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THE TOXICITY OF VESICANTS AND SOME OTHER COMPOUNDS TO THE PYRUVATE OXIDASE SYSTEM* (BRAIN)

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The pyruvate oxidase system in pigeon brain tissue is known to be delicate and sensitive to the tissue inhibitors: fluoride, iodoacetate, and phloridzin (Peters and Sinclair, 1934; Peters, Rydin, and Thompson, 1935). During the war it was used as a test system in Chemical Defence Research during the course of a planned investigation, the origin and trend of which has been described elsewhere (Peters, 1947). The fundamental postulates were that vesication could be initiated by damage to an enzyme with the pyruvate oxidase system as a leading string, and that the attack was upon a sulphydryl grouping; these originated in the earlier finding (Peters, 1936) that dichlorodiethylsulphone (HO_2) (as well as arsenite) had a selective action on the lactate oxidase system in brain, poisoning it at the pyruvate stage. There was a close analogy with the action of iodoacetate, also a vesicant.† At the outbreak of war the researches described here developed simultaneously with the arsenical investigation (Peters, Stocken, and Thompson, 1945) by a team which included E. Holiday, A. G. Ogston, J. St. Philpot, and L. A. Stocken; the objective was a better understanding of the vesicant action and the hope of more effective therapy.

In examining the suitability of the pyruvate oxidase system in brain as a test enzyme its behaviour to several substances allied to mustard gas (H) was carefully investigated; some comparisons were also made with other enzymes. It was found that there was some correspondence between the toxicity of many non-arsenical vesicants to the pyruvate oxidase system and their potency as vesicants, but that this relation failed for H; even so, the enzyme system was still sufficiently sensitive to the latter to be used as a test agent. It is interesting to note in regard to our observations (Peters and Wakelin, 1946) made also at this time upon the -SH nature of some components of the pyruvate system that G. Barron (1936), in the course of observations upon the inhibitors of pyruvate oxidase from gonococcus, noted its sensitivity to oxygen; also more recently Mann and Quastel (1946) and Dickens (1946) consider that it is the enzyme preferentially attacked in high pressure oxygen poisoning. When taken together this work and that upon the biochemical lesion in aneurin deficiency and in arsenical poisoning from this laboratory emphasize the pharmacological importance of this enzyme system.

The present paper embodies the gist of 5 reports written early in the war (Peters and Wakelin, 1940, 1941) and is divided into sections upon the water soluble and water insoluble substances and observations upon other enzymes.

I. Water Soluble Substances

Experimental methods

Brain brei and dispersion and pyruvate dehydrogenase.—These were prepared as described previously (Kinnersley, O'Brien, and Peters, 1935; Banga, Ochoa, and Peters, 1939; Peters and Wakelin, 1946). Brei and

* This paper was planned to be the initial publication of a series, which includes two by the same authors elsewhere (*Biochem J.*, 1946, **40**, 513, and 1947, **41**, 545); its publication has been delayed through unforeseen circumstances. Though some figures are included which are given in one of the other papers, it has been thought desirable to publish the data upon which they are based, because they indicate the kind of accuracy obtainable in this type of pharmacological enzyme investigation.

† Berenblum, Kendall, and Orr (1936) found that mustard gas was more toxic to the whole glycolytic process in tissue than to respiration.

slice experiments were made with oxygen in the bottles, and dispersion experiments with air.

Experiments with the pyruvate oxidase system in pigeon's brain tissue can be made with tissue prepared in three different ways, as slices, as brei (a mashed preparation), and as a dispersion, made by grinding the brain finely in a mortar under ice-cold conditions. The slice resembles the organized tissue best: in this H. M. Carleton has shown that the cell outlines are still intact (unpublished results). In the brei the cell outlines have been mainly destroyed, though large numbers of nuclei are still intact: though the brei approximates more to a preparation of enzymes, some phosphoric esters still fail to penetrate to the active centres present; for instance, vitamin B_1 will penetrate, whereas its pyrophosphoric ester (cocarboxylase) will not (Peters, 1937). In the dispersions we have an enzyme system sufficiently homogeneous to reduce permeability phenomena to a minimum; this has been produced, however, at the expense of stability and a dispersion is only stable for about 30 min. at 38°; it can therefore be used only for rapidly acting substances. It is not necessarily identical with the so-called "homogenates" (Potter and Elvehjem, 1936; Elliott, Scott, and Libet, 1942). As several of the poisons concerned react rather slowly, the most useful information was obtained with the more stable " brei."

Any uncertainties produced by the residual respiration could have been largely eliminated, if desired, by a preliminary washing of the brei with ice-cold Ringer-phosphate solution (Long and Peters, 1939); the delay which would have been caused by this refinement would have made little difference to the result and was not thought worth while. Most experiments were therefore made with unwashed brei.

Respiration was studied in Barcroft Dixon or Warburg type respirometers in Ringer-phosphate *p*H 7.3, 3.0 ml. per bottle ± Na pyruvate ($1.8 \times 10^{-2}M$); the poison was added last after division of the tissue (approx. 100 mg. pigeon brain " brei "). Each figure quoted in Table I and elsewhere represents the average rate of respiration in μl./g./hr. moist tissue (water content approx. 80 per cent) for the time interval given, excluding the initial period of 12 min. required for equilibration. Temp. 38°. Gas: O_2 or air. One pigeon brain (cerebrum + optic lobes) can be distributed conveniently among 12 bottles. In this series 8 bottles (4 duplicates) were devoted to the values for pyruvate alone and those of pyruvate + poison, and the remaining 4 were used to obtain residual values.

A typical experiment is given (Exp. 1939) in Table I; it confirms the previous finding (Peters, 1936) that HO_2 has a marked toxic action upon this system, and that there is no protection even with a relatively high concentration of vitamin B_1 (1 μg. would be a maximum dose for an avitaminous brain). It will be noticed that the effect of HO_2 appears slowly with these concentrations, even with 100 μg./3 ml. not until after 15 min. have elapsed.

The falling rate of respiration is due largely to the decrease in residual respiration: much work (Peters, 1938) has shown that the extra rate of respiration over the period 30–120 min. forms the most reliable estimate in experiments of this type. This period was therefore used to compare the inhibitory effects.

TABLE I

EFFECT OF HO_2 (100 μg. = 675 μM ($10^{-6}M$) UPON THE OXYGEN UPTAKE OF PIGEON BRAIN BREI IN RINGER-PHOSPHATE *p*H 7.3. SUBSTRATE: Na PYRUVATE ($0.018M$)

Exp. conditions	O_2 uptake in μl./g./hr. during respiration periods (min.)					Average	
	0–15	15–30	30–60	60–90	90–120	0–30	30–120
(*a*) No addition	1550	1276	903	790	490		
(*b*) + 50 μg. HO_2*	1405	1090	750	437	222		
(*c*) + 100 μg. HO_2*	1280	1040	640	246	162		
(*d*) Na pyr.	3315	3060	2710	2305	1845		
(*e*) Na pyr. + 50 μg. HO_2	3285	2875	2080	1325	956		
(*f*) Na pyr. + 100 μg. HO_2	3130	2340	1450	764	361		
(*g*) Same + 10 mg. vit. B_1	3185	2465	1623	917	431		
DIFFERENCES FOR							
Pyr. alone (*d*)–(*a*)	1765	1784	1807	1515	1355	1780	1559
Same + 50 μg. HO_2 (*e*)–(*b*)	1880	1785	1330	888	734	1832	984
Same + 100 μg. HO_2 (*f*)–(*c*)	1850	1300	810	518	199	1675	509

% change in respiration during	0–30 min.	30–120 min.
50 μg. HO_2	—	−37%
100 μg. HO_2	−9%	−67.5%

* Single observations. Remainder are average duplicates. HO_2 = Dichlorodiethylsulphone.

RESULTS

Table II gives the results obtained for a number of water soluble substances. The 50 per cent inhibition values (ET50, expressed in μM) were estimated by inspection of curves drawn through the known points.

In ordinary experiments of this type the standard deviation of a single observation is c. 3.5 per cent, but in the present work substantial further errors undoubtedly arose from difficulties in getting satisfactory solution of some of the substances used, and from uneven actions of the poisons in the early stages before the brei was properly divided.

TABLE II

SUMMARY OF AVERAGE PERCENTAGE INHIBITIONS IN OXYGEN UPTAKE OF PIGEON BRAIN BREI PRODUCED BY SEVERAL WATER SOLUBLE VESICANTS AND OTHER SUBSTANCES. SUBSTRATE: Na PYRUVATE (0.018M)

Respiration period 30–120 min. No. of experiments in brackets

Substance	Conc. $M \times 10^{-4}$	% change	μM for 50% inhibition
Dichlorodiethylsulphone (HO_2) (5)	0.437	−22.7	90
	0.875	−48.3	
	1.75	−69.4	
Trichlorotriethylamine (4)	1.91	−35.9	380*
	4.77	−55.1	
	9.55	−63.7	
Divinylsulphone (3) ..	0.29	−3.2	75
	0.86	−52.7	
	2.66	−92.5	
Dichlorodiethylsulphoxide (3)	1.9	+6.5	Nil at 572
	5.72	+7	
	15.2	+6.5	
	57.2	−4	
	95.2	−11	
Phenyl-2-chloroethylsulphone (2)	3.3	+1	900
	4.07	−25.2	
	8.15	−48	
	16.3	−61	
Iodoacetic acid (4) ..	2.16	−46.8	250
	4.32	−69.5	
	5.4	−60.3	
Iodoacetamide (4) ..	0.54	−51.8	50
	1.08	−67.7	
	1.78	−85.7	
Thiodiglycol† and dihydroxydiethylsulphone† (1)	55 and 43	Nil	Nil at 40

* The methyl bis(2-chloroethyl)amine was found by Peters, Thompson, and Wakelin (1942) to have 50 per cent toxicity at 1.040 μM.

† Non-vesicant.

A few comments are necessary upon the method of dissolving some of the substances and upon their behaviour.

Dichlorodiethylsulphone (HO_2)

Table II gives values for the last four experiments and for a previous one considered to be reliable. Several earlier experiments were excluded after discovery that solution was apt to be incomplete at a concentration of 1 mg./ml. Ringer-phosphate solution owing to the formation of small glass-like globules of the compound which were difficult to see. For these purposes the best way was to add c. 0.5 ml. Ringer-phosphate to about 3.0 mg. HO_2 in a test tube, warm until the crystals went into solution, and then add up to 6.0 ml. to make 0.1 ml. = 50 μg. HO_2. The solution should then be examined for small transparent globules of HO_2 and, if these are suspected, the whole warmed. As hydrolysis is slow in presence of salt (Peters and Walker, 1923) any slight loss is completely offset by the certainty of complete solution. Three separate solutions were used in getting the results of Table II. Upon the same solution, results for different brains agreed closely.

2 : 2′ : 2″-trichlorotriethylamine

Solutions of the pure hydrochloride (kindly supplied by Sir Robert Robinson and Dr. Mason) were used: any necessary small additions of alkali were previously made to the bottles to compensate for changes in *p*H produced by the hydrochloride. The free base is liberated as an oil at *p*H 7·3.

Divinylsulphone (DVS)

The experiments quoted were done with a pure specimen from Dr. McCombie, supplied through Dr. Fell. A few mg. stirred with appropriate volumes of water (c. 5.0 ml.) appeared to give satisfactory solution.

Phenyl-2-chloroethylsulphone

This compound (supplied by Porton) was dissolved as far as possible by warming. It does not hydrolyse to any extent.

Iodoacetic acid and iodoacetamide

It was noted that the poisoning induced by iodoacetamide in the first 30 min. is relatively greater than that with other compounds, showing that the condensation is very rapid.

Thiodiglycol and dihydroxydiethylsulphone

In this experiment the values given for the O_2 uptake are those of the bottles containing pyruvate and pyruvate + substances added, without subtraction of the residuals. These substances had a depressant effect on the residual respiration whereas they will be seen to have none on the values in presence of pyruvate. This is interpreted to mean that there is competition for the active centres in the brei between the last traces of pyruvate present in the residue and these substances.

In order to get a better picture of the accuracy of these comparisons, the behaviour of HO_2, divinylsulphone, and trichlorotriethylamine was examined more closely. Dr. R. B. Fisher, of this Department, kindly tested the figures for HO_2 and trichlorotriethylamine (Table II) statistically upon the assumption that the relation between percentage inhibitions and log. concentrations is linear. This was more true for HO_2 than for trichlorotriethylamine, owing to the hydrolysis of the latter during the 2 hr. of experiment. The analysis showed a rather wide variance for the figures as they stood; the probable value for the ratio between means at the point of 50 per cent toxicity was 4.3, with a 1/20 chance of the ratio lying outside the limits 2.6 to 7.3.

In view of this, which was due mainly to the difficulty of standardizing the initial rate of action of the poison in the brei, we carried out a few further experiments, making a direct comparison at the molarities required to give approximately 50 per cent inhibition in order to get more information upon the accuracy of this type of experiment. Experiments were made with 16 bottles as follows: two 0 (residual), two + pyruvate, three + HO_2, three + trichlorotriethylamine, three pyruvate + HO_2, and three pyruvate + trichlorotriethylamine. The results are given in Table III.

The statistical estimate made the probable value for the ratio 4.43, with limits of 2.72 – 7.21 (1/20 chance) in agreement with the estimate in Table II. This gives the possible limits of accuracy of this type of experiment.

Table III (*b*) confirmed the previous figures. On a molar basis divinylsulphone was the most toxic substance of the series.

Since the first object of this work was a comparison of toxicity to the pyruvate system with vesication, the values for enzyme inhibition (50 per cent toxicity) were compared with the Porton data for vesication (upon a weight basis). In Table IV HO_2 is put as 100 and some correspondence between vesicant action and toxicity will be seen. The only marked discrepancy was in the values for divinylsulphone; this is known to be highly toxic to animals by injection, and on the arm of one of us (R.A.P.) small amounts readily formed vesicles. We are inclined to consider the available Porton estimate too low, though could not press this in the absence of further data. So far as the hypothesis of an attack upon the -SH group in the enzyme is concerned, the data given so far are in agreement; HO_2 and divinylsulphone have a μM toxicity (ET50) of 90 and 75 respectively, which is to be compared with the trivalent arsenical toxicity (Peters, Sinclair, and Thompson, 1946) of 17–30 μM, where two -SH groups are attacked per mol. The case is different for mustard gas now to be considered.

TABLE III

COMPARISON OF TOXIC EFFECTS UPON THE PYRUVATE OXIDASE SYSTEM OF THE SAME PIGEON BRAIN BREI TISSUE RESPECTIVELY OF (*a*) HO_2 AND TRICHLOROTRIETHYLAMINE AND (*b*) HO_2 AND DIVINYLSULPHONE (DVS). SUBSTRATE: Na PYRUVATE, 0.018*M*. RESIDUALS SUBTRACTED

(*a*)	Conc. $M \times 10^{-4}$		Average resp. rate in μl./g./hr.		% inhibition	
Exp.	HO_2	Trichloro-triethyl-amine	0–30 min.	30–120 min.	0–30 min.	30–120 min.
1970	—	—	1980	1849	—	—
	0.9	—	1831	901	−7.5	−54.8
1971	—	—	1636	1378	—	—
	0.9	—	1728	766	—	−44.4
	—	3.87	1311	727	−19.5	−47.2
1972	—	—	1636	1542	—	—
	0.9	—	1339	645	−18	−58.2
	—	3.87	1214	712	−26	−53.9

(*b*) HO_2 AND DVS. DIRECT COMPARISON. NO CONTROLS WITH PYRUVATE ALONE. TRIPLICATE ESTIMATIONS. HO_2, $0.91 \times 10^{-4}M$; DVS, $0.74 \times 10^{-4}M$; 52 μg. AND 26 μg. PER 3 ML. RESPECTIVELY

Pyruvate	HO_2	DVS			Differences	
—	+	—	1263	521		
—	—	+	1296	539		
+	+	—	2204	902	941	381
+	—	+	2115	985	819	446

TABLE IV

COMPARISON OF "OXIDASE" TOXICITY AND VESICANT ACTION; $HO_2 = 100$

Substance	Oxidase toxicity	Vesicant action
Iodoacetamide	186	Unknown*
Divinylsulphone (DVS)	198	2.5
Dichlorodiethylsulphone (HO_2)	100	100
Iodoacetic acid	37	Vesicant
Trichlorotriethylamine (hydrochloride)	19.8	10–20
Phenyl-2-chloroethylsulphone	9	nil
Dichlorodiethylsulphoxide	nil	nil
Thiodiglycol and dihydroxydiethylsulphone	nil	nil

* One of us (R.W.) produced on the left forearm a large erythematous patch 8 × 8 cm. and vesicle 3.5 × 6.0 cm. with an application of 1.5 mg. iodoacetamide in ethanol.

II. Water Insoluble Substances

Mustard gas (H)

Some early experiments were done by the method used by Berenblum *et al.* (1936), in which the substance was mixed with the tumour tissue thoroughly before it was added to the respiration bottles. Table V gives some results so obtained.

TABLE V

CONCENTRATIONS* FOR IMMISCIBLE SUBSTANCES AND THIODIGLYCOL GIVING APPROX. 50 PER CENT DECREASE IN O_2 UPTAKE OF PYRUVATE OXIDASE SYSTEM IN PIGEON BRAIN BREI

Substrate: Na pyruvate, 0.018*M*. No. of experiments in parentheses

Substance	Conc. for −50%(μ*M*)	Conc. for −10%(μ*M*)
2:2′-Di(β-chloroethylthio)-diethyl ether	330 (3)	—
Mustard gas	770 (3)	—
Phenyl-2-chloroethylsulphide	1000 (3)	—
Thiodiglycol	—	150 (2)
Butyl chloride (tertiary)	—	200 (3)
Butanol (tertiary)	—	240 (1)

* Concentrations in this series of experiments only are reckoned in terms of the amount of test substance per 100 mg. tissue used.

It will be seen that although H is toxic it is much less so than the arsenicals and divinylsulphone. Thinking that this might be due to the inefficient method of introduction of the poison, we tried other solvents. Table VI gives the results of the early and some later experiments using *iso*propanol and ethyl and methyl cellosolve.

It was possible to use these solvents with a correction, and in fact this was done later in the experiments in this laboratory upon BAL (Stocken, Thompson, and Whittaker, 1947); but it was then thought advisable to find some other method, and the use of lecithin was explored. Commercial lecithin (B.D.H.) cannot be used for the emulsification as it is toxic in itself. Egg lecithin was therefore prepared as follows (we are indebted to Professor J. B. Leathes for the details of this preparation):

Preparation and use of lecithin.—The yolks of 6 eggs were stirred with 3 volumes of acetone and the solution filtered; the solid residue was stirred with a further 3 volumes of acetone. The residue was then shaken with

TABLE VI

TOXIC EFFECT OF SMALL CONCENTRATIONS OF SOLVENTS UPON PYRUVATE OXIDASE SYSTEM AND THE COMBINED EFFECT OF SOLVENT AND MUSTARD GAS. BREI AND DISPERSIONS, PIGEON BRAIN. SUBSTRATE: Na PYRUVATE, 0.018*M* (BREI); 0.011*M* (DISPERSION)

Me.cs. = Methyl cellosolve, 0.025 ml./3 ml. Et.cs. = Ethyl cellosolve. H = Mustard gas. L = Lecithin

Exp.	Substrate	Qo_2 0–30 min.	Averate rate 30–120 min.	Decrease	Dispersion (D) or brei (B)
2212	Pyr. alone	13.05	9.98		B
	Pyr. + *iso*propanol 0.84%	10.78	8.61	1.37	
	Same + H	9.90	6.21	3.77	
2223	Pyr. alone	19.46	11.58		B
	Pyr. + *iso*propanol	14.08	9.22	2.36	
2224	Pyr. alone	16.36	11.43		B
	Pyr. + *iso*propanol	14.30	9.56	1.87	
2226	Pyr. alone	16.90	11.57		B
	Pyr. + propanol	13.50	9.00	2.57	
2227	Pyr. alone	17.01	11.34		B
	Pyr. + *iso*propanol	12.96	9.51	1.83	
	Pyr. + *iso*propanol + 800 μg. H	11.37	5.88	5.46	
2323	Pyr. alone	15.53			D (dialysed)
	Pyr. + Me.cs.	6.21		9.32	
2327	Pyr. alone	9.59			D (dialysed)
	Pyr. + Et.cs.	7.52		2.07	
	Pyr. + Et.cs. + 800 μg. H	5.48		4.11	(extra for H −2.03)
	Pyr. + L	10.30			
	Pyr. + L + H	8.13		2.17	

acetone for 2 hr. in a mechanical shaker, the suspension filtered, and the residue suspended in absolute ethanol overnight. After filtration the residue was again suspended in absolute ethanol and shaken mechanically for 2 hr. The ethanolic filtrates were combined and concentrated *in vacuo* at 38–40° to a small volume and made up so that 1 ml. = 100 mg. When this solution is kept in the ice chest, a deposit of sphingomyelin gradually forms. We have found the product from this relatively crude ethanolic solution quite suitable for our purpose, and it gives no inhibition of respiration with a brei.

For experiment, weighed amounts of H (c. 8 mg.) were added to 50 mg. lecithin (after removal of ethanol *in vacuo* in a small test tube) and an intimate mixture was made with a glass rod. Immediately before being added to the respiration bottles, the emulsion was treated with 0.5 ml. Ringer-phosphate solution with vigorous stirring, followed by a further 1.5 ml. to a volume of 2.0 ml. From this a usual addition to the bottles was 0.2 ml. (5 mg. lecithin).

Experiment 2254 (Table VII) shows that the amount of lecithin added to the 3.0 ml. fluid in the respirometer bottles can be varied from 1–5 mg. without change, but that 10 mg. may reduce the effect of the H. In this experiment the H (50 μg.) was potentiated by addition of diethanol dithiocarbamate (Peters and Wakelin, 1947) (1 mg. per bottle).

TABLE VII

EXP. 2254. INHIBITORY EFFECT OF POTENTIATED H (420 μM) UPON Qo_2 OF PYRUVATE OXIDASE SYSTEM OF PIGEON. BRAIN BREI WITH VARYING AMOUNTS OF LECITHIN PRESENT. Na DIETHANOL DITHIOCARBAMATE (1.9 mM) ALSO PRESENT

Substrate: Na pyruvate, 0.018M

Lecithin	Qo_2	%
+ 1 mg. ..	−4.35	−59
+ 5 mg. ..	−4.62	−62
+ 10 mg. ..	−3.67	−46

We have employed this method of adding oily substances extensively and have confidence in the results obtained. The arguments in favour of the use of lecithin may be summarized:

(*a*) Lecithin is a normal constituent of brain and other tissue; its addition therefore only increases slightly the amount already present in the tissue.

(*b*) When properly purified and added alone, it has no effect upon the Qo_2.

(*c*) It has no competitive action with H (Holiday, Ogston, Philpot, and Stocken, 1940; Ogston *et al.*, 1948) but merely reduces the rate of hydrolysis, as has been proved experimentally by Ogston (personal communication).

(*d*) There is no increased toxicity if the combined emulsion of lecithin and H is allowed to stand before addition of the Ringer-phosphate, or even if it is warmed to 38°; hence it does not form a toxic addition compound. Experiment 2304 shows this (Table VIII).

TABLE VIII

EXP. 2304. BRAIN BREI (PIGEON). Qo_2 FOR PERIOD 35–120 MIN. SUBSTRATE: Na PYRUVATE (0.018M); LECITHIN IN ALL BOTTLES

Additions	Qo_2
No addition	10.45
800 μg. H*	5.89
800 μg. H stood for 1 hr. at room temperature after thorough mixing*	6.18
800 μg. H warmed in bath for 20 min. after thorough mixing*	5.97

* The Ringer-phosphate was added to the thoroughly stirred lecithin immediately before addition to the bottles.

Tables IX, X, and XI give a selection of results obtained for mustard gas with dispersion, with brei, and with slices; these are given separately because there are differences in the detail of the effect. The toxicity (ET50) works out at more than 1,000 μM. This is clearly a relatively low toxicity compared with those of divinylsulphone and the arsenicals, even if we allow for a slight loss by preliminary hydrolysis before the poison has time to act; it is of the same order of toxicity as that of iodoacetic acid to this enzyme.

Dispersion.—The action upon pyruvate respiration (Table IX) is noticeable in the first 10 min. period of observation and fully established after 20 min.; the maximum effect was seen in the respiration period 10–20 min. Only 3 out of 15 experiments showed significant inhibition in the residual respiration. The protocol of 2 typical experiments out of the large number performed are given.

TABLE IX

DISPERSION (PIGEON BRAIN). CHANGE IN RATE OF RESPIRATION IN μl./g./hr. O_2 UPTAKE, DUE TO ADDITION OF LECITHIN + H (800 μg. H IN 5 MG. LECITHIN PER BOTTLE). SUBSTRATE PYRUVATE. RESPIRATION PERIOD IN MIN.

Exp. No.	0–10	10–20	20–30	30–40	Average	% change
2047	−660	−670	−580	−485	−599	−18
2061	−225	−795	−835	−353	−560	−16

The values given represent the decreased rate of O_2 uptake over the periods mentioned, i.e., that for pyruvate minus pyruvate + H; for the reason why this procedure is used see Peters (1938).

Brei.—Fig. 1 shows the rate of poisoning of brei in a typical experiment; Table X gives the percentage inhibitions observed in a series of experiments with different concentrations of H. The lowest amount producing a noticeable change is 100 μg. H/3 ml.; the effect depended much upon the efficiency of emulsification and the rapidity with which the emulsion was added to the bottles. The effect was noticeable in the first 15 min. but did not become maximal until the 30–60 min period. Experiment 1993 shows that, if first allowed to hydrolyse in an aqueous solution, the H produced no action, as is readily understood from consideration of the kinetics (Ogston *et al.*, 1948).

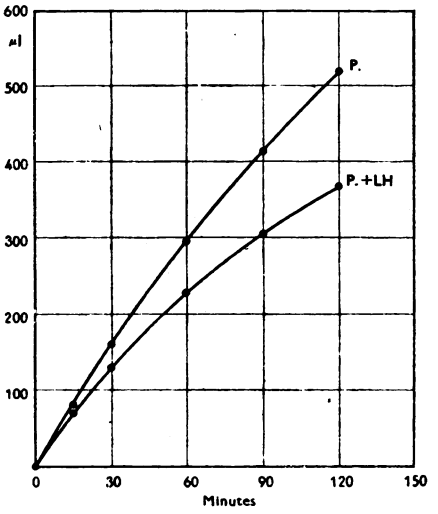

FIG. 1.—The effect of H in lecithin (LH) upon the respiration of brain brei with sodium pyruvate (P) as substrate. Ordinate ml./g./hr. Abscissae min. Pigeon brain brei.

In the final experiments, the changes produced by addition of 800 μg. of H to each bottle (1.67 m*M*) were of the order of −16 per cent for period 0–30 min. and −30 per cent for 30–12 min.—i.e., the concentration required to produce a 50 per cent change was greater than 1,000 μM.

Slices.—Exp. 2065 (Table XI) confirms that in slices too lecithin has no influence on the respiration; H here inhibited the residual respiration. In Exp. 2068 it will be seen that with glucose the poisoning effect is increasing up to 3 hr. Exp. 2072 was a comparison between the action of H with pyruvate and glucose as substrates, in which it will be noticed again that poisoning of the respiration in presence of glucose is increasing up to 3 hr.

TABLE X

BREI (PIGEON BRAIN). CHANGES IN RATE OF RESPIRATION GIVEN IN PER CENT, DUE TO ADDITION OF H IN LECITHIN IN VARIOUS CONCENTRATIONS. SUBSTRATE: Na PYRUVATE, 0.018*M* (residuals subtracted)

Exp.	Amount H per bottle (μg.)	% change	
		0–30 min.	30–120 min.
1987	750	−4.5	−50.1
1988	650	+5	−19
	325	+11	0
1990	750	−22.3	−29.8
1991	750	−16	−43.5
1992	515	−12.6	−23.5
1994	1180	−20	−41.7
	590	+1	−23.5
1995	400	−34.2	−45.2
1997	660	−10.7	−27.5
	330	+3	−9.4
1998	510	−6	−30.7
1999	900	−26.5	−47.8
2000	760	−24.3	−47
2003*	360	−7.5	−23.4
	120	−44	−6.5
2004*	50	−12.3	−17.5
	30	−2	—
2006*	50	−7.8	−12

* Residuals not subtracted

H-lecithin added after hydrolysis in Ringer-phosphate for 30 min. at 38°

1993	1080	−8.2	0

NOTE: In most recent experiments, as the result of further emulsification and quicker working, the changes produced by 800 μg. H/bottle ($1.67 \times 10^{-3} M$) were of the order of −16 per cent for 0–30 min., and −30 per cent for 30–120 min.

DISCUSSION

In a comparison of the rates of poisoning and of hydrolysis for H it is to be noted that in accord with the theory of the kinetics of replacement of chlorine in H (Ogston *et al.*, 1948) any substitution with a group in the enzyme must take place during the hydrolysis; most of this occurs in these experiments in the first 15 min. and is complete in 30 min. The rate of action upon the dispersion was consistent with this; that upon brei and slices was too slow. One conceivable explanation for this difference was conversion of H to an oxidation product; in a direct test of this we could find no increased O_2 uptake owing to the addition of H which might suggest oxidation to sulphoxide. It seems more likely that there is adsorption into inactive lipoid parts of the brei or slice with slower penetration to the active centres; this could explain most of the effect in slices when pyruvate

TABLE XI

SLICES (PIGEON CEREBRAL HEMISPHERES). CHANGES IN RATE OF RESPIRATION DUE TO ACTION OF H IN LECITHIN (L) (800 μg./ml.)

L = Lecithin. Gl. = Glucose. H = Mustard gas. Pyr. = Pyruvate

Exp.	Substrates	Respiration rate in μl./g./hr. during periods (min.)						
		0–15	15–30	30–60	60–90	90–120	120–150	150–180
2065	None (*a*)	1851	1585	1290	976	809	698	
	L (*b*)	1846	1555	1240	967	761	604	
	L + H (*c*)	1670	1406	1055	689	471	307	
	(*b*)–(*c*)	−176	−149	−185	−278	−290	−297	
2068	Gl. only (*d*) ..	—	3350	3255	3255	3247	3147	3057
	Gl. + L (*e*)	—		3320	3135	3275	2990	3000
	Gl. + L + H (*f*) ..	—		2875	2630	2465	2155	1995
	(*e*)–(*f*)			−445	−505	−810	−835	−1005
2072	Pyr. + L (*g*)		4260	3794	3006	2910	2630	2320
	Pyr. + L + H (*h*) ..	3625	3485	2997	2362	2055	1745	1341
	(*g*)–(*h*)		−775	−797	−646	−855	−885	−979
	Gl. + L (*j*)	3720	3785	3650	3445	3500	3340	3265
	Gl. + L + H (*k*) ..	3085	3070	2514	2440	2400	2120	1915
	(*j*)–(*k*)	−640	−715	−836	−1005	−1100	−1220	−1350

is the substrate; it would leave unexplained the gradual increase in poisoning observed with slices in glucose solutions. Since this work was reported in 1940, Dixon and Needham and colleagues (for review see 1946) have produced much evidence in support of the poisoning of hexokinase by H, which would explain the latter effects better. Other factors contributing to the slower action may be the adsorption and half product formation (Peters and Wakelin, 1947) and also perhaps sulphonium salt formation (Stahmann *et al.*, 1946).

III. Selective Action of H

In 1936 (Peters) it was shown that HO_2 poisoned the lactate oxidase system of brei selectively at the pyruvate stage; since Keilin's cytochrome system is common to the oxidation of both lactic acid and pyruvic acid, this experiment indicated that the cytochrome system and the lactate dehydrogenase were much less sensitive to HO_2 than pyruvate dehydrogenase; it also excluded poisoning of cozymase. Though the sulphone combined with glutathione, the latter did not reactivate the poisoned system; except for the more powerful action of HO_2, the effect resembled that of iodoacetate (Peters, Rydin, and Thompson, 1935).

After showing that H could poison the pyruvate oxidase system we thought it advisable to confirm the earlier conclusions for HO_2 by direct experiment and to extend them to H. Direct experiments were therefore done (1) upon the vitamin B_1 component, (2) upon the cytochrome system, (3) upon the total succinate oxidase system in brain, and (4) upon amino-acid oxidase, especially because this contained the adenine flavine dinucleotide as a component.

Aerobic experiments

Cocarboxylase.—It had been shown previously that addition of vitamin B_1 did not stop the poisoning effect of HO_2. In Exp. 1875 (Table XII) it was shown that addition of large amounts of cocarboxylase, now known to be the active component (Banga, Ochoa, and Peters, 1939), does not restore the poisoned respiration.

Cytochrome system.—Cytochrome oxidase and cytochrome C were prepared by the method of

TABLE XII

SEPT. 6, 1939. FAILURE OF THE ADDITION OF COCARBOXYLASE TO RESTORE ACTIVITY OF PYRUVATE OXIDASE SYSTEM POISONED BY DICHLORODIETHYLSULPHONE (HO_2). PIGEON BRAIN DISPERSION; SUBSTRATE: PYRUVATE, 0.018*M*

HO_2 μ*M*	Cocarboxylase μg.	O_2 uptake μl./g./hr. (20 min.)	% change
—	—	2645	
100	—	2242	−15%
100	18	2242	−15%

0.2 μg. cocarboxylase should produce a maximum effect under these conditions in a vitamin B_1 deficient brain.

Keilin and Hartree (1938), and hydroquinone was used as substrate. Table XIII gives the results.

Arsenite, HO_2, and divinylsulphone, all of them vesicant or potentially vesicant substances, produced no effect; 10 μg. As_2O_3 poisoned the pyruvate system to the extent of 85 per cent and 25 μg. divinylsulphone, 50 per cent. H (emulsified in lecithin) showed inhibitions up to 7 per cent, when cytochrome C was present in maximum concentration, and up to 20 per cent when the latter was present in submaximal amounts. That this effect was mainly on the cytochrome C component was suggested by two other experiments: in one (Exp. 6) the action upon the cytochrome oxidase, to which cytochrome C was added before mustard gas-lecithin, was compared with the effect when cytochrome C was added after incubation for 15 min. with H; by this time much of the H would have been hydrolysed or rendered ineffective by combination with phosphate, etc.; in another (Exp. 7) cytochrome oxidase was incubated for 15 min. with and without lecithin, the residue centrifuged and tested with cytochrome C. There was no essential difference from the experiment carried out in the presence of the poison; where much cytochrome C was present, the effect was reduced.

TABLE XIII

EFFECT OF MUSTARD GAS, SOME SULPHONES, AND ARSENITE UPON KEILIN'S "CYTOCHROME SYSTEM"

All bottles contained cytochrome oxidase (0.3 ml.); cytochrome C (0.7 per cent solution as stated); 5 mg. hydroquinone in 0.2 ml. ($1.5 \times 10^{-2}M$) was added after equilibration. L = Lecithin. H = Mustard gas. HO_2= Dichlorodiethylsulphone. DVS = Divinylsulphone

Exp.	Cyt. (ml.)	Additions	Time in min.	μl.O_2	% change
1	0.02	None	20	224	
24.4.40	0.02	+ L (7.5 mg.) ..	20	243	
	0.02	+ L + H (1,200 μg.)	20	250	−17.8
	0.02	+ HO_2 (200 μg.) ..	20	248*	
2	0.2	None	30	262	
25.4.40	0.2	+ As_2O_3 (200 μg.) ..	30	265	+1.5
3	0	None	20	344	
7.8.40	0	+ L	20	336	
	0	+ L + H (1,200 μg.)	20	286	−15
	0	+ L + DVS (150 μg.)	20	334	—
4	0	+ L (5 mg.) ..	20	325	
7.8.40	0	+ L + H (800 μg.)	20	292	−11
	0.02	+ L (5 mg.) ..	20	353	
	0.02	+ L + H (800 μg.)	20	328	−7
5	0	+ L	15	249	
8.8.40	0	+ L + H (800 μg.)	15	207	−20†
	0.1	+ L	15	351	
	0.1	+ L + H (800 μg.)	15	343	−2‡
6	0.1	+ L	15	405	
8.8.40	0.1	+ L + H (800 μg.)	15	386	−5
	0.1	+ L	15	420	
	0.1	+ L + H (800 μg.)	15	383	−7§

* Single observation. † Addition of extra cytochrome C reduced the toxicity. ‡ Cytochrome C added before L + H. § Cytochrome C added after 1 min. incubation with L + H.

NOTE: Vessels containing H had 0.05 ml. N/10 NaOH. per 800 μg. to neutralize the acid formed from H.

EXP. 7. Equal amounts of cytochrome oxidase suspension were incubated (A) with lecithin only and (B) with H in lecithin for 20 min.; N/10 NaOH was added at 10 min. to B to adjust the pH. After being centrifuged, aliquot amounts were compared for activity with and without cytochrome C (0.1 ml.).

	Cyto. C	μl.O_2	% change
A	0	292	
B	0	259	−13
A	+	363	
B	+	341	−6.5

These inhibitions of cytochrome C indicate that H can combine with it; they are of no significance in the experiments upon brain brei as the cytochrome C is not a limiting factor; this was tested directly in one experiment 8 where there was no significant change.

EXP. 8. Brain dispersion from the cerebrums of two pigeons was treated with lecithin (L) and H (800 μg.) in lecithin as usual; after 10 min. preliminary incubation period and a further period of 5 min., 0.15 ml. cytochrome oxidase (cyto. ox.) + 0.05 ml. cytochrome C (cyto. C) were added from a dangling tube to see whether this improved respiration. Substrate: Na pyruvate, 0.023 M. Average of duplicates.

Addition	μl.O_2/g./hr. 30 min.	Change
L only	2933	
HL	2622	−311
L+cyto. ox.+cyto. C ..	2780	
HL+cyto. ox.+cyto. C	2452	−328

Succinate oxidase system.—Table XIV is a summary of tests made upon the relative toxicity of these compounds to succinate and pyruvate respiration in the same brei. In each test there is 2–3 times larger inhibition of the pyruvate than that of the succinate respiration; the effect is therefore selective. There is a sporadic slight poisoning of the succinoxidase system, not yet explained, which is connected with the state of the tissue, because in ground muscle preparations the succinodehydrogenase is not poisoned by H.

Exp. 2027 is a striking variation of the same theme. We have shown separately (Peters and

TABLE XIV

COMPARISON OF TOXICITY TO PYRUVATE AND SUCCINATE SYSTEMS. SUMMARY OF CHANGES (INHIBITIONS) DUE TO POISON IN μl./g./hr. FOR PERIOD (2) 30–120 MIN.

Poison*	Exp.	Residual	Pyruvate	Succinate
HO_2 (0.173 m*M*) ..	1893	−221	−1120	−282
H (as oil) (0.592 m*M*)	1932	−167	−696	−273
H (in lecithin) (1.31–1.72 m*M*)	2032	−92	−631	−264
,, ,,	2099	−251	−698	−139
,, ,,	2034	−120	—	−125
,, ,,	2036	−100	—	−341
H (0.315 m*M*) in lecithin and diethyldithiocarbamate (1.56 m*M*)	2027	−346	−1136	0

* Per 100 mg. tissue

Wakelin, 1943, 1947) that diethanoldithiocarbamate potentiates the toxicity of H. Here an amount of H, which would have had no action upon pyruvate oxidation by itself, had a large effect in presence of diethanoldithiocarbamate ; under the same conditions succinate oxidation was unaffected. Since the cytochrome system is common to both oxidations, we have a remarkably clear selective inhibition.

Amino-acid oxidase.—This was prepared from pig's kidney by the method of Krebs (1935). The system employed for study consisted of 2.0 ml. enzyme extract and 1 ml. buffer (*p*H 7.3) with or without additions made up in buffer solution. The enzyme extract was made by shaking 1 g. dry powder with 40 ml. water at room temperature for 10 min. and then centrifuging. Table XV gives the results and shows that none of the poisons tested interfered with the amino-acid oxidase

TABLE XV

EFFECT OF SOME POISONS UPON THE AMINO-ACID OXIDASE FROM KIDNEY

Substances	O_2 uptake (μl.) in 55 min.
No addition	25
dl-Alanine (0.22*M*)	382
,, ,, + 5 mg. lecithin..	394
,, ,, + 700 μg. H in lecithin	394
,, ,, + 200 μg. iodoacetic acid	382
,, ,, + 200 μg. dichlorodiethylsulphone	367

system ; they cannot therefore inactivate the adenine flavine dinucleotide component.

The experiments in this section indicate that the pyruvate oxidase system is selectively poisoned by H at concentrations which do not affect the other parts of the system, though the concentration of H required for this toxic effect is much larger than that of arsenicals and the two relevant sulphones. Since this work was reported Dixon and Needham (1941) have shown that H is toxic to the pyruvate system of *B. coli,* an enzyme obtained by Still (1940) in clear cell-free solutions ; the optimal *p*H for their enzyme was *p*H 6.2, differing from that of the brain experiments, *p*H 7.3.

Dehydrogenase experiments.—So far the experiments described have been upon partly organized tissue systems, which is reasonable when the effect upon the living cell is being especially considered. But it was desirable to know how the isolated dehydrogenases behaved. Many experiments were therefore done upon the actions of several of these compounds upon standard dehydrogenase preparations ; only representative experiments are given in Table XVI for the 3 substances H, HO_2, and divinylsulphone. In muscle preparations H had no appreciable effect upon succinate, malate, or lactate dehydrogenase ; with brain tissue H had a slight and variable effect on succinodehydrogenase, but the action of H upon pyruvate dehydrogenase was always greater. Nevertheless, as with the arsenicals, the action of H upon the pyruvate dehydrogenase is less than upon the total oxidase, suggesting that there is a factor in the aerobic system, perhaps the phosphokinase (Dixon and Needham, 1946), which is more sensitive.

The toxic action of HO_2 and of divinylsulphone was greater upon the pyruvate dehydrogenase than upon the other dehydrogenases ; on the former the effect of comparatively low concentrations was considerable, which is in agreement with the investigation upon the total oxidase.

Discussion

HO_2, divinylsulphone, and H show a selective action upon the component of the pyruvate system dealing specifically with pyruvate ; after completion of these experiments R. van Heyningen (1941) and workers in the U.S. considerably extended the number of enzymes tried ; very few were sensitive to H, hexokinase (Dixon and Needham, 1946 ; van Heyningen, 1941) being an outstanding exception. Like the arsenicals, HO_2 and divinylsulphone appear to be combining with an -SH group ; this is consistent not only with the toxicity values but

TABLE XVI

EFFECT OF DICHLORODIETHYLSULPHONE (HO_2), DIVINYLSULPHONE (DVS), AND MUSTARD GAS (H) UPON PYRUVATE AND SOME OTHER DEHYDROGENASES. PYRUVATE, 0.018*M*; Na SUCCINATE, 0.082*M*; MALATE, 0.0585*M*; LACTATE, 0.069*M*; BOTH THE LATTER HAD COZYMASE ADDED; TOTAL VOLUME 1.5 ML.

Exp.	Source and type	Incubation time (min.) for poison	Substrate	Decolorization time (in min.) for methylene blue			
				Substrate alone•	HO_2 100 μg.	HO_2 200 μg.	DVS 25 μg.
1	Brain	40	Pyruvate	15	40	140	—
			Succinate	13½	24	22	—
2	Brain	40	Pyruvate	16	37½	70	—
			Lactate	6½	9	10	—
3	Brain	40	Pyruvate	23	35	—	40
			Succinate	15	17	—	17½
4	Brain	40	Pyruvate	12½	—	—	α (100 μg.)
5	Brain	40	Pyruvate	17	—	—	80 (50 μg.)
6	Muscle	0	Malate	10	—	10+	—
		0	Succinate	19	—	—	19 (240 μg.)
7	Muscle	40	Malate	7½	—	—	7½
					H (800 μg.)		
8	Brain	20	Pyruvate	13½	22½	200 μg. HO_2 = 0.70 m*M*	
			Succinate	11¼	12½		
9	Brain	40	Pyruvate	28	42	25 μg. DVS = 0.141 m*M*	
			Succinate	28	30		
10	Muscle	20	Succinate	9½	9½	800 μg. H = 3.28 m*M*	
11	Muscle	15	Malate	50	50		
			Succinate	20	22	emulsified with lecithin	
12	Muscle	40	Malate	4	4		
				9	9½		

also with the earlier work (Peters and Walker, 1924–5) on the abolition of -SH reactions in skin slices, etc., by some of these substances, and with other facts known now about the action of the sulphones (Ford-Moore and Lidstone, 1940; Banks, Boursnell, Francis, Hopwood, and Wormall, 1946; Stahmann, Golumbic, Stein, and Fruton, 1946); on the other hand, the idea that H combines as well with the -SH groups in the proteins as it does with thiol groups in compounds like glutathione must be abandoned. In the earlier work (1924–5) it had been found that H, unlike its sulphone when free from dithio compounds, did not abolish the nitroprusside reaction of the fixed -SH groups in tissues; this was set aside at the start of the war, erroneously as it turned out. Other workers (van Heyningen, 1941; Bailey and Webb, 1944; Banks *et al.*, 1946) as well as ourselves (Peters and Wakelin, 1947) have concluded that in proteins the attack of H is only partly upon the -SH groups and that it combines with other groups like COOH. In view of this, it cannot yet be decided how H attacks the pyruvate oxidase system. In another communication (Peters and Wakelin, 1947) the suggestion was made that potentiation by diethyl dithiocarbamate directs the attack of H to the -SH groups. In this relation, with work upon lacrimators, vesicants, and -SH groups, the work of Z. Bacq and his colleagues (for summary see Z. Bacq, 1946) is of interest.

In regard to the toxicity of HO_2 and of the arsenicals to the pyruvate oxidase system, it is interesting to note that Fell and Allsopp (1939, 1946) have found for the lethal doses to tissue culture preparations, 11–18 μ*M* for lewisite oxide and 75 μ*M* for HO_2; these amounts are surprisingly close to those which inactivate this enzyme. If it is not the pyruvate system which is attacked, it must be some as yet unknown enzyme of equal sensitivity.

Note: Since completion of this report, it has been concluded that the pyruvate oxidase system as studied here includes the tricarboxylic acid cycle (Coxon, Liébecq, and Peters; see Peters, Dixon lecture, 1948).

SUMMARY

1. The toxicity of several non-arsenical vesicants and related substances to the pyruvate oxidase system in pigeon brain has been determined.

2. The most toxic substances were dichlorodiethylsulphone (HO_2) and divinylsulphone; it is

suggested that these compounds attack an -SH group.

3. Mustard gas (H) was less toxic ; but with the help of emulsification with lecithin it could serve as a test for antidotes. There is evidence that it is not attacking the -SH group alone.

4. All three substances attacked the pyruvate system selectively, and the pyruvate components of the total oxidase system ; the toxic action upon succinic dehydrogenase, malate dehydrogenase, and lactate dehydrogenase was much less marked, and no toxic action upon amino-acid oxidase was observed with the concentrations used.

We wish to acknowledge the help of members of the team during this work, which was carried out during the war for the Ministry of Supply. We are grateful to the Chief Scientific Officer for permission to publish, to Dr. Ing and Mr. Philpot for diethanoldithiocarbamate, and to Prof. Keilin for preparations of cytochrome C and succinodehydrogenase.

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