Reactivation by Dithiols of Enzymes Inhibited by Lewisite

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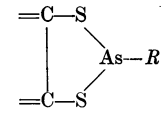
The protein component of a number of enzyme systems contains SH groups which are either freely reacting or sluggish. The freely reacting SH groups give the nitroprusside test in the native protein, are easily oxidized by mild oxidizing agents and alkylating agents in low concentrations (iodoacetate, mustard gas, diphosgene, cyanogen chloride), and readily form mercaptides. On denaturation, the sluggish SH groups become freely acting. Sulphydryl enzymes are widely distributed, existing among the enzymes concerned with the metabolism of proteins, fats and carbohydrates. Since the metabolism of foodstuffs is performed by a series of enzymic reactions which in some steps are linked to each other, it follows that inhibition of sulphydryl enzymes will create profound disturbances in the metabolism of the body. Inhibition of any of these SH enzymes by oxidizing agents or mercaptide-forming agents can be counteracted by addition of thiol compounds. In fact, thiols were used successfully years ago by Voegtlin, Dyer & Leonard (1923), Voegtlin, Rosenthal & Johnson (1931), and by Eagle (1939), in the treatment of arsenical poisoning in animals. Monothiols, however, were often found inefficacious. The introduction of dithiols (Peters, Stocken & Thompson, 1945) has marked a great progress in the reactivation of SH enzymes inhibited by oxidizing or mercaptide-forming agents, and hence in the treatment of intoxications produced by these substances.

This paper is concerned with the inhibition of SH enzymes by lewisite and their reactivation by dithiols.

EXPERIMENTAL AND RESULTS

Inhibitions produced by arsenic and subsequent reactivation

It is well known that trivalent arsenic combines with thiol compounds giving thioarsinites which are soluble and can be dissociated quite easily (Cohen, King & Strangeways, 1931). If the arsenical combines with the SH groups of sulphydryl enzymes, this combination may be broken by addition of thiol compounds. Stocken & Thompson (1941) postulated on the basis of their studies on kerateine solutions treated with lewisite that the thioarsinites thus formed had the configuration:



Bartlett, Cohen, Dauben, Rosen & Ryan (personal communication) confirmed the cyclic nature of the dithiol compounds with lewisite. It was thought then that dissociation of the thioarsinites formed with the SH groups of the protein molecule could be more easily performed by thiols possessing the property of forming more stable cyclic compounds. In fact, Stocken & Thompson (1941) found that brain pyruvate oxidase inhibited by lewisite could be reactivated by 2:3-dimercaptopropanol (BAL), while equal concentrations of cysteine had no effect at all.

Succinoxidase is a sulphydryl enzyme (Hopkins & Morgan, 1938). It was inhibited 96% by 5×10^{-5} M-lewisite and was completely reactivated on addition of $2{\cdot}5 \times 10^{-4}$ M-BAL. On addition of $1{\cdot}25 \times 10^{-4}$ M-dithiol there was 83% reactivation. (To produce this reactivation, 2·5 mol. of dithiol were thus necessary against 1 mol. of lewisite.) Addition of 5×10^{-4} M-glutathione (GSH) (equivalent in SH groups to $2{\cdot}5 \times 10^{-4}$ M-dithiol) produced only 23% reactivation. Liver choline oxidase was inhibited 74% by 5×10^{-4} M-lewisite; on addition of $1{\cdot}25 \times 10^{-3}$ M-dithiol there was 37% inhibition, i.e. the enzyme was reactivated 37%. This partial reactivation is probably due to rapid oxidation of dithiol by the ground liver preparation. The oxidation of ethanol by yeast alcohol oxidase was 40% inhibited on addition of $2{\cdot}5 \times 10^{-4}$ M-lewisite; 1×10^{-3} M-dithiol produced 20% reactivation (Table 1).

In Table 2 is given a list of the enzyme systems which were tested with lewisite. It can be seen that not all SH enzymes were inhibited by lewisite, striking examples being yeast carboxylase and *d*-amino-acid oxidase; both, however, are inhibited by *p*-chloromercuric benzoic acid and reactivated by glutathione (Barron & Singer, 1945; Singer & Barron, 1945).

Table 1. *Reactivation by thiols of enzymes inhibited by lewisite*

Enzyme	Lewisite (M)	Inhibition (%)	GSH Molarity	GSH Reactivation (%)	BAL Molarity	BAL Reactivation (%)
Succinoxidase	5×10^{-5}	96	5×10^{-4}	23	$2{\cdot}5 \times 10^{-4}$	Complete
Choline oxidase	5×10^{-4}	74	—	—	$1{\cdot}25 \times 10^{-3}$	37
Alcohol oxidase (yeast)	$2{\cdot}5 \times 10^{-4}$	40	—	—	1×10^{-3}	20

Table 2. *Effect of lewisite on the activity of isolated enzyme systems*

Enzyme system	Lewisite concentration (M)	Substrates	Determination	Inhibition (%)
Arginase (liver)	1×10^{-4}	Arginine	Urea formation	None
	1×10^{-3}	Arginine	Urea formation	87
Alcohol oxidase, yeast (activating protein)	5×10^{-4}	Ethanol	Diphosphopyridine nucleotide reduction	60
Alcohol oxidase, liver (activating protein)	1×10^{-3}	Ethanol	Diphosphopyridine nucleotide reduction	None
Lactic oxidase, liver (activating protein)	1×10^{-3}	Lactate	Diphosphopyridine nucleotide reduction	None
Uricase	1×10^{-3}	Uric acid	O_2 uptake	None
Pepsin	1×10	Haemoglobin	Tyrosine formation	None
Trypsin	1×10	Haemoglobin	Tyrosine formation	None
Choline oxidase, liver	5×10^{-4}	Choline	O_2 uptake	72
Choline esterase, serum	1×10^{-4}	Acetylcholine	Acetylcholine hydrolysis	85
Cytochrome oxidase, muscle	1×10^{-3}	Hydroquinone	O_2 uptake	None
Succinoxidase, muscle (activating protein)	5×10^{-5}	Succinate	O_2 uptake	96
Catalase	1×10^{-3}	H_2O_2	H_2O_2 left undestroyed	None
Transaminase, muscle	1×10^{-3}	Pyruvate + glutamate	Pyruvate disappearance	33
Diamine oxidase, kidney	1×10^{-3}	Histamine	O_2 uptake	None
Carboxylase, yeast	1×10^{-3}	Pyruvate	CO_2 production	None
Urease	1×10^{-3}	Urea	CO_2 production	None
Citric dehydrogenase, liver	1×10^{-3}	Citrate	Cresyl blue reduction	None
Pyruvate oxidase (gonococci)	1×10^{-4}	Pyruvate	O_2 uptake	Complete
Starch phosphorylase	1×10^{-3}	Hexose-1-phosphate	Inorganic P formation	None
Acid phosphatase	1×10^{-3}	β-Glycero-phosphate	Inorganic P formation	24
Alkaline phosphatase	1×10^{-3}	β-Glycero-phosphate	Inorganic P formation	None
d-Amino-acid oxidase	1×10^{-4}	*dl*-Alanine	Pyruvate formation	None

Table 3. *Effect of lewisite on tissue respiration*

(Tissue slices; lewisite added after neutralization in water, final concentration 1×10^{-4} M. Buffer, Ringer-phosphate pH 7·4. Temp. 38°. Figures give μl./hr./mg. dry wt.)

Tissue	Substrate	O_2 uptake Control (μl.)	O_2 uptake Inhibitor (μl.)	Inhibition (%)
Kidney (rat)	None	18·47	6·62	64
Kidney (rat)	Succinate	27·8	11·05	60
Kidney (rat)*	None	20·6	0·61	97
Kidney (rat)	Pyruvate 1st hr.	30·0	4·2	86
Kidney (rat)	Pyruvate 2nd hr.	32·8	0·5	98·5
Liver (pigeon)	None	7·13	3·87	46
Liver (pigeon)	Pyruvate	8·17	3·99	51
Testis (rat)	Glucose 1st hr.	9·04	5·76	36
Testis (rat)	Glucose 2nd hr.	8·80	2·47	72
Spleen (rat)	Glucose 1st hr.	10·92	6·82	37·5
Spleen (rat)	Glucose 2nd hr.	10·89	3·39	69
Lung (rat)	Glucose 1st hr.	6·96	3·53	49
Lung (rat)	Glucose 2nd hr.	7·05	1·56	78
Brain (rat)	Pyruvate 1st hr.	12·98	1·80	86
Brain (rat)	Pyruvate 2nd hr.	12·08	0·25	98
Kidney (rat)	*dl*-Alanine 90 min.	42·05	12·7	70
Kidney (rat)	*l*(+)-glutamate 1st hr.	45·9	19·6	57
Kidney (rat)	*l*(+)-glutamate 2nd hr.	34·9	4·8	86
Skin (lewisite) applied to skin of living rat)	Glucose	0·47	0	Complete

* Inhibitor concentration 1×10^{-3} M.

Table 4. *The effect of arsenicals on the oxidation of succinate by succinic oxidase (from pigeon breast muscle), and on the respiration of brain slices (rat)*

Inhibitor	Concentration (M)	O_2 uptake: Control (μl.)	O_2 uptake: Inhibitor (μl.)	Inhibition (%)
Succinic oxidase:				
Arsenite	5×10^{-3}	232·5	197	15
p-Carboxyphenyl arsine oxide (I)	5×10^{-5}	217·5	5·3	63
Lewisite	5×10^{-5}	236	10	96
Brain:				
Arsenite	1×10^{-4}	13·9	10·3	26
(I)	1×10^{-4}	13·9	12·5	9·5
Lewisite	1×10^{-4}	13·9	4·6	66·6
Kidney, 1st hr.:				
Arsenite	1×10^{-4}	45·9	33·2	27·6
(I)	1×10^{-4}	45·9	38·3	16·5
Lewisite	1×10^{-4}	45·9	19·6	57·4
Kidney, 2nd hr.:				
Arsenite	1×10^{-4}	34·9	21·9	37
(I)	1×10^{-4}	34·9	20·7	40
Lewisite	1×10^{-4}	34·9	4·8	86

Lewisite previously hydrolyzed in water and neutralized to pH 7·4 inhibited to a great extent the respiration of tissue slices; the degree of inhibition in every case increased with time. Thus, for instance, the respiration of rat testis was inhibited 36 % in the first hour after addition of 1×10^{-4} M-lewisite and 72 % in the second hour. Lewisite applied to the skin of a living rat which was killed 2 hr. later inhibited the respiration of skin slices completely (Table 3).

A comparison of the inhibiting power of arsenite (arsenious oxide neutralized to pH 7·4), of organic arsenicals used in the treatment of syphilis, and lewisite shows that the divinyl group of lewisite greatly enhanced the inhibiting action of arsenic on tissue respiration (Table 4). The same results were obtained with succinoxidase. Arsenite (5×10^{-3} M) inhibited 15 %; *p*-carboxyphenylarsine oxide (5×10^{-5} M) inhibited 63 %; and lewisite (5×10^{-5} M) inhibited 96 %. In yeast alcohol dehydrogenase, arsenite at a concentration of 5×10^{-4} M produced only a slight inhibition; $2{\cdot}5 \times 10^{-4}$ M-*p*-carboxyphenylarsine oxide inhibited by 30 %, and $2{\cdot}5 \times 10^{-4}$ M-lewisite by 60 %. Addition of BAL reduced the inhibition to 40 % (Fig. 1).

With kidney slices and pyruvate as the oxidizable substrate, 5×10^{-5} M-lewisite produced 67 % inhibition on the O_2 uptake. On addition of BAL ($2{\cdot}5 \times 10^{-4}$ and $1{\cdot}25 \times 10^{-4}$ M) 20 min. after the addition of lewisite there was complete reactivation of the rate of respiration. On the other hand, the addition of $2{\cdot}5 \times 10^{-3}$ M-glutathione produced only 17 % reactivation, while 5×10^{-4} M had no effect at all (Table 5). Similar results were obtained when the utilization of pyruvate by kidney slices was measured.

Lewisite (5×10^{-5} M) inhibited the utilization of pyruvate by 88 %. This inhibition was completely released on addition of $2{\cdot}5 \times 10^{-4}$ M-BAL; a concentration of $1{\cdot}25 \times 10^{-4}$ M, i.e. 2·5 times the concentration of lewisite, released the inhibition by 94 %.

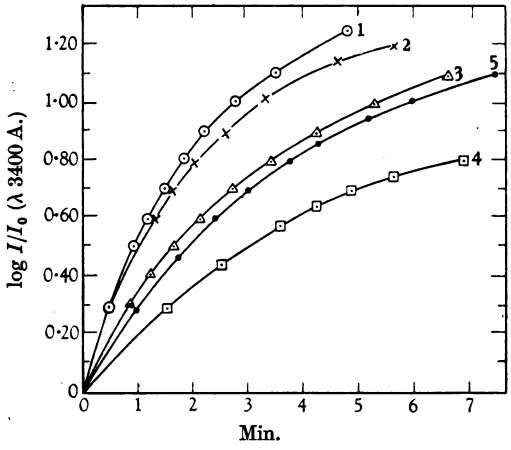


Fig. 1. Inhibition of yeast alcohol dehydrogenase by arsenicals, as measured by the rate of reduction of diphosphopyridine nucleotide (DPN). Temp. 25°, pH 7·7, DPN added at time 0.

1, control; 2, Na arsenite, 5×10^{-4} M; 3, *p*-carboxyphenylarsine oxide, $2{\cdot}5 \times 10^{-4}$ M; 4, lewisite, $2{\cdot}5 \times 10^{-4}$ M; 5, lewisite + BAL, 1×10^{-3} M.

Glutathione, however, produced only slight reactivation, a concentration of $2{\cdot}5 \times 10^{-3}$ M reactivating by 20 %. Similar results were obtained with brain tissue by Peters, Sinclair & Thompson (1946).

Table 5. *Effect of lewisite on the O_2 uptake and pyruvate utilization by the kidney: reactivation with glutathione and BAL added 15 min. after lewisite*

(Buffer, Ringer-phosphate, pH 7·4; pyruvate, 0·01 M; lewisite, 5×10^{-5} M; temp. 38°; O_2 uptake in μl./mg. dry wt./hr; pyruvate utilization, μmol./mg./90 min.)

Exp. conditions	Reactivator conc. (M)	O_2 uptake	Inhibition (%)	Pyruvate utilization	Inhibition (%)
Control	—	28·3	—	2·28	—
Lewisite	—	9·3	67	0·28	88
Lewisite + BAL	$2·5 \times 10^{-4}$	31·5	None	2·63	None
Lewisite + BAL	$1·25 \times 10^{-4}$	32·9	None	2·15	6
Lewisite + GSH	$2·5 \times 10^{-3}$	14·2	50	0·47	79·5
Lewisite + GSH	5×10^{-4}	8·6	70	0·35	84·5

Table 6. *Inhibition by lewisite of the oxidation of α-ketoglutarate by kidney slices*

(Lewisite, 1×10^{-4} M; reactivation by BAL ($2·5 \times 10^{-3}$ M) and glutathione (5×10^{-3} M); O_2 uptake in μl./mg. dry wt./hr.)

			Ketoglutarate	
Inhibitor	O_2 uptake	Inhibition (%)	Utilization (%)	Inhibition (%)
Control	21	—	98	—
Lewisite	5·9	72	14	87
Lewisite + glutathione	13·7	34	52	47
Lewisite + BAL	19·5	6	83	15·5

The oxidation of α-ketoglutarate by kidney slices, as determined by the oxygen uptake and the utilization of α-ketoglutarate, was inhibited by lewisite (1×10^{-4} M). The inhibition of the O_2 uptake was 72 %; the inhibition of α-ketoglutarate utilization, 87 %. BAL (5×10^{-3} M) produced a reactivation on the O_2 uptake of 66 %, and of 72 % on the utilization of α-ketoglutarate. Glutathione at the same concentration was less effective (38 % reactivation of the O_2 uptake and 40 % of α-ketoglutarate utilization) (Table 6).

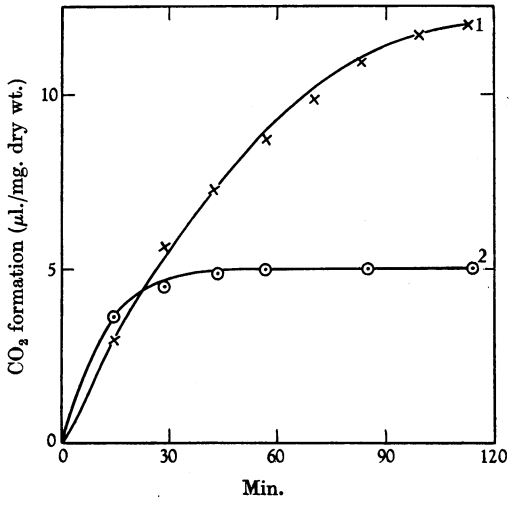


Fig. 2. Effect of lewisite on brain glycolysis when added immediately before measurement of the rate of glycolysis (CO_2 formation). Buffer, Ringer-bicarbonate, pH 7·4; N_2-CO_2 as gas phase. 1, control; 2, effect of 1×10^{-4} M-lewisite.

When lewisite (neutralized) was added immediately before measurement of the rate of glycolysis in brain slices (CO_2 production in Ringer-bicarbonate buffer) inhibition did not start until 15 min. later, reaching its maximum at the end of 45 min. (Fig. 2). The inhibition produced on addition of 1×10^{-5} M-lewisite was completely released by $2·5 \times 10^{-4}$ M-BAL. Glutathione, on the other hand, prevented the inhibition when added before the addition of lewisite; it produced a reactivation of 37 % when added 10 min. later, and no effect at all when added 30 min. later (Table 7).

The synthesis of carbohydrate from pyruvate by rat kidney slices was completely inhibited with 1×10^{-4} M-lewisite; 1×10^{-5} M inhibited by 50 %, and 5×10^{-6} M by 15 %. There are many steps in the series of enzymic reactions from pyruvate to carbohydrate where the protein moiety contains SH groups susceptible to inhibition by lewisite. The synthesis of acetoacetate from pyruvate by minced pigeon liver in the presence of malonate was inhibited by lewisite (Table 8).

DISCUSSION

Of the numerous arsenicals used for the inhibition of SH enzymes, lewisite was usually the most effective as shown in experiments with succinoxidase, yeast alcohol dehydrogenase and in experiments where the respiration of tissue slices was measured; the organic trivalent arsenicals came next in inhibitory power; and the arsenites were least effective. This greater affinity of lewisite for the SH groups of

Table 7. *Inhibition of the anaerobic glycolysis of brain slices (rat) by lewisite and reactivation with glutathione and BAL*

(Buffer, Ringer-bicarbonate; gas phase, N_2:CO_2; pH 7·4; temp. 38°; glucose concentration, 0·01 M; lewisite, 1×10^{-4} M.)

Exp. conditions	Reactivation concentration (M)	$Q^{N_2}_{CO_2}$ (µl.)	Inhibition (%)
Control	—	7·78	—
Lewisite	—	0·82	89
Lewisite + BAL	5×10^{-4}	10·14	None
Lewisite + BAL	$2{\cdot}5 \times 10^{-4}$	11·68	None
Lewisite + GSH added 10 min. later	5×10^{-3}	3·76	51·6
Lewisite + GSH added 30 min. later	5×10^{-3}	1·59	80
GSH before lewisite	5×10^{-4}	7·80	None

Table 8. *Effect of lewisite on the synthesis of carbohydrate by kidney slices (rat) and the synthesis of acetoacetate by minced pigeon liver*

Carbohydrate synthesis: Buffer, Ringer-$NaHCO_3$; gas phase, O_2:CO_2; pH 7·4; pyruvate concentration, 0·02 M; temp. 38°; incubation time, 3 hr.

Acetoacetate synthesis: Minced pigeon liver suspended in 0·121 M-KCl + 0·02 M-phosphate, pH 7·4; 0·02 M-pryuvate + 0·02 M-malonate. (1 µmol. acetoacetate = 22,400 µl.)

	Carbohydrate synthesis		Acetoacetate synthesis	
Lewisite conc. (M)	(µmol. glucose/ 100 mg. tissue)	Inhibition (%)	(µl./hr.)	Inhibition
None	3·62	—	274	—
1×10^{-4}	0·35	97	—	—
1×10^{-5}	1·18	50	—	—
5×10^{-6}	3·08	15	—	—
5×10^{-4}	—	—	0	Complete

enzymes must be due to the presence of the vinyl groups in the molecule, for the lewisite used in all the experiments was previously hydrolyzed and made neutral, existing presumably as Cl.CH:CH.AsO. In general, lewisite combines rapidly with the freely reacting SH groups while it has no action on the sluggish SH groups of the protein molecule. There were, however, three notable exceptions: *d*-amino-acid oxidase and yeast carboxylase which are reversibly inhibited by *p*-aminophenyldichloroarsine-HCl (Barron & Singer, 1945) were not inhibited by lewisite; and transaminase, which was largely inhibited by *p*-carboxyphenylarsine oxide (81 % inhibition), was only 33 % with the same concentration of lewisite. Possibly these differences are due to the spatial distribution of the SH groups in the protein molecule. Among the inhibitions produced by lewisite, that of carbohydrate synthesis from pyruvate is extremely interesting, not only because it is produced at very low concentrations but also because it may help us to understand the mechanism of synthesis. Of all the reactions where pyruvate takes part and where SH enzymes are required (oxidation, dismutation, carboxylation, decarboxylation, transamination) it is the oxidative reaction which is the most sensitive to the inhibiting action of lewisite as was demonstrated for the first time by Peters *et al.* (1940, 1946). Since the amounts of lewisite required to produce complete inhibition of carbohydrate synthesis have only a partial effect in all pathways of pyruvate metabolism except the oxidative pathway, it is reasonable to postulate that the synthesis of carbohydrate from pyruvate starts with the formation of an active acetyl group produced by the oxidation of pyruvate. Synthesis would proceed via condensation of this acetyl group. The great superiority of BAL on producing reactivations of enzyme inhibition by lewisite shown by the Oxford workers has been confirmed and extended in this paper.

SUMMARY

1. Among the trivalent arsenical compounds (organic arsenicals, arsenite, lewisite) able to form reversible thioarsinites, lewisite has in general the greatest affinity for the SH groups of the protein moiety of enzymes requiring those groups for activity. It inhibited all the sulphydryl enzymes with the exception of *d*-amino-acid oxidase, yeast carboxylase, and transaminase. In every case enzyme inhibition was reversed by the addition of BAL, which was found to be a better reversal agent than glutathione.

2. Lewisite inhibited the respiration of tissue slices, the inhibition being reversed on addition of BAL and to a lesser degree on addition of glutathione. It inhibited anaerobic glycolysis, this inhibition

being reversed completely by BAL and only partially by glutathione. Lewisite inhibited also the synthesis of carbohydrate from pyruvate by kidney slices.

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* Information available on application to Ministry of Supply, London.

The Action of British Anti-lewisite (BAL) on Enzyme Systems*

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It has been shown by Peters and others that 2:3-dimercaptopropanol (BAL) can be used as an antidote to lewisite, and can prevent vesication of human skin by lewisite if applied within 1 hr. after contamination. It was also shown not only to protect the arsenic-sensitive pyruvic oxidase from the inhibitory action of the arsenical *in vitro*, but to reverse poisoning which had already occurred, owing to its high affinity for arsenic. The work on BAL has been briefly reviewed by Peters, Stocken & Thompson (1945). BAL is itself toxic in large doses, with an LD_{50} of 110 mg./kg. by subcutaneous injection in rats. In an attempt to study the cause of this toxicity the action of BAL on a large number of enzymes was investigated *in vitro*.

EXPERIMENTAL

Methods. For each enzyme the conditions of estimation (amount of enzyme, etc.) were so arranged that the enzyme concentration was limiting, i.e. such that the rate of reaction was proportional to the amount of active enzyme present.

A pure specimen of BAL, obtained from Prof. Peters, was used throughout this work as a stock M/4 solution in ethylene glycol monoethyl ether. The required concentration of BAL was added to the enzyme solution in buffer and incubated with it for 15 min. before starting the reaction by addition of the substrate. Zymohexase and creatine phosphokinase† were tested in veronal buffer at pH 7·4, pyrophosphatase in veronal buffer at pH 7·9, and *d*-amino-acid oxidase in phosphate buffer at pH 7·7. All other experiments were carried out at pH 7·4 in phosphate or bicarbonate buffer.

Enzymes. These were prepared from the following materials: polyphenol oxidase from mushrooms (Keilin & Mann, 1938); carbonic anhydrase from mammalian erythrocytes (Keilin & Mann, 1940); catalase from ox liver (Sumner & Dounce, 1939); peroxidase from cow's milk (Elliott, 1932); aldehyde mutase from ox liver (Dixon & Lutwak-Mann, 1937); phosphorylase from potato (Hanes, 1940); xanthine oxidase from milk (Dixon & Thurlow, 1924); *d*-amino-acid oxidase from ox kidney (Krebs, 1935); cholinesterase from horse serum (Stedman & Stedman, 1935) and from rabbit brain (a strained tissue-suspension in bicarbonate-Ringer); glyoxalase from ox liver (a dialyzed extract of acetone-dried tissue); pyro-

* This work formed a part of the research programme carried out for the Ministry of Supply by an Extra-Mural Research team under the direction of Dr M. Dixon, and was reported to the Ministry in 1943 (Webb & van Heyningen, 1943).

† The term phosphokinase has been suggested by Needham & Dixon (1942) as a general name for enzymes of the hexokinase group which catalyze reactions of the type

$$X + \text{Adenosinetriphosphate} \rightleftharpoons X\text{-phosphate} + \text{Adenosinediphosphate}.$$