (1943). *Biochem. J.* **37**, 215.
Long, C. & Peters, R. A. (1939). *Biochem. J.* **33**, 759.
Morgan, E. J. & Friedmann, E. (1938). *Biochem. J.* **32**, 862.
Peters, R. A. (1936*a*). *Nature, Lond.*, **138**, 327.
Peters, R. A. (1936*b*). *Curr. Sci.* **5**, 207.
Peters, R. A. (1937). *Perspectives in Biochemistry*, p. 41. Cambridge.
Peters, R. A., Sinclair, H. M. & Thompson, R. H. S. (1946). *Biochem. J.* **40**, 516 and Reports to Ministry of Supply (1940/41).
Peters, R. A., Stocken, L. A. & Thompson, R. H. S. (1945). *Nature, Lond.*, **156**, 616.
Stocken, L. A. & Thompson, R. H. S. (1940–1). Report to Ministry of Supply by Peters, R. A., nos. 20 and 29.
Voegtlin, C., Dyer, A. & Leonard, C. S. (1923). *U.S. Publ. Hlth Rep.* **38**, 1882.
Walker, E. (1925). *Biochem. J.* **19**, 1085. (Also Peters, R. A. & Walker, E. (1924). Government Report.)
Walker, E. (1928). *Biochem. J.* **22**, 292.
Waters, L. L. & Stock, C. (1945). *Science*, **102**, 601.
Weil-Malherbe, H. (1938). *Biochem. J.* **32**, 2257.

An Analysis of the Inhibition of Pyruvate Oxidation by Arsenicals in Relation to the Enzyme Theory of Vesication

By R. A. PETERS, H. M. SINCLAIR and R. H. S. THOMPSON, *Department of Biochemistry, Oxford*

(*Received* 5 *April* 1946)

This research was planned as the first step in an attempt to find an antidote to arsenical vesicants, such as lewisite, and a brief account of the work as a whole has appeared already (Peters, Stocken & Thompson, 1945).

The vesicant action of arsenicals (as of other substances) on human skin will be conditioned by their ability to penetrate the keratin layer of the epidermis, and so reach the site, whether it be small blood vessels or living cell layers, which after

CORRIGENDA

An Analysis of the Inhibition of Pyruvate Oxidation by Arsenicals in Relation to the Enzyme Theory of Vesication.
By R. A. Peters, H. M. Sinclair and R. H. S. Thompson

Volume 40 (1946), No. 4, p. 517, col. 1, line 43

for **Rona & Szent-Györgyi** ***read*** **Rona & Gyorgy**

injury give rise to the blister. Thus, although casual contact of the skin with arsenious oxide is not injurious in man, prolonged and intimate contact with this compound may produce erythema and vesication (Cushny, 1924). The arsenical vesicants such as lewisite (chlorovinyldichloroarsine) on the other hand, being lipid-soluble, penetrate the epidermis rapidly, and on reaching the aqueous medium of the cellular layer of the skin will be rapidly hydrolyzed to the corresponding oxides, which are well known to be toxic to Protozoa (McCleland & Peters, 1919; Walker, 1928). These oxides will likewise have general systemic effects if they reach the circulation.

Once the toxic substance has reached the cellular layer of the skin, vesication might theoretically be initiated by several possible mechanisms and may involve selective poisoning of some enzyme as a first step. This hypothesis inspired our work upon this problem, which was based upon two ideas: (*a*) that the pyruvate oxidase system was specially sensitive to arsenicals, and (*b*) that the attack was primarily upon —SH groups in this system. This latter point will be reported separately (Peters & Wakelin, 1946). Evidence for an essential —SH group was early obtained, and eventually made definite by Stocken & Thompson's 'dithiol theory' (1941, 1946). The idea that an attack upon an enzyme may initiate vesication is not new. It certainly occurred to Flury (1921) who thought that the universal cell poisoning by arsenical compounds might be explained by injury to ferments; this was supported by the statement that inhibitory effects of some arsenicals (diphenylchloroarsine) on blood catalase had been found. Rona and colleagues in a series of papers compared the action of several arsenicals upon enzymes; attention was early drawn by them to the possibility of a connexion between the minute amounts of chemical warfare agents causing damage and the small amounts of enzymes; but their work became oriented more to the use of these poisons to throw light upon enzyme constitution. Rona & Szent-Györgyi (1920) found diphenylarsenoxide and phenylarsenoxide very toxic to urease; methylarsenoxide produced about 50% inhibition at 0·01 mM; arsenite was less effective, and atoxyl without action. In contrast, serum lipase was strongly inhibited by atoxyl and arsenite and little affected by methylarsenoxide. Again, Rona, Airila & Lasnitski (1922) found methylarsenoxide to inhibit maltase and α-methylglucosidase *c.* 50% at approximately 0·25 mM, whereas invertase was unaffected. Peters & Walker (1923–5), on the other hand, could obtain no support for the enzyme theory upon the above lines.

Though it has been known for some years, therefore, that trivalent compounds of arsenic are potent inhibitors of some enzyme systems, much of the work fails in essential respects for our problem. For example, solid grounds for believing in an intimate connexion between poisoning of an enzyme and vesication can be based only upon the proof that the action is selective in nature, that the arsenical is active in high dilution, and that some enzyme of more consequence to higher organisms than urease, etc., is inhibited.

Onaka (1911), working in Warburg's laboratory, first linked the poisonous action of arsenic upon whole animals with inhibition of tissue respiration, by showing that 0·029 mM-sodium arsenite inhibited the respiration of nucleated red blood corpuscles. Later, Warburg (1925) suggested that arsenious oxide combines chemically with the iron-containing respiratory ferment, a view not now tenable. Dresel (1926) showed that the rates of respiration of normal rat tissues (liver, kidney and testis) and of Jensen sarcoma were powerfully inhibited by low concentrations of arsenite; both respiration and anaerobic glycolysis of yeast were also sensitive, but the anaerobic glycolysis of Jensen sarcoma was only slightly inhibited. Subsequently (1928), he noted that glucose could protect yeast cells against the inhibitory influence of arsenite on oxygen consumption.

Szent-Györgyi (1930) stated that the oxidation of succinate by minced rabbit liver was relatively insensitive to arsenite, though he found that the respiration of liver pulp was 50% inhibited by 0·13 mM. His general conclusion that 'it had no effect upon oxygen activation and little upon hydrogen activation' was supported by his finding that *p*-phenylenediamine oxidation was insensitive to 10·0 mM-arsenite. In a continuation of this work (Banga, Schneider & Szent-Györgyi, 1931) in which no specific enzyme systems were investigated, they concluded that 'biological oxidations can be classed into two groups, those sensitive to As_2O_3, comprising the greater number, and those unaffected'.

In connexion with Voegtlin's well-known views that the toxic action of arsenic on living cells is due to its reaction with certain essential thiol compounds present in protoplasm, Voegtlin, Rosenthal & Johnson (1931) found that 10 mM-arsenite inhibited the residual respiration of testis by about 25%, and that this inhibition was partially abolished by the addition of excess glutathione. Based only upon the observation that 1·0 mM-arsenite allowed deamination of amino-acids by kidney slices with accumulation of the corresponding α-keto acid, Krebs (1933) claimed that it specifically inhibited α-keto acid oxidation. In 1935, Jowett & Quastel showed that 1·0 mM-arsenite inhibited the formation of acetoacetate from fatty acids by guinea-pig liver, and also decreased the respiration; Quastel & Wheatley (1935) later found that

0·15 mM-arsenite caused 70% inhibition of the breakdown of acetoacetate by kidney slices. Crasnaru & Gavrilescu (1935) found an inhibition of the respiration of brain by novarsenobenzol, in concentrations which, they claimed, stimulated that of other tissues. Arsenite has also been used by Oelkers (1937), Das (1937), Krebs & Johnson (1937), Green & Brosteaux (1936), Ochoa (1941) and Long (1945).

The above review shows that up to the time of our earliest observations in 1936, none of the work upon arsenic and enzymes, except for that of Rona, was upon chemical warfare arsenicals. In general, rather high concentrations of arsenical had been used, and there was little evidence of specificity.

Turning to work in this laboratory, Peters & Thompson (1934) had reported an inhibition with iodoacetate of pyruvate oxidation in brain tissue respiring in the presence of lactate, which was specific for the vitamin B_1-catalyzed system. This was investigated in more detail by Peters, Rydin & Thompson (1935). Later, Peters (1936*a*), drawing attention to the similar vesicant action of iodoacetate and dichlorodiethyl-sulphone, reported that the latter had a similar selective action upon lactate oxidation. The poisoning caused by small concentrations (0·087 mM) gave an accumulation of pyruvate without interfering with the step, lactate → pyruvate; from this it could be inferred that the cytochrome system was untouched. Also in preliminary experiments (1936*b*) Peters found that traces of arsenite exerted a similar effect (0·05 mM producing definite inhibition); using avitaminous brain it was proved that there was genuine inhibition of the pyruvate oxidation. This work was rapidly confirmed and extended in 1939 in experiments described below which place the specific poisoning of the pyruvate oxidase system upon a firm basis.

EXPERIMENTAL

Enzyme preparations. In the aerobic experiments involving enzymes present in the brain, the method has been to compare manometrically the oxygen consumption of pigeon's brain tissue respiring in a Ringer-phosphate solution at pH 7·3 and at 38°, both in the presence and absence of the substrate and of a suitable dilution of the arsenical.

Two methods of preparing the tissue have been used. In the first the cerebrum is finely minced on a porcelain plate. This type of preparation will be referred to hereafter as a 'brain *brei*'. In the second method the whole of the brain except the cerebellum is ground in an ice-cold mortar with the slow addition of 24 ml. ice-cold KCl (0·9%) and 4 ml. phosphate buffer (0·5 M), pH 7·3. It is then pressed through muslin and 2 ml. samples pipetted into the Warburg bottles, which have already been filled with substrate, 0·005 M-Na fumarate and arsenical, and are standing in ice. In the 10 min. interval between death of the bird and immersion of the first bottle in the bath the brain dispersion is maintained at 0–2°. The first reading is taken 20 min. after death, and subsequent readings every 10 min. for the next half-hour. This type of preparation will be referred to as a 'brain dispersion' (Banga, Ochoa & Peters, 1939).

Other enzyme preparations used will be described in a later section.

Arsenicals. Sodium arsenite was prepared by dissolving arsenious oxide (B.D.H., A.R.) in water by warming and addition of N/2 NaOH, subsequently adjusting to pH 7·3.

The lewisite and phenyl- and ethyldichloroarsine (kindly supplied by the Ministry of Supply Experimental Station, Porton) were dissolved directly in Ringer-phosphate; lewisite oxide was dissolved in alkali and neutralized. In each case the dilution was such that the appropriate amount of arsenical was contained in 0·1 ml. of the solution.

RESULTS

Pyruvate oxidation. The result of a typical *brei* experiment with 0·02 M-sodium pyruvate is shown below (Table 1); the inhibitions produced are expressed as percentages of the extra respiration due to added pyruvate. This method of expressing percentage decreases is used throughout the manometric experiments described in this paper.

Table 1. *Effect of* 0·017 mM-*sodium arsenite on pyruvate oxidation in brain* brei

	O_2 uptake (μl. O_2/g. tissue/hr.)			
	0–30 min.	30–60 min.	60–90 min.	
Residual	1025	618	610	
Residual + arsenite	865	592	514	
Pyruvate	2140	1883	1677	
Pyruvate + arsenite	1363	1218	1065	
(Pyruvate-residual)	1115	1265	1067	
(Pyruvate-residual) arsenite	498	626	551	
				Mean
Inhibition (%)	55	50	48	51

The residual respiration, i.e. in the absence of any added substrate, is only slightly affected, whereas the extra respiration due to the added pyruvate is inhibited to the extent of 51% by 0·017 mM-sodium arsenite.

The inhibition produced by arsenite in this system is a rapid process, since the maximum effect is always obtained in the first half-hour of the experiment, and the degree of inhibition remains relatively constant throughout 3 or 4 succeeding half-hour periods. With brain dispersions the inhibition of pyruvate oxidation is almost maximal in the first 10 min. period.

Table 2 shows the percentage inhibition of the oxidation of pyruvate by the brain system in the presence of varying concentrations of sodium arsenite and of three arsenoxides derived from the corresponding dichloro-compounds.

Table 2. *Percentage inhibition of pyruvate oxidation in brain produced by varying concentrations of trivalent arsenicals*

(A) Brain *brei* (mean percentage inhibition over 1½ hr.)

Arsenical	Concentration of As (mM)						
	0·003	0·008	0·017	0·034	0·05	0·068	0·102
Sodium arsenite	—	—	53	75	—	86	95
Chlorovinylarsenoxide	40	60	69	—	76	—	—
Ethylarsenoxide	—	34	—	—	—	—	—
Phenylarsenoxide	—	64	—	—	—	—	—

(B) Brain dispersion (percentage inhibition in first 30 min.)

Arsenical	Concentration of As (mM)									
	0·001	0·002	0·005	0·006	0·008	0·01	0·02	0·04	0·06	0·12
Sodium arsenite	—	—	—	—	—	26	50	88	93	100
Chlorovinylarsenoxide	—	35	100	—	—	100	100	—	—	—
Ethylarsenoxide	—	—	—	65	—	100	100	—	—	—
Phenylarsenoxide	30	58	—	—	98	100	100	—	—	—

The oxides derived from the three arsenical vesicants lewisite and ethyl- and phenyldichloroarsine, are all strongly inhibitory in low concentrations, being even more potent in their effects than sodium arsenite. The dispersion is more sensitive than brei to these compounds.

The inhibition of oxygen uptake is associated with a diminished removal of pyruvate, and is therefore a genuine effect upon pyruvate oxidation. Table 3 gives data for pyruvate, as estimated by bisulphite-binding capacity (Clift & Cook, 1932).

Table 3. *Effect of sodium arsenite upon the aerobic removal of pyruvate by brain dispersions*

	Concentration of As (mM)					
	0·0	0·01	0·02	0·04	0·06	0·12
Pyruvic acid removed (μg./hr.)	470	450	280	150	140	60
Inhibition (%)	0	4	40	68	70	87
Oxygen uptake (μl.)	110	83	58·5	20·5	10	0
Inhibition (%)	0	25	47	81	91	100

Having confirmed the sensitivity of the pyruvate oxidase system to arsenite and to some of the chemical warfare arsenicals, the selectivity of the arsenicals was further defined by investigating their effect on the oxidation of certain other intermediaries in carbohydrate metabolism.

Succinate oxidation. In view of the conflicting results reported in the literature sodium succinate was chosen first. In agreement with the earlier work of Szent-Györgyi (1930), the oxidation of succinate by pigeon brain was found to be relatively insensitive to the presence of arsenic, amounts of arsenite or lewisite that produce an almost maximum inhibition of pyruvate oxidation showing only a small effect on succinate (Table 4). In each experiment additional bottles were set up containing pyruvate to give a direct comparison of the

Table 4. *Inhibition of succinate oxidation by brain systems in the presence of arsenite and lewisite*

(Pyruvate given for comparison.)

Arsenical	Concentration As (mM)	Inhibition (%) Succinate (0·055 M)	Inhibition (%) Pyruvate (0·02 M)
(A) Brain *brei* (percentage inhibition of extra respiration during 1½ hr.)			
Arsenite	0·034	18	79
	0·034	9	68
	0·068	17	86
	0·102	16	95
	0·34	7	100
Lewisite	0·017	5	89
(B) Brain dispersion (percentage inhibition over 0–30 min.)			
Arsenite	0·04	15	44
	0·04	0	46
	0·04	0	73

Table 5. *Inhibition by arsenicals of α-glycerophosphate oxidation by brain*

Arsenical	Concentration As (mM)	Inhibition (%) α-Glycerophosphate (0·037 M)	Inhibition (%) Pyruvate (0·02 M)
(A) Brain *brei* (percentage inhibition of extra respiration during 1½ hr.)			
Arsenite	0·034	1	89
	0·034	4	75
	0·034	0	76
Lewisite	0·017	6	—
	0·017	0	—
(B) Brain dispersion (percentage inhibition over 0–30 min.)			
Arsenite	0·04	0	40
	0·04	0	46
	0·04	0	73

relative toxicities. From these experiments it may by inferred that cytochrome oxidase is also relatively insensitive, again confirming Szent-Györgyi's conclusions.

α-Glycerophosphate oxidation. Similar experiments have been carried out using sodium α-glycerophosphate (Johnson, 1936), and it will be seen (Table 5) that the oxidation of this compound by brain is also unaffected by amounts of arsenite or lewisite that produce a very substantial effect on pyruvate oxidation.

Lactate oxidation. It was found that low concentrations of arsenic produced a marked inhibition of oxygen uptake by pigeon brain *brei* in the presence of lactate. If pyruvate accumulates as a result of the presence of the arsenical, this result is not surprising, since Green & Brosteaux (1936) have shown that the immediate product of oxidation of lactate (pyruvate) is itself an inhibitor of the lactate dehydrogenase.

The amount of pyruvate present in the various bottles at the end of the respiration period was therefore estimated by the bisulphite-binding method of Clift & Cook (1932), thus giving a measure of the amount of pyruvate that had accumulated or escaped oxidation throughout the respiration period. The results of these experiments are shown in Table 6.

The considerable accumulation of bisulphite-binding substance found in the presence of the arsenical may be assumed from the earlier work of Peters *et al.* (1935) to consist largely of pyruvate (*c.* 85%), defective oxidation of which would account in large measure for the inhibition of oxygen uptake. The same result occurs with the avitaminous pigeon brain *brei*, where there exists a specific fault in pyruvate oxidation. Table 6 records an experiment in which the rather high concentration of arsenite used inhibits the removal of pyruvate catalyzed by aneurin. It also shows that the catatorulin effect of aneurin is abolished by arsenite.

Confirming and extending the earlier results of Green & Brosteaux (1936) with the lactate dehydrogenase of heart muscle, it appears, therefore, that this system in brain is also not appreciably affected by arsenicals in concentrations that produce an almost maximal inhibition of pyruvate oxidation.

Effect of arsenicals on anaerobic systems. From earlier work in this laboratory it is known that the pyruvate oxidase system of brain is less stable than the brain pyruvate dehydrogenase which is only capable of carrying out simultaneous decarboxylation and oxidation to acetic acid (Lipmann, 1937; Long & Peters, 1939). Since less pyruvate is removed anaerobically than aerobically, we next determined which system was more sensitive to arsenite. Using the Thunberg methylene blue technique the effect of arsenite on the pyruvate dehydrogenase was investigated (Table 7), the enzyme preparation being obtained from pigeon brain by the method described by Long & Peters (1939).

A concentration of arsenite (0·12 mM) which produces almost complete inhibition of the total pyruvate oxidase system only produces 30% inhibition of pyruvate dehydrogenase.

In confirmation of this, the rate of removal of pyruvate by a brain dispersion was measured both aerobically and anaerobically. It was found that in 1 hr. 280 μg. of pyruvate were removed anaerobically and 390 μg. aerobically, as judged by estimations of bisulphite-binding capacity; 0·12 mM-arsenite inhibited the anaerobic removal of pyruvate by about 40% and the aerobic removal by about 90%.

Table 6. *Effect of arsenite and lewisite on the oxidation of lactate by pigeon brain* brei

I. *Normal brain*

(A) Total extra O_2 uptake in 2 hr. (μl. O_2/g. tissue) in presence of 0·026 M-lactate as substrate

Exp.	Arsenical	Concentration As (mM)	Lactate	Lactate + As	Difference	Inhibition (%)
1	Arsenite	0·034	1726	487	1239	72
2		0·034	1748	850	898	51
3	Lewisite	0·017	1847	758	1089	59
4		0·017	1686	420	1266	75
5		0·017	1979	643	1336	68

(B) Extra bisulphite-binding substances (as mg. pyruvic acid/g. tissue) accumulating in 2 hr. in presence of 0·026 M-lactate

Exp.	Lactate	Lactate + As	Extra accumulation due to As
1	1·07	3·25	2·18
2	0·40	2·25	1·86
3	0·44	2·09	1·65
4	0·58	1·97	1·39

II. *Aneurin-deficient brain*

Effect of 0·168 mM-arsenite on lactate oxidation and pyruvate accumulation in avitaminous pigeon brain *brei* during a 2 hr. period of respiration

Lactate	Lactate + As	Inhibition (%)	Lactate + aneurin	Lactate + aneurin + As	Inhibition (%)
O_2 uptake (μl./g./2 hr.)					
2884	1300	45	4102	1286	32

Extra pyruvate (as mg. pyruvic acid/g. tissue) accumulating in 2 hr. due to arsenite

No added vitamin	With added vitamin
1·25	2·98

Table 7. *Effect of arsenite on the pyruvate dehydrogenase of brain*

		Concentration Na arsenite (mM)							
	Control	0	0·014	0·02	0·04	0·12	0·18	0·32	0·4	0·8
Time taken to reduce MB (min.)	78	23·5	28	30·5	33	33·5	38	39	44·5	80
Inhibition (%)	—	0	8	13	17	18	27	28	39	100

(In calculating percentage inhibition two methods were used to correct for the residual reduction; each gave substantially the same result.)

The pyruvate dehydrogenase preparation from brain was also tested with other substrates, and the effects of both arsenite and lewisite studied. The percentage inhibitions obtained with these substrates are shown in Table 8. Using this system it will be seen that pyruvate dehydrogenase is more sensitive than any of the other dehydrogenases tested, though with lewisite the triosephosphate dehydrogenase is equally sensitive in brain.

Table 8. *Percentage inhibition of the brain dehydrogenase preparation by arsenite and lewisite in the presence of various substrates*

Concentration As (mM)	Pyruvate	Lactate	Succinate	α-Glycerophosphate	Hexosediphosphate
			(A) Arsenite		
0·24	60	10	0	0	20
0·48	100	15	0	27	39
			(B) Lewisite		
0·006	50	6	0	0	58
0·12	100	46	100	100	100

Using a dehydrogenase preparation made from pigeon breast muscle it was found that 0·24 mM-arsenite produced no inhibition with succinate, α-glycerophosphate, lactate, fumarate, malate or hexosediphosphate, while an acetone-dried rabbit muscle preparation was not inhibited by 0·8 mM-arsenite in the presence of succinate, glucose, hexosediphosphate, malate or fumarate. This relatively enormous concentration also did not affect the triosephosphate dehydrogenase of pig heart muscle, and 1·2 mM produced no inhibition of pig-kidney amino-acid oxidase (using alanine as substrate).

On account of the importance of the triosephosphate dehydrogenase system, two experiments with this enzyme are given in detail (Table 9). The source of the enzyme was an acetone-dried rabbit muscle powder prepared in collaboration with Dr L. A. Stocken. The activity of the preparation was determined by estimating the CO_2 produced from bicarbonate solution resulting from the catalyzed coenzyme-linked reaction occurring between triosephosphate and pyruvate (Green, Needham & Dixon, 1937). Sodium hexosediphosphate, freshly prepared from the Ca salt by Dr Stocken, was added as a source of triosephosphate which is formed from hexosediphosphate by the zymohexase present in the muscle extract (Meyerhof & Lohmann, 1934).

Using this unpurified preparation, relatively large amounts of lewisite (20 times the amount required to produce a marked effect on the unpurified pyruvate oxidase system) cause no inhibition of triosephosphate dehydrogenase. With pigeon breast muscle preparation, 0·006 mM-lewisite gave no inhibition of lactate, succinate, α-glycerophosphate or triosephosphate dehydrogenases, and actually stimulated the malate dehydrogenase, as was also found by Green (1936) using 30 mM-arsenite.

Lewisite oxide (0·48 mM) had no effect on fumarate, and caused only 20% inhibition of succinate, 0·95 mM producing 35% inhibition of the latter. In general, these other dehydrogenases were very resistant to the arsenicals tried, though succinodehydrogenase was poisoned by high concentrations.

We have seen that the complete pyruvate oxidase system is particularly sensitive to the action of arsenite, and the dehydrogenase, or anaerobic phase, less so. It was probable, therefore, that yeast

Table 9. *Effect of lewisite on triosephosphate dehydrogenase*

(1 ml. enzyme (5% muscle powder), 0·5 ml. 0·3% cozymase, 0·3 ml. 0·05M-hexosediphosphate and 0·1 ml. M-pyruvate. Hexosediphosphate (HDP) tipped in from side-bulbs after temperature equilibration.)

	Concentration As (mM)	μl. CO_2 evolved in 20 min. Exp. 1	Exp. 2
Enz. + cozymase + pyruvate	—	23	42
,, ,, ,, + HDP	—	148	110
,, ,, ,, ,, + lewisite	0·03	161	—
,, ,, ,, ,, ,,	0·3	—	177

carboxylase would also be less sensitive to arsenite since there is no oxidative phase. Experiments were therefore carried out anaerobically at 28° using alkaline-washed yeast as the apo-enzyme and excess vitamin B_1, following the technique of Ochoa & Peters (1938). Cocarboxylase was added as coenzyme. It was found that a concentration of arsenite (0·12 mM) that completely inhibited the oxidation of pyruvate by pigeon brain had no effect at all on yeast carboxylase, and the same result was obtained with 0·02 mM-lewisite and ethylarsenoxide.

Relation of arsenicals to coenzymes. Banga *et al.* (1939) proved that in addition to cocarboxylase, an adenine nucleotide and cozymase were necessary for the pyruvate oxidase system. Table 10 shows that, using the brain dispersion, an excess of cocarboxylase, up to 100 times the necessary amount, has no restorative action on the inhibition.

Table 10. *Effect of excess cocarboxylase upon the inhibition of pyruvate oxidase by arsenite or lewisite*

	Concentration arsenical (mM)	Concentration cocarboxylase (μg./2·5 ml.)	Percentage inhibition of pyruvate oxidation: Without cocarboxylase		Percentage inhibition of pyruvate oxidation: With cocarboxylase	
Arsenite	0·17	40	100		100	
	0·02	40	86	Mean = 60 %	86	Mean = 54 %
	0·02	50	46		28	
	0·02	50	55		52	
	0·02	18	63		42	
	0·02	—	50		64	
Lewisite	0·014	4	100		100	
	0·010	50	100		100	
	0·001	18	50		47	

It was shown in Table 6 that aneurin does not restore the inhibition. Further, Table 11 shows that adenylic acid is unaffected and that the part of the oxidase system catalyzed by an adenine nucleotide is sensitive to lewisite oxide. Since it is already known that the malate and lactate dehydrogenases for which cozymase is necessary are insensitive to arsenic, it can be concluded that the point of attack lies in the protein part of the enzyme.

Table 11. *Effect of adenylic acid upon the rate of pyruvate respiration in pigeon brain dispersion dialyzed for 2 hr. at 2°*

(Pyruvate, 11 mM; lewisite, 0·04 mM; adenylic acid, 0·28 mM)

O_2 uptake μl./g./hr.			
Pyruvate	Pyruvate + adenylic acid	Pyruvate + lewisite	Pyruvate + adenylic acid + lewisite
699	2212	177	197

The blood pyruvate level in arsenic intoxication. In the failure of pyruvate oxidation that occurs in animals fed on a vitamin B_1-deficient diet it has been shown (Thompson & Johnson, 1935) that in the terminal stages of the deficiency, when the animals are showing signs of opisthotonus, there is a marked elevation in the bisulphite-binding capacity of the blood due to an accumulation of pyruvate. To determine whether the effect of arsenic on the pyruvate oxidase system is also an *in vivo* action leading to a demonstrable metabolic disorder in the whole animal, the bisulphite-binding capacity and pyruvate level of normal animals have been compared with those of animals at varying stages after the administration of arsenic.

The animals were beheaded and the blood taken directly into 25 % (w/v) trichloroacetic acid. Total bisulphite-binding substances were then estimated in the protein-free filtrates by the method of Clift & Cook (1932), and in some experiments the pyruvate level determined by the method of Lu (1939). In the experiments with pigeons, the arsenic was administered intramuscularly in the form of a solution of sodium arsenite (pH 7·3). In the 'acute' experiments the birds received one dose of 8 mg. As_2O_3/kg.; in the 'chronic' experiments the daily dosage was varied according to the condition of the birds.

The results of these blood estimations are given in Table 12. Both the bisulphite-binding capacity and the blood pyruvate level itself are raised by poisoning with arsenite, this increase showing itself

Table 12. *Effect of injected arsenite on the blood-pyruvate level of pigeons*

Normal	Acute	Chronic	Total dose (mg. As_2O_3)
(A) Bisulphite-binding capacity (as mg. pyruvic acid/100 g. blood)			
(Duration of intoxication given in brackets.)			
4·17	7·32 (2 hr.)	20·52 (13 days)	35·5
3·76	8·85 (2 hr.)	9·60 (15 days)	35
3·90	8·33 (3 hr.)	24·98 (1 day)	8
4·03	10·32 (3 hr.)	21·29 (2 days)	10
3·33		21·87 (2 days)	11
3·36		18·66 (4 days)	8·5
5·24		14·99 (4 days)	8·5
5·26		17·10 (43 days)	7
4·06			
Mean 4·01	8·81	18·63	
(B) Pyruvate, determined by 2:4-dinitrophenylhydrazone method			
(mg. pyruvic acid/100 g. blood)			
1·28	4·02 (2 hr.)	12·39 (4 days)	8·5
0·94	3·00 (2 hr.)	14·15 (4 days)	8·5
0·94	3·12 (2 hr.)	13·85 (43 days)	7
	6·30 (3 hr.)		
Mean 1·06	4·11	13·47	

as early as 2 hr. after injection of the arsenic. Earlier workers (Johnson & Edwards, 1937; Lu & Platt, 1939) have shown that exercise raises the blood pyruvate level of normal animals, but as the poisoned birds invariably remained resting in their cages, usually in the sitting position, for at least 1 hr. before killing, exercise can safely be excluded as a contributory cause of the findings.

That a similar state of affairs exists in mice and rats after poisoning with arsenite is shown in Table 13.

Table 13. *Blood pyruvate (mg./100 g. blood) of mice and rats poisoned with a single intraperitoneal injection of* As_2O_3

	Mice				Rats	
	Blood pyruvate (mg./100 g.) for As_2O_3 dosage (in μg./g.) of				Blood pyruvate (mg./100 g.) for As_2O_3 dosage (in μg./g.) of	
	0	8	12	15	0	7
Duration of poisoning (hr.)	1·23	—	—	—	1·18	—
	1·23	—	—	—	—	—
	1·38	—	—	—	—	—
1	—	—	—	—	—	2·44*
1½	—	—	—	8·1	—	—
1¾	—	—	2·88	—	—	—
2	—	—	2·32	—	—	1·21
2	—	—	6·00	—	—	—
3½	—	2·12	—	—	—	2·28
4½	—	2·16	—	—	—	—
5	—	1·66	—	—	—	—
5	—	2·33	—	—	—	—
5	—	2·70	—	—	—	—
5	—	3·00	—	—	—	—
7	—	4·45	—	—	—	—
11	—	—	—	—	—	1·75
22½	—	6·55	—	—	—	3·37

* This animal received 10% (w/v) lewisite in ethanol on the shaved skin instead of the As_2O_3.

It will be noted that one experiment is included in Table 13 in which the rat was contaminated with lewisite. This result was confirmed by carrying out bisulphite-binding titrations (Table 14) on the blood of three normal rats and two that had received $4 \times LD_{50}$ of lewisite on the clipped skin of the back.

Table 14. *Bisulphite-binding capacity of blood of lewisite-poisoned rats*

(Duration of intoxication given in brackets.)

	Pyruvic acid (mg./100 g. blood)	
Rat no.	Normal	Poisoned
1	4·52	—
2	6·61	—
3	5·84	—
4	—	7·40 (½ hr.)
5	—	12·93 (1 hr.)

DISCUSSION

The accumulated evidence shows that, among the enzyme systems studied, the pyruvate oxidase system is specially sensitive to poisoning by arsenicals. Though isolated enzymes were studied, the emphasis in this work is laid upon results with unpurified enzyme systems approximating more to whole cell preparations. In this way it was thought that the differential affinity of the different possible 'arsenic acceptors' within the cells could be better determined.

That inactivation of the pyruvate oxidase system is one of the outstanding biochemical effects brought about *in vivo* by arsenicals is supported by two lines of reasoning:

(1) the increase in the blood pyruvate level in poisoned animals;

(2) the similarity between the clinical manifestations of arsenical neuritis and the neuritis accompanying vitamin B_1 deficiency (Sinclair, 1939).

On present evidence the pyruvate oxidation system is found to be outstandingly sensitive, both *in vivo* and *in vitro*, among the enzymes tested with low concentrations of arsenite or lewisite. Hence, we postulate that inactivation of this system is responsible for the primary effect in the lewisite lesion; with higher concentrations there is evidence that other enzymes are attacked, but from the work reported here it was logical to use the pyruvate oxidase system as a test system for arsenical poisoning, a view which subsequent work thoroughly substantiated.

With regard to the application of these results to the original problem of vesication, they provide grounds for the first time for a belief in inactivation of an enzyme as a first step in cell damage, and so support the 'enzyme theory' as one mode at least of inflicting damage in skin. The application of these results to skin itself will be discussed elsewhere (Thompson, 1946). The increased capillary permeability which is the result of damage by lewisite (Cameron, Short & Calder, 1941), may be due either to direct action upon some cell surface, to liberation of capillary-dilating substances from skin cells, or to interference with the metabolism of the capillary endothelial cells; both of these two latter effects could be initiated by enzyme damage; the actual formation of the vesicle clearly involves other stages.

Our results on the pyruvate system have been substantiated and considerably extended by parallel work carried out in U.S.A. by Barron and his colleague (cf. Barron & Singer, 1945). In addition to unreported observations (R. A. P.) much of the work described above was contained in reports submitted earlier to the Ministry of Supply (Sinclair & Thompson, 1940; Sinclair, 1940).

SUMMARY

1. The sensitivity to trivalent arsenicals of several respiratory enzyme systems related to carbohydrate metabolism has been investigated in relation to the 'enzyme theory' of vesication.

2. Of the enzymes studied the pyruvate oxidase system has proved the most sensitive.

3. The pyruvate dehydrogenase is more sensitive than the other dehydrogenases tested, but less so than the whole oxidase system of brain.

4. Lewisite has in general proved more toxic than arsenite.

5. None of the coenzymes at present known to be concerned with the pyruvate oxidase system is sensitive; we therefore conclude that the point of attack is on a protein component of the system.

6. Finally, *in vivo*, the blood pyruvate level is significantly raised in animals poisoned with arsenite or lewisite.

7. It is concluded that solid grounds have been obtained for the belief in an enzyme theory of vesication, and that the first point of attack of arsenicals is on pyruvate oxidation.

This work was initiated at the outbreak of the war for the Ministry of Supply. Our thanks are due to the Chief Scientific Officer, Ministry of Supply, for permission to publish. We are also grateful to Messrs R. W. Wakelin, L. C. Waters, and C. Dear for technical assistance.

REFERENCES

Banga, I., Ochoa, S. & Peters, R. A. (1939). *Biochem. J.* **33**, 1109.

Banga, I., Schneider, L. & Szent-Györgyi, A. (1931). *Biochem. Z.* **240**, 462.

Barron, E. G. & Singer, T. P. (1945). *J. biol. Chem.* **157**, 221.

Cameron, G. R., Short, R. D. H. & Calder, R. M. (1941). Porton Report.

Clift, F. P. & Cook, R. P. (1932). *Biochem. J.* **26**, 1788.

Crasnaru, L. & Gavrilescu, N. (1935). *C.R. Soc. Biol., Paris*, **120**, 226.

Cushny, A. R. (1924). *Pharmacology and Therapeutics*, 8th ed. London.

Das, N. B. (1937). *Biochem. J.* **31**, 1116.

Dresel, K. (1926). *Biochem. Z.* **178**, 70.

Dresel, K. (1928). *Biochem. Z.* **192**, 351.

Flury, F. (1921). *Z. ges. exp. Med.* **13**, 523.

Green, D. E. (1936). *Biochem. J.* **30**, 2095.

Green, D. E. & Brosteaux, J. (1936). *Biochem. J.* **30**, 1489.

Green, D. E., Needham, J. & Dixon, M. (1937). *Biochem. J.* **31**, 2327.

Johnson, R. E. (1936). *Biochem. J.* **30**, 30.

Johnson, R. E. & Edwards, H. T. (1937). *J. biol. Chem.* **118**, 427.

Jowett, M. & Quastel, J. H. (1935). *Biochem. J.* **29**, 2159.

Krebs, H. A. (1933). *Hoppe-Seyl. Z.* **217**, 191.

Krebs, H. A. & Johnson, W. A. (1937). *Enzymologia*, **4**, 148.

Lipmann, F. (1937). *Skand. Arch. Physiol.* **76**, 255.

Long, C. (1945). *Biochem. J.* **39**, 143.

Long, C. & Peters, R. A. (1939). *Biochem. J.* **33**, 759.

Lu, G. D. (1939). *Biochem. J.* **33**, 249.

Lu, G. D. & Platt, B. S. (1939). *Biochem. J.* **33**, 1538.

McCleland, N. & Peters, R. A. (1919). *J. Physiol.* **53**, xxiv.

Meyerhof, O. & Lohmann, K. (1934). *Biochem. Z.* **271**, 89.

Ochoa, S. (1941). *J. biol. Chem.* **138**, 751.

Ochoa, S. & Peters, R. A. (1938). *Biochem. J.* **32**, 1501.

Oelkers, H. A. (1937). *Arch. exp. Path. Pharmak.* **184**, 276.

Onaka, M. (1911). *Hoppe-Seyl. Z.* **70**, 433.

Peters, R. A. (1936*a*). *Nature, Lond.*, **138**, 327.

Peters, R. A. (1936*b*). *Curr. Sci.* **5**, 207.

Peters, R. A., Rydin, H. & Thompson, R. H. S. (1935). *Biochem. J.* **29**, 63.

Peters, R. A., Stocken, L. A. & Thompson, R. H. S. (1945). *Nature, Lond.*, **156**, 616.

Peters, R. A. & Thompson, R. H. S. (1934). *Biochem. J.* **28**, 916.

Peters, R. A. & Wakelin, R. (1946). *Biochem. J.* **40**, 513.

Peters, R. A. & Walker, E. (1923–5). Government Reports (unpublished).

Quastel, J. H. & Wheatley, A. H. M. (1935). *Biochem. J.* **29**, 2773.

Rona, P., Airila, Y. & Lasnitski, A. (1922). *Biochem. Z.* **130**, 582.

Rona, P. & György, P. (1920). *Biochem. Z.* **111**, 115.

Sinclair, H. M. (1939). *Proc. IIIrd Cong. Neur. internat. Copenhagen*, p. 890.

Sinclair, H. M. (1940). Report to Ministry of Supply by Peters, no. 27.

Sinclair, H. M. & Thompson, R. H. S. (1940). Report to Ministry of Supply by Peters, no. 4.

Stocken, L. A. & Thompson, R. H. S. (1941). Report to Ministry of Supply by Peters, no. 33.

Stocken, L. A. & Thompson, R. H. S. (1946). *Biochem. J.* **40**, 535.

Szent-Györgyi, A. (1930). *Biochem. J.* **24**, 1723.

Thompson, R. H. S. (1946). *Biochem. J.* **40**, 525.

Thompson, R. H. S. & Johnson, R. E. (1935). *Biochem. J.* **29**, 694.

Voegtlin, C., Rosenthal, S. M. & Johnson, J. M. (1931). *U.S. Publ. Hlth Rep.* **46**, 339.

Walker, E. (1928). *Biochem. J.* **22**, 292.

Warburg, O. (1925). *Science*, **61**, 576.