2. In the case of benzoic acid, 2:5-dinitrobenzoic acid appears to be mainly responsible for the development of the red colour on reduction. The preparation of this substance by nitration of o-nitrobenzoic acid is described.

3. The colour formed with phenylacetic acid is considered to be due to a mixture of the 2:5- and 3:4-dinitro compounds.

4. The violet colour given by nitration, followed by alkaline reduction, of phenylalanine appears also to be due to a mixture of products, which probably include 2:5- and 3:4-dinitro derivatives of phenyl-

alanine. The main nitration product was shown by analysis to be a non-chromogenic dinitrophenyl-alanine.

5. All of the above chromogens are relatively minor products of nitration, and as such do not appear to provide a really satisfactory basis for quantitative estimation of these aromatic substances.

We wish to thank Dr H. H. Hodgson for his kindness in supplying samples of 2:5- and 2:3-dinitrotoluene and Dr O. L. Brady for samples of 2:6- and 3:4-dinitrotoluene, from which the corresponding dinitrobenzoic acids were prepared. All analyses were made by Drs Weiler and Strauss.

REFERENCES

- Block, R. J. (1938). *The Determination of the Amino-acids*, p. 75. Minneapolis: Burgess Publishing Co.
 Block, R. J. & Bolling, D. (1939). *J. biol. Chem.* **129**, 1.
 Borsche, W. (1909). *Ber. dtsh. chem. Ges.* **42**, 1313.
 Dickens, F. & Pearson, J. (1951). *Biochem. J.* **48**, 216.
 Gilman, H. (1932). *Organic Synth.* Collective Vol. 2, p. 385.
 Griess, P. (1874). *Ber. dtsh. chem. Ges.* **7**, 1225.
 Grossfeld, G. (1927). *Z. Untersuch. Nahr.- u. Genussm.* **53**, 467.
 Kapeller-Adler, R. (1932). *Biochem. Z.* **252**, 185.
 Meisenheimer, J. (1903). *Ber. dtsh. chem. Ges.* **36**, 4174.
 Meisenheimer, J. & Patzig, E. (1906). *Ber. dtsh. chem. Ges.* **39**, 2526.
 Mohler, M. E. (1890). *Bull. Soc. Chim., Paris*, **3**, 414.
 Sidgwick, N. V. (1942). (Revised by Taylor, T. W. J. & Baker, W.) *The Organic Chemistry of Nitrogen*. Oxford: Clarendon Press.
 Waelsch, H. & Klepetar, G. (1935). *Hoppe-Seyl. Z.* **236**, 92.

The Action of Sulphydryl Inhibitors upon *iso*Citric Dehydrogenase with Especial Reference to the Behaviour of Some Trivalent Arsenicals*

BY W. D. LOTSPEICH AND R. A. PETERS
Department of Biochemistry, University of Oxford

(Received 22 February 1951)

The —SH nature of *isocitric* dehydrogenase is still in some doubt because Adler, Euler, Günther & Plass (1939) stated that the enzyme from pig heart was sensitive to iodoacetic acid, whereas Barron & Singer (1945) said that the enzyme from liver was not affected by —SH reagents. It seemed important to examine the effect of arsenical substances on this enzyme in relation to other arsenical studies in this laboratory (Peters, Sinclair & Thompson, 1946). This paper records some observations of the effect of several —SH and other inhibitors upon the enzymes prepared from pig heart and from kidney tissue. A new difference between the action of mono- or di-substituted arsenical compounds has been found, which is thought to be of theoretical interest. Observations upon this enzyme are also related to the recent work in this laboratory (Buffa, Lotspeich,

Peters & Wakelin, 1950), in which fluoroacetate was found to inhibit the tricarboxylic acid cycle at the citrate stage.

EXPERIMENTAL

*iso*Citric dehydrogenase. The preparation used in most experiments was a dialysed phosphate extract of acetone dried powder of pig heart. In making the acetone powder of pig heart certain modifications of previous methods (Straub, 1942; Ochoa & Weisz-Tabori, 1948) shortened the procedure and increased the enzymic activity of the resulting phosphate extract; the procedure was as follows. A pig heart, obtained as soon as possible after the death of the animal, was immediately freed of fat and gross connective tissue, and cut into pieces which were placed in ice for transport back to the laboratory. All further operations were carried out in the cold room. The pieces of chilled heart muscle were placed in a Waring blender with three times their volume of acetone which had been previously chilled to -10° . After blending for 30 sec. the pulp was pressed out through muslin. These procedures were repeated twice more. The resulting heart

* A preliminary account of these experiments was given to the XVIIIth International Congress of Physiology, Copenhagen, in August, 1950 (Lotspeich & Peters, 1950).

pulp was then spread out on a large square of filter paper and allowed to dry in the air, after which it was placed in a vacuum desiccator over CaCl_2 until absolutely dry. The fibrous connective tissue was next separated from the fine powder by shaking through muslin. The very fine dry powder which resulted from this sifting process was then used as the starting material for preparation of the phosphate extract, made according to Ochoa (1948), i.e. 1 g. of the powder was extracted successively with 10 ml. and 2 ml. of 0.1 M-phosphate buffer, pH 7.2, at room temperature. After centrifuging, the supernatant fluid was dialysed against running tap water for 6 hr. The precipitate which formed during dialysis was removed by centrifugation. The dialysate was much more active than that described by Ochoa (1948) and had to be diluted tenfold for use; 0.05 ml. of this dilution was used for each test. In some later experiments a similar preparation was made from ox kidney, which had about one-quarter the activity of the heart preparation.

Coenzyme II (triphosphopyridine nucleotide). This was prepared from ox liver by a modification of the method of Warburg, Christian & Griese (1935), the details of which were kindly supplied by Dr S. Ochoa. As used the purity was approx. 10% as checked with a sample of Co II of known purity kindly sent by Dr Ochoa.

DL-isocitric acid was prepared in this laboratory by Mr R. W. Wakelin, following the directions of Krebs & Eggleston (1944). The DL form was used in all experiments described; the tables show the calculated amount of D-isocitrate present.

Measurement of isocitric dehydrogenase activity. The details given by Ochoa (1948) were followed; the activity of the isocitric dehydrogenase was measured by the rate of reduction of Co II as observed in the Beckman spectrophotometer at 340 m μ . at room temp. (14.5–18°). In all cases the reaction was started by adding isocitrate to the mixture already prepared in the Beckman glass cell, and followed for 10–20 min. Unless specially mentioned all experiments were carried out in Sørensen's 0.05 M-phosphate buffer, pH 7.2, made up from mixtures of KH_2PO_4 and Na_2HPO_4 ; the final total volume was 3 ml. The amount of Co II preparation used was equivalent to 40 μg . of pure Co II. Using 0.05 ml. of enzyme/3.0 ml., 0.1 ml. 0.2% $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.99 μmol .) was required to produce maximum activity. The optimum pH was found to be between 6.5 and 7.0, confirming Adler *et al.* (1939).

RESULTS

Effect of phosphate and pyrophosphate

Adler *et al.* (1939) stated that inorganic phosphate inhibited the action of the enzyme, and mentioned that it might precipitate the manganese. Fig. 1 shows the effect at pH 7.2 of increasing phosphate concentrations in the presence of a constant amount of Mn. The inhibitory effect produced by phosphate did not appear until approx. 0.1 M-phosphate was reached. Addition of extra manganese removed this inhibition. Grafflin & Ochoa (1950), working at pH 7.4, recommended that phosphate concentrations higher than 0.3 mM should not be used, because of precipitation of manganese phosphate. Evidently, at pH 7.2, the precipitation is much reduced. The inhibitory effect of phosphate, when it arises, is

completely explained by its removal of manganese. The inhibition produced by pyrophosphate (Adler *et al.* 1939) was marked at pH 7.2, 3.33 mM producing inhibition with the manganese concentration used; it was confirmed that the addition of more Mn^{++} relieved this inhibition as well.

Fig. 1. Effect of inorganic phosphate on isocitric dehydrogenase at pH 7.2. Each cell contained phosphate buffer, pH 7.2, of final concentration indicated on the graph; MnCl_2 1.01 μmol .; Co II approx. 0.0819 μmol .; D-isocitrate 0.104 μmol .; enzyme extract diluted 1:10, 0.05 ml.; water to final volume of 3 ml.

Relation of Mn^{++} to activity of isocitric dehydrogenase

On the basis of the observation that the first step in the oxidation of isocitrate (eqn. 1) proceeds without added manganese, Ochoa & Weisz-Tabori (1948) considered that manganese was not necessary for this stage, but was needed for the second step, namely decarboxylation of oxalosuccinate (eqn. 2).

This conclusion would be justified only if the experimental system were completely free of manganese. Accordingly all reagents used in the experiments were tested for manganese. It was found that there were appreciable quantities of manganese in the enzyme used, employing Feigl's (1947) method which uses periodate and 'tetrabase', whereas the Co II (both the specimen used and that from Dr Ochoa) and the isocitrate showed none. Since manganese was present, there was no evidence that it was not necessary for the dehydrogenase stage; in fact, it seemed to be essential, because, using the diluted enzyme, practically no activity was found unless manganese was added, whereas with the undiluted enzyme (as used by Ochoa, 1948) there was an appreciable activity.

Effect of substrates of the tricarboxylic acid cycle

DL-isoCitric acid. In confirmation of previous work, the enzyme was saturated at 0.02 mM using the amount of CoII stated.

α -Ketoglutarate was slightly inhibitory if present in a concentration of 2 mM acting for 20 min.

Oxalosuccinate and oxaloacetate showed inhibition presumably attributable to the carbon dioxide formed during their spontaneous decarboxylation; all other substrates of the tricarboxylic cycle had no inhibitory effects when tested at a concentration of 1 mg./ml. (approx. 0.2 mM).

Inhibitors

Non-arsenical substances. Table 1 summarizes the results for various —SH inhibitors, including certain metallic compounds other than the arsenicals, which are considered below. The results show that the

enzyme is only sensitive to *o*-iodosobenzoate, copper salts and mercury compounds; the inhibition produced by the two metallic compounds was reversible by British anti-lewisite (BAL) and glutathione (Fig. 2). With recrystallized iodoacetic acid no inhibitions were found in concentrations as high as 10^{-3} M; the inhibitory effect observed by Adler *et al.* (1939) was therefore not confirmed. Initially we found that a discoloured preparation of this acid may cause inhibition which disappeared on recrystallization. A typical lachrymator (chloroacetophenone) had no action.

Arsenicals. The experiments with arsenical compounds have given such unexpected results that a short account of the types of the trivalent compounds used is required. In all cases the arsenicals were allowed to act for 15 min. at room temperature before addition of the *isocitrate*. The very insoluble disubstituted compounds were dissolved in 95 % ethanol;

Table 1. *Effect of inhibitors upon isocitric dehydrogenase*

(In all cases the inhibitor was allowed to act on the enzyme for 15 min. at room temperature before addition of *isocitrate*. Temperature of test, 15°.)

Inhibitor	Concentration (mM)	Time (min.)	Inhibition (%)
Non-arsenical inhibitors			
Iodine compounds:			
Iodoacetate	1.08	20	0
Iodoacetamide	0.54	20	0
Iodosobenzoate	0.40	20	16
	0.81	20	50
	1.21	20	68
Metallic compounds:			
CuSO ₄	0.026*	10	0
	0.052	10	50
	0.52†	10	100
<i>p</i> -Chloro-mercuribenzoate	0.02	20	88
<i>p</i> -Chloro-mercuribenzoate + BAL	0.02	20	0
<i>p</i> -Chloro-mercuribenzoate + GSH	0.02	20	21
	2.0	20	100
<i>p</i> -Chloro-mercuribenzoate	0.04	20	100
	0.002	20	19
	0.004	20	77
Phenylmercuric nitrate	0.04	20	100
Other SH inhibitors:			
Sodium maleate	2.08	15	0
Chloroacetophenone	0.65	10	0
CH ₃ N(C ₂ H ₅ Cl) ₂	1.28	10	0
(C ₂ H ₅ Cl) ₃ N	2.85	10	0
Alloxan	4.0	10	0
Other substances:			
<i>p</i> -Aminobenzoate	1.2	20	0
Sodium fluoroacetate	1.66	20	0
Arsenical inhibitors			
Disubstituted arsenicals:			
Diphenylchloroarsine	0.031‡	20	31
	0.063‡	20	100
Other arsenicals:			
Arsenite	1.01	20	0
Lewisite oxide	0.655	20	0

* Rate slowed during first 5 min.

† Effects of Cu were reversed by BAL and GSH (Fig. 2).

‡ For effect of SH compounds, see Fig. 3.

0.1 ml. of this was added to 2.9 ml. of test solution. Controls showed that the amount of ethanol used had no action on the enzyme. Arsenite and the mono-substituted (primary) compound lewisite oxide were studied, and the following disubstituted (secondary) compounds: diphenylchloroarsine (DA), and four dihydrophenarsazines, namely 10-chloro-2-methyl-, 2:8-dichloro-, 10-chloro- and 10-bromo-5:10-dihydrophenarsazines.

Fig. 2. Inhibition of isocitric dehydrogenase by copper and reversal by GSH and BAL. Each cell contained 0.3 ml. 0.1 M-phosphate buffer, pH 7.2; $MnCl_2$, 1.01 μ mol.; Co II approximately 0.0819 μ mol.; D-isocitrate 0.104 μ mol.; enzyme extract 0.1 ml. of 1:10 dilution; water to 3 ml. $CuSO_4$ (final concentration 0.052 mM) was allowed to stand with enzyme and other reactants for 15 min. before addition of GSH or BAL and isocitrate to start the reaction. Curve A, control; curve B, copper 0.05 mM, BAL 0.5 mM; curve C, copper 0.05 mM, GSH 0.5 mM; curve D, copper 0.05 mM.

Contrary to the results with the pyruvate oxidase system which is markedly sensitive to 0.017 mM-lewisite and 0.03 mM-arsenite, the isocitric dehydrogenase from pig heart (Table 1) was insensitive to these compounds in a range of concentrations of 0.6–1 mM; it is to be noted that 0.1 mM-As would affect even relatively insensitive enzymes such as succino-dehydrogenase. Any thiol groups present apparently cannot form a stable >As— ring. The preparation from ox kidney was equally insensitive to arsenite, but was found to be slightly inhibited by lewisite oxide at 0.65 mM. This inhibition appeared to differ in some way because it was irreversible and the activity could not be restored by adding BAL. It is

difficult to be certain that, when tested at this strength, traces of lewisite II ($ClCH=CH$) $_2$ AsCl were not concerned; these might be present as an impurity.

Fig. 3. Inhibition of isocitric dehydrogenase by diphenylchloroarsine and reversal by monothiols. All cells contained 0.3 ml. 0.1 M-phosphate buffer, pH 7.2; $MnCl_2$, 1.01 μ mol.; Co II approximately 0.0819 μ mol.; D-isocitrate, 0.104 μ mol.; enzyme extract, 0.1 ml. 1:10 dilution; 95% ethanol 0.1 ml.; water to 3 ml. Experimental cells contained diphenylchloroarsine (in 0.1 ml. 95% ethanol) and freshly prepared monothiol to give stated concentrations. Inhibitor stood with enzyme for 15 min. in all experiments. This was also the case before adding monothiol. MTEG=monothioethylene glycol. Curve A, control; curve B, DA 0.063 mM, GSH 1.3 mM; curve C, DA 0.063 mM, MTEG 0.63 mM; curve D, DA 0.063 mM.

In strong contrast to the results with the mono-substituted arsenicals, the disubstituted were as toxic as the mercury and copper compounds.

Diphenylchloroarsine

Phenarsazine

Suitable concentrations of BAL and of the monothiol compounds, monothioethylene glycol and glutathione, reversed the inhibition (Fig. 3). The interest of this new fact, namely, that there exist —SH groups in one enzyme at least, which can form a stable monothioarsinite, will be discussed below.

This exceptional behaviour of diphenylchloroarsine was explored further, using some other aromatic disubstituted arsenicals, derivatives of phenarsazine.

Fig. 4. *iso*Citric dehydrogenase from ox kidney; effect of substituted 5:10-dihydrophenarsazines and comparison with effect of diphenylchloroarsine. Final concentration of arsenicals 0.06 mM. Each cell contained 0.3 ml. 0.1M-phosphate buffer, pH 7.2; $MnCl_2$, 1.01 μ mol.; Co Π approx. 0.0819 μ mol.; D-isocitrate 0.109 μ mol., undiluted kidney phosphate extract 0.1 ml.; water to 3 ml. Control cell contained 0.1 ml. 95% ethanol. Experimental cells contained 0.1 ml. 95% ethanol containing inhibitor in sufficient quantity to give final concentration of 0.06 mM. In all cases inhibitor stood at room temperature with reactants for 15 min. before starting reaction with D-isocitrate. Curve A, control; curve B, 10-chloro-2-methyl-5:10-dihydrophenarsazine; curve C, 2:8-dichloro-5:10-dihydrophenarsazine; curve D, 10-chloro-5:10-dihydrophenarsazine; curve E, 10-bromo-5:10-dihydrophenarsazine; curve F, diphenylchloroarsine.

Fig. 4 shows that these were all toxic though less so than diphenylchloroarsine.

DISCUSSION

The —SH problem. Upon the generally accepted assumption that only —SH groups will respond to these inhibitors, the —SH nature of the *isocitric* dehydrogenase appears to be established by the sensitivity to *o*-iodosobenzoate, to mercury and copper compounds, and to at least one class of arsenicals. The behaviour of the —SH enzymes to the various inhibitors is known to be somewhat capricious, in that the order of activity of inhibitors for a given enzyme is often quite different from the

order for another enzyme. This suggests different reactivities of the related —SH groups. It is significant that the —SH group concerned in these *isocitric* dehydrogenase preparations must differ from most of those known at present; considering the order of sensitivity to —SH reagents, it is different in behaviour from those inhibited by lachrymators (Dixon, 1948; Bacq & Desreux, 1946), from myosin (Bailey & Perry, 1947), from triosephosphate dehydrogenase (Dixon, 1937), from succinodehydrogenase (Hopkins & Morgan, 1938) and from the lewisite-sensitive components of the pyruvate oxidase system from brain (Peters *et al.* 1946), which includes the tricarboxylic acid cycle (Peters, 1948), at any rate in part. It is possible that the differences found here between the heart and kidney *isocitric* dehydrogenases might disappear upon further purification.

Relation to the toxicity of arsenical compounds. The work upon lewisite and BAL has cleared up one of the major problems in regard to the biochemical attack made by arsenicals. It has left unsolved one, which is of at least 30 years' standing. McClelland & Peters (1919) found that the toxicity to ciliates of disubstituted trivalent arsenicals was 5–10 times greater than that of the monosubstituted compound; e.g. dimethylchloroarsine > methylchloroarsine, and diphenylchloroarsine (DA) > phenyl-dichloroarsine; also diphenylarsine oxide was found to be very toxic to urease by Rona & György (1920). The preliminary observations on Protozoa were placed upon an accurate basis for the ciliates, *Colpidium colpoda* and *Glaucoma scintillans*, by Walker (1928) in this laboratory, who also showed a temporary reversal of the toxicity to ciliates of DA by monothioethylene glycol (MTEG) ($HS.CH_2CH_2.OH$). It was also early shown that diphenylchloroarsine abolished the nitroprusside reaction for 'fixed' —SH, as well as that given by —SH glutathione; in this respect its action is in line with that of the monosubstituted trivalent arsenicals, as well as arsenite.

In the more recent research in this laboratory unpublished experiments (by Peters & Wakelin) have shown that whereas 0.017 mM-lewisite oxide gave a 50% poisoning of the pyruvate oxidase system from brain under our conditions, DA was without effect in concentrations which it was practicable to try (the substance is relatively insoluble in aqueous solutions). Since this trivalent arsenical is disubstituted it could only form a monothioarsinite and hence could not make a 'dithiol' attack. The question therefore arose as to whether there could exist monothioarsinites formed with DA sufficiently stable to account for the toxicity to ciliates (as also to the irritation caused to the sensitive tissue in the tracheal system in man). If this were true then the non-toxicity to the pyruvate oxidase system could be due to removal of DA by

combination with some other monothiol substances. In pursuance of the idea that thioarsinites with diphenylchloroarsine might be relatively stable owing to the two benzene rings, Peters & Stocken in one unpublished experiment found that the thioarsinite formed by mixing diphenylchloroarsine and a monothiol was relatively stable to the porphyrindin in the test used by Stocken & Thompson (1946). The suggestion, however, lacked biochemical support; the experiments recorded in this paper have now provided this, and have also shown that glutathione can act as an antidote, presumably by forming a more stable monothioarsinite than that of the enzyme-diphenylchloroarsine compound. This leads to the speculation that the animal may be protected systemically against the toxicity of diphenylchloroarsine by the circulating glutathione, in which case compounds like lewisite would be expected to be more successful in penetrating to the tissues owing to the greater instability of their monothiol thioarsinites.

SUMMARY

1. The effect of —SH inhibitors upon isocitric dehydrogenase from pig heart has been studied. Some experiments were made with an enzyme prepared from ox kidney.

2. The isocitric dehydrogenase preparations needed manganese ions for activity and the inhibitory effect of phosphate was explicable as due to precipitations of manganese.

3. Copper and mercury compounds were inhibitory and the inhibition was reversed by monothiols.

4. With the heart enzyme, the monosubstituted arsenical compounds, arsenite and lewisite oxide, were non-toxic in the concentrations tried; but the disubstituted compounds diphenylchloroarsine and some phenarsazines were toxic. Their toxicity to the enzyme could be reversed by —SH glutathione and by British anti-lewisite (BAL).

5. Maleate, chloroacetophenone, nitrogen mustard, alloxan, iodoacetate and iodoacetamide produced no inhibition, nor did *p*-aminobenzoate and sodium fluoracetate; some effect was found with iodosobenzoate.

6. With the kidney enzyme, lewisite oxide showed some toxicity not reversible by BAL.

7. Some implications of these facts are discussed; it is concluded that isocitric dehydrogenase possesses an —SH grouping of unusual properties.

We are grateful to the late Prof. C. S. Gibson, F.R.S. (Guy's Hospital), for the supply through Prof. R. H. S. Thompson of the phenarsazine compounds used; also to Dr L. A. Stocken for advice.

REFERENCES

- Adler, E., von Euler, H., Günther, G. & Plass, M. (1939). *Biochem. J.* **33**, 1028.
 Bacq, Z. M. & Desreux, V. (1946). *Acta biol. Belg.* **3**, 369.
 Bailey, K. & Perry, S. V. (1947). *Biochem. J.* **41**, xxii.
 Barron, E. S. G. & Singer, T. P. (1945). *J. biol. Chem.* **157**, 221.
 Buffa, P., Lotspeich, W. D., Peters, R. A. & Wakelin, R. W. (1950). *Biochem. J.* **47**, xvii.
 Dixon, M. (1937). *Nature, Lond.*, **140**, 806.
 Dixon, M. (1948). *Biochem. Soc. Symp.* **2**, 42.
 Feigl, F. (1947). *Qualitative Analysis by Spot Tests*, p. 134. 3rd English ed. trans. by R. E. Olsper. N.Y., Amsterdam: Elsevier Publ. Co. Inc.
 Grafflin, A. L. & Ochoa, S. (1950). *Biochim. Biophys. Acta*, **4**, 205.
 Hopkins, F. G. & Morgan, E. J. (1938). *Biochem. J.* **32**, 611.
 Krebs, H. A. & Eggleston, L. V. (1944). *Biochem. J.* **38**, 426.
 Lotspeich, W. D. & Peters, R. A. (1950). *Int. physiol. Congr. Abstr.* p. 341.
 McClelland, N. & Peters, R. A. (1919). *J. Physiol.* **53**, xv P.
 Ochoa, S. (1948). *J. biol. Chem.* **174**, 115. And personal communication.
 Ochoa, S. & Weisz-Tabori, E. (1948). *J. biol. Chem.* **174**, 123.
 Peters, R. A. (1948). *Proc. R. Soc. Med.* **41**, 781.
 Peters, R. A., Sinclair, H. M. & Thompson, R. H. S. (1946). *Biochem. J.* **40**, 516.
 Rona, P. & György, P. (1920). *Biochem. Z.* **111**, 115.
 Stocken, L. A. & Thompson, R. H. S. (1946). *Biochem. J.* **40**, 535.
 Straub, F. B. (1942). *Hoppe-Seyl. Z.* **275**, 63.
 Walker, E. (1928). *Biochem. J.* **22**, 292.
 Warburg, O., Christian, W. & Griesse, W. (1935). *Biochem. Z.* **282**, 157.