pH) most of this lipid again became extractable only with difficulty.

5. The parallelism between the two criteria of decomposition used, namely dispersibility in sodium chloride solution-ether and extractability of the lipid with ether, was not complete.

The authors are indebted to Dr K. McQuillen for assistance with the mobility measurements. The work described in this paper has been carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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The Effects and the Fate of Malononitrile and Related Compounds in Animal Tissues

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(Received 29 December 1951)

It is generally accepted that the toxicity of aliphatic nitriles in the animal body is mainly due to a gradual release of cyanide. The evidence on which this view is based consists (a) in the similarity of the symptoms of intoxication to those of cyanide poisoning, with due consideration of the different rates at which cyanide is liberated from different nitriles, (b) in the antidotal effect of thiosulphate, and (c) in the increased excretion of thiocyanate (cf. Hunt, 1923). The second and third facts are to be attributed to the action of the enzyme rhodanese which catalyses the reaction (Lang, 1933):

The formation of cyanide from nitriles has, however, not yet been demonstrated *in vitro*. Lang (1933) investigated the formation of SCN^- from acetonitrile and propionitrile by minced tissues with negative results. Warburg (1911; Warburg & Wiesel, 1912), who found an inhibition of the respiration of avian erythrocytes and of the fermentation of yeast cells by high concentrations of mononitriles, came to the conclusion that this was an unspecific 'narcotic' effect.

Recently, Hydén & Hartelius (1948) described an effect of malononitrile which they could not reproduce with cyanide. Using the microspectrographic method they found, in experiments on rabbits, an increase in the concentration of cytoplasmic protein and nucleoprotein of ganglion cells 1-48 hr. after an injection of malononitrile. Their observations are at variance with the results of Hicks (1950), Liu (1951) and Reale & D'Argenio (1951) using histological techniques, and with those of Samuels, Boyarsky, Gerard, Libet & Brust (1951) using chemical methods. Nevertheless, the general importance of the claims of Hydén & Hartelius and the particular interest arising from their recommendation of malononitrile therapy in mental diseases suggested an investigation into the effects of malononitrile and other nitriles on the metabolism of tissue slices, and into the correlation of any such effects with the release of cyanide. As Heymans & Masoin (1897) have shown, the toxic action of malononitrile is fast; it seemed therefore well suited for short *in vitro* experiments.

Another class of compounds from which cyanide is gradually liberated, in the body as well as in

presence of tissue preparations, is the group of alkyl thiocyanates, especially the lower homologues of the series (Oettingen, Hueper & Deichmann-Gruebler, 1936). Methyl and ethyl thiocyanates were therefore included amongst the substances studied.

EXPERIMENTAL

Methods

Measurement of tissue metabolism. Tissue slices were used for metabolism experiments. They were cut free-hand with a safety-razor blade moistened with Ringer solution, pooled and kept on ice. The manometer vessels were also kept on ice until they were attached to the manometers. All results were calculated on the basis of the initial dry weight: the slices, freed from adhering moisture by filter paper, were weighed on the torsion balance and the dry weight calculated from the ratio determined on a separate sample which was removed from the pool simultaneously with the other slices and dried at 100°. Owing to the swelling of the slices in Ringer solution the ratio for brain slices varied between 5.5 and 7.0.

When respiration alone was measured, 'medium III', a saline low in phosphate and bicarbonate, but including organic salts (Krebs, 1950), was used. In other experiments glycolysis was determined by chemical estimation of lactic acid while respiration was recorded manometrically. The medium used for these experiments differed from medium III only by the omission of the three organic salts and their replacement by isotonic NaCl; it will be referred to as 'medium III, modified'. Respiration was followed in conical Warburg flasks containing NaOH in the inner well and O₂ in the gas space. When lactic acid was determined chemically, 0.1 vol. of 30% (w/v) trichloroacetic acid was added from the side bulb at suitable intervals. The first vessel was acidified at the time of starting the readings and served as initial value. Lactic acid estimations were usually carried out on 0.3 ml. of filtrate by the method of Barker & Summerson (1941) as modified by Umbreit, Burris & Stauffer (1945).

In another series of experiments respiration and glycolysis were determined simultaneously by the two-vessel method of Warburg (1924) in a bicarbonate-glucose medium (Krebs & Henseleit, 1932) with O₂-5% CO₂ as gas phase.

Respiratory quotients (R.Q.) were determined in bicarbonate medium by the method of Dickens & Šimer (1931).

Anaerobic glycolysis was measured manometrically in bicarbonate medium in an atmosphere of N₂-5% CO₂. The values for the aerobic glycolysis recorded in Figs. 1 and 2 have been expressed as a percentage of the anaerobic glycolysis. In these experiments slices were incubated in medium III, modified, in N₂, for 30 min. and lactic acid was determined by chemical estimation. This was done in order to measure aerobic and anaerobic glycolysis under identical conditions.

For the investigation of the effects of cyanide, Dickens-Šimer vessels (bicarbonate type) were used. The outside circular trough contained 1 ml. of alkaline KCN solution (Umbreit *et al.* 1945). Medium and tissue were introduced into the inner chamber and, after the vessels had been gassed, they were quickly removed from the manometers for the addition of the requisite amount of neutral KCN to the medium. All tissue slice experiments were done at 37°.

Tissue homogenates and extracts. Tissues were homogenized in an all-glass homogenizer (Potter & Elvehjem, 1936) with ice-cooled, glass-distilled water, usually in the ratio 1:10. For the preparation of extracts, the homogenates were centrifuged for 10 min. in an angle centrifuge at 3500 rev./min. and the supernatant was used. When experiments were carried out with boiled preparations, the required volume was pipetted into the empty reaction flask, which was stoppered and put in a boiling-water bath for 10 min. After cooling, the other constituents were added.

Homogenates and extracts of liver were prepared from rats which had been starved for 24 hr. before death.

Estimation of cyanide. The method of Epstein (1947), as modified by Boxer & Rickards (1951), has been adapted for our purpose. Aeration was carried out in 4-5 trains connected in series to a water-jet pump. Before entering the train the air stream was passed through 2N-NaOH; it was then led through the sample containing up to 2 μ moles CN⁻, through a trap containing 20 ml. acid ceric sulphate (0.5N-solution in N-H₂SO₄) and finally through two collection tubes each containing 2 ml. 0.1N-NaOH. Entrainment of spray was prevented by suitable traps. Fresh ceric sulphate solution was used for each run and the train was aerated for about 1 hr. before the addition of the cyanide and NaOH solutions. Thereafter aeration was continued, at a moderate rate, for 90 min. The cyanide samples usually contained 5% trichloroacetic acid and required no further acidification.

Various modifications were also applied to the colorimetric procedure. The following stock solutions were used: (1) chloramine T, 1%; (2) 3-methyl-1-phenyl-5-pyrazolone (recrystallized three times), 0.4% solution in pyridine; (3) bis(3-methyl-1-phenyl-5-pyrazolone), 0.1% solution in pyridine. Solutions 2 and 3 were stored in a freezing compartment. Immediately before the analysis, 1 ml. of solution 1 was mixed with 3 ml. m-NaH₂PO₄ (solution 4; cf. Boxer & Rickards, 1951). Further, a pyrazolone-pyridine reagent (solution 5) was prepared by mixing 1 part of solution 2, 1 part of solution 3 and 6 parts of water. A 1 ml. sample containing not more than 0.3 μ mole CN⁻ was cooled in ice, 0.2 ml. of solution 4 were added and, after thorough mixing, the tube was left at 0° for 2 min. Then 6 ml. of solution 5 were added and the mixture was kept at 30° for 30 min. Ilford spectrum filter no. 608 was used for the photoelectric measurement.

Malononitrile, methyl and ethyl thiocyanate did not interfere with the recovery of CN⁻; they did, however, slightly increase the reagent blank, probably owing to the presence of traces of CN⁻ in the solutions.

Estimation of thiocyanate. 2 ml. of ferric nitrate solution (Cosby & Sumner, 1945) were added to 8 ml. of solution containing 0.2-4.0 μ mole SCN⁻. The red colour was determined photoelectrically after 15 min. with Ilford spectrum filter no. 601. A calibration curve was constructed with solutions containing the same concentration of buffer, thiosulphate and trichloroacetic acid as the unknown sample, and a standard solution and reagent blank were included with each series of estimations. Neither malononitrile nor the alkyl thiocyanates interfered with the reaction.

For deproteinization, the sample was mixed with an equal amount of 10% (w/v) trichloroacetic acid containing 0.14% formaldehyde (Rosenthal, Rogers, Vars & Ferguson, 1950).

Estimation of cozymase. The deproteinized filtrates were freed from trichloroacetic acid by three extractions with

peroxide-free ether (about 5 ml. each time). Ether was removed from the aqueous solution *in vacuo* at about 30°. The pH was adjusted to 6.4 and the volume made up to about ten times the weight of fresh tissue used. Cozymase concentration in the extract was determined by the apozymase test at 20°, as described by Schlenk & Schlenk (1947).

Cozymase, prepared by the method of LePage (1947), was standardized spectrophotometrically according to Kornberg (1950) and had a purity of 48.5%.

Materials

Dimethylmalononitrile. Dimethylcyanacetamide was prepared from cyanacetamide (Corson, Scott & Vose, 1932) according to Errera (1896), and was dehydrated with PCl_5 as described for the preparation of malononitrile (Corson, Scott & Vose, 1943). Owing to the tendency of dimethylmalononitrile to sublime, the distillate was collected at -20° and the solidified product was repeatedly washed with ice-cold water and dried over P_2O_5 at atmospheric pressure; m.p. 30–32°.

All other substances were commercial products which were used without further purification. Malononitrile, redistilled *in vacuo* immediately before use, gave results which

in no way differed from those obtained with the preparation before distillation. The crystals of malononitrile (m.p. 32°) were protected from light and stored at 2°. We did not notice any signs of decomposition under these conditions for many months.

RESULTS

The effects of nitriles and related compounds on tissue metabolism

The respiration of brain, kidney and liver slices is strongly inhibited by 0.01 M-malononitrile (Tables 1 and 2). The inhibition is progressive with time and is practically complete at the end of the second hour. In a bicarbonate medium, the respiration of brain and kidney slices is not significantly inhibited by 0.01 M-malononitrile during the first hour of the experiment, but in the second hour the effect is as strong as in phosphate medium. At the same time the aerobic glycolysis of brain slices is increased; it reached, in some experiments, 80–90% of the level of the anaerobic glycolysis. Malononitrile, in

Table 1. *Effect of malononitrile on the respiration of tissue slices*

(Media: III = medium III (Krebs, 1950); III mod. = medium III, modified (cf. Methods section, p. 115); Additions: MN = malononitrile (0.01 M); 0 = none. Q_{O_2} values are in $\mu\text{l./mg. dry wt./hr.}$)

No.	Species	Tissue	Medium	Addition	Q_{O_2}		Inhibition (%)	
					1st hr.	2nd hr.	1st hr.	2nd hr.
1	Rabbit	Brain	III	0	-12.7	-10.1	—	—
				MN	-8.3	-4.5	35	55
2	Rat	Brain	III	0	-15.6	—	—	—
				MN	-10.3	—	34	—
3	Rat	Kidney	III	0	-27.0	-26.0	—	—
				MN	-20.0	-12.0	26	54
4	Rat	Kidney	III	0	-29.0	-24.0	—	—
				$\text{Na}_2\text{S}_2\text{O}_3^*$	-27.0	-21.0	7	12
				MN	-19.0	-5.7	35	76
				$\text{MN} + \text{Na}_2\text{S}_2\text{O}_3^*$	-23.0	-4.0	15‡	81‡
5	Rat	Liver	III	0	-11.6	-11.9	—	—
				MN	-6.8	-1.8	41	85
6	Rat	Liver	III	0	-11.4	-10.4	—	—
				$\text{Na}_2\text{S}_2\text{O}_3^*$	-10.1	-8.8	11	15
				MN	-6.4	-1.5	44	85
				$\text{MN} + \text{Na}_2\text{S}_2\text{O}_3^*$	-7.6	-2.3	25‡	74‡
7	Rat	Kidney	Bicarbonate	0	-20.3	-16.8	—	—
				$\text{Na}_2\text{S}_2\text{O}_3^\dagger$	-22.2	-11.8	0	30
				$\text{MN} + \text{Na}_2\text{S}_2\text{O}_3^\dagger$	-7.9	-2.2	64‡	81‡
8	Rat	Liver	Bicarbonate	0	-13.2	-12.4	—	—
				$\text{Na}_2\text{S}_2\text{O}_3^\dagger$	-13.3	-12.9	0	0
				MN	-4.9	-1.5	63	88
				$\text{MN} + \text{Na}_2\text{S}_2\text{O}_3^\dagger$	-3.0	-2.3	77‡	82‡
9	Rat	Kidney	III mod.	0	-16.7	-16.2	—	—
				$\text{Na}_2\text{S}_2\text{O}_3^\dagger$	-12.3	-11.7	26	28
				MN	-8.8	-5.9	47	64
				$\text{MN} + \text{Na}_2\text{S}_2\text{O}_3^\dagger$	-10.7	-5.7	13‡	51‡
10	Rat	Liver	III mod.	0	-9.4	-9.2	—	—
				$\text{Na}_2\text{S}_2\text{O}_3^\dagger$	-8.4	-6.8	10	26
				MN	-4.8	-2.6	49	72
				$\text{MN} + \text{Na}_2\text{S}_2\text{O}_3^\dagger$	-7.2	-1.1	14‡	84‡

* 0.05 M.

† 0.035 M.

‡ Calculated on control + $\text{Na}_2\text{S}_2\text{O}_3$.

0.01 M-concentration, had little effect on the anaerobic glycolysis during the first hour (Table 3), but during the second hour both aerobic and anaerobic glycolysis tended to decrease. With lower concentrations of malononitrile there was no falling-off of the aerobic glycolysis during the second hour.

the inhibition of respiration caused by malononitrile was often partly relieved, but in the second hour respiration was equally inhibited in the presence and in the absence of thiosulphate (excepting Exp. 6, Table 2). Thiosulphate did not prevent the rise of the aerobic (Table 2) or the inhibition of the

Table 2. *Effect of malononitrile, methyl and ethyl thiocyanates on respiration and aerobic glycolysis of brain slices*

(Experiments in bicarbonate medium were performed by Warburg's two-vessel method. Where phosphate medium (medium III, modified) was used, lactic acid was determined chemically. Inhibitor concentration 0.01 M.)

No.	Species	Medium	Addition	Q_{O_2}		Inhibition (%)		$Q_G^{O_2}$	
				1st hr.	2nd hr.	1st hr.	2nd hr.	1st hr.	2nd hr.
1	Rat	Bicarbonate	0	-9.1	-9.2	—	—	+1.5	-0.2
			Malononitrile	-11.5	-2.5	0	73	+10.9	+7.2
2	Rat	Phosphate	0	-9.1	-9.5	—	—	+0.7	+0.3
			Malononitrile	-6.2	-3.3	31	65	+5.6	+5.6
3	Rat	Bicarbonate	0	-11.2	—	—	—	+2.7	—
			Methyl thiocyanate	-13.2	—	0	—	+15.2	—
			Ethyl thiocyanate	-12.0	—	0	—	+12.3	—
4	Rat	Phosphate	0	-11.3	-10.2	—	—	+2.2	+1.4
			Methyl thiocyanate	-6.3	-4.0	44	61	+8.8	+6.1
			Ethyl thiocyanate	-6.7	-5.2	41	49	+7.3	+5.1
5	Rat	Phosphate	$Na_2S_2O_3^*$	-8.5	-9.2	—	—	+2.5	+4.1
			Malononitrile + $Na_2S_2O_3^*$	-6.9	-3.5	19	62	+7.5	+8.0
6	Guinea pig	Bicarbonate	0	-8.9	-7.2	—	—	+2.6	+1.8
			Malononitrile	-6.9	-3.2	22	56	+18.0	+9.9
			$Na_2S_2O_3^*$	-7.6	-7.0	15	3	+0.9	+0.7
			Malononitrile + $Na_2S_2O_3^*$	-6.9	-5.5	9†	21†	+14.9	+12.4

* 0.035 M.

† Calculated on control + $Na_2S_2O_3$.

Similar effects were observed with 0.01 M-methyl and ethyl thiocyanates (Table 2), but not with any of the following substances (all in a concentration of 0.01 M): acetonitrile, propionitrile, butyronitrile,

anaerobic glycolysis (Table 3) produced by malononitrile in brain slices. Thiosulphate itself has a slight inhibitory effect on respiration and may somewhat increase the aerobic glycolysis of brain slices, especially in the second hour.

Table 3. *Effects of malononitrile and of thiosulphate on the anaerobic glycolysis of guinea pig brain slices*

Addition	$Q_G^{O_2}$	
	1st hr.	2nd hr.
0	24.8	24.8
Malononitrile (0.01 M)	19.6	11.5
$Na_2S_2O_3$ (0.035 M)	23.7	29.6
Malononitrile + $Na_2S_2O_3$	18.7	13.5

isovaleronitrile, capronitrile, isocapronitrile, acrylonitrile, succinonitrile, glutaronitrile, dimethyl-malononitrile, ethyl cyanoacetate. A slight inhibition of respiration and increase of aerobic glycolysis was found in some cases, but the effects are of doubtful significance. The numerical results of these experiments are therefore omitted.

Thiosulphate, which has such a dramatic effect as an antidote to malononitrile poisoning *in vivo*, has only a comparatively slight action *in vitro* (Tables 1 and 2). In the first hour of the experiment

For comparison, the antagonism between cyanide and thiosulphate was investigated in experiments with tissue slices (Table 4). Although the effects of cyanide were not completely reversed by thiosulphate, they were clearly alleviated. The inhibition of respiration by cyanide, unlike that caused by malononitrile, is not progressive with time; this is probably the reason why the antagonistic effect of thiosulphate is maintained in the second hour in the experiments with cyanide, but not in those with malononitrile.

The determination of respiration and aerobic glycolysis of brain slices by the Dickens-Simer method confirmed the effects of malononitrile found with other methods. The R.Q. was not affected (Table 5).

Concentration effects. Fig. 1 shows the effects of increasing concentrations of malononitrile on the respiration and aerobic glycolysis of rat-brain slices in phosphate medium during the first hour. It appears that concentrations which inhibit

respiration by only about 10% raise the level of aerobic glycolysis to about 50% of that of the anaerobic glycolysis. It is noteworthy that respiratory inhibitions of this order were observed with

100 times smaller than those of malononitrile (Fig. 2). Here, too, aerobic glycolysis is inhibited at high concentrations, possibly owing to cyanohydrin formation.

Table 4. *Effect of cyanide and thiosulphate on the metabolism of rat-tissue slices*

(Phosphate saline (medium III, modified); cyanide 10^{-4} M, thiosulphate 0.035 M.)

No.	Tissue	Addition	Q_{O_2}		Inhibition (%)		$Q_{O_2}^a$ 1st hr.
			1st hr.	2nd hr.	1st hr.	2nd hr.	
1	Kidney	0	-18.8	-16.2	—	—	—
		$Na_2S_2O_3$	-16.2	-16.2	14	0	—
		KCN	-8.3	-7.3	56	55	—
		KCN + $Na_2S_2O_3$	-15.0	-12.4	7.4	23	—
2	Kidney	0	-17.9	-17.2	—	—	—
		$Na_2S_2O_3$	-17.7	-16.7	1	3	—
		KCN	-7.9	-7.0	56	59	—
		KCN + $Na_2S_2O_3$	-15.5	-14.2	12	15	—
3	Liver	0	-8.3	-8.3	—	—	—
		$Na_2S_2O_3$	-8.9	-7.5	0	10	—
		KCN	-4.7	-4.6	43	45	—
		KCN + $Na_2S_2O_3$	-6.8	-5.3	23	29	—
4	Liver	0	-8.4	-7.9	—	—	—
		$Na_2S_2O_3$	-8.7	-6.8	0	14	—
		KCN	-4.8	-3.4	43	57	—
		KCN + $Na_2S_2O_3$	-6.7	-5.4	23	21	—
5	Brain	0	-9.7	—	—	—	2.1
		$Na_2S_2O_3$	-8.9	—	8	—	4.6
		KCN	-3.1	—	68	—	12.1
		KCN + $Na_2S_2O_3$	-5.0	—	44	—	6.3

several of the other nitriles tested without the effect on aerobic glycolysis, suggesting a specific mechanism for the effect of malononitrile in contrast to an unspecific, 'narcotic' effect of other nitriles.

At concentrations of malononitrile greater than 0.03 M, the curve of the aerobic glycolysis shows no further rise, but rapidly declines until, at a concentration of 0.1 M, aerobic glycolysis is completely inhibited. This may be due, at least in part, to a direct reaction of malononitrile with glucose indicated by the appearance of a yellow colour, but, in addition, the unspecific, 'narcotic' action of malononitrile may be expected to become manifest.

Table 5. *Respiratory quotient of guinea pig brain slices in bicarbonate medium (Dickens & Šimer, 1931)*

(Duration of experiment, 2 hr.)

No.	Addition	Q_{O_2}	$Q_{O_2}^a$	R.Q.
1	0	-8.3	+0.6	1.01
	Malononitrile (0.01 M)	-4.5	+12.0	1.08
2	Malononitrile (0.01 M)	-5.7	+8.6	0.96

The curves of respiratory inhibition and aerobic glycolysis are of a similar shape and show a similar relationship to each other when cyanide is used instead of malononitrile, except that the effects occur at concentrations of cyanide which are about

The effect of malononitrile injections on the concentration of lactic acid and cozymase in rat brain

Male white rats were injected subcutaneously with 14 mg./kg. of malononitrile in 0.35% aqueous solution. Though this is a near-lethal dose, none of the injected rats succumbed within 2 hr. The animals were dyspnoeic and cyanotic and were lying quietly in their cages. Convulsions were occasionally seen. In some rats the effects had largely worn off after 2 hr. A second injection, equal to the first, was given to three animals after 1 hr.

The rats were killed after 2 hr. by immersion in liquid oxygen. The brains were dissected while frozen and crushed in a cooled steel mortar (LePage, 1948). The frozen powder was transferred to tared weighing bottles containing 10% trichloroacetic acid; these were then re-weighed. A series of control values was obtained by analysing the brains of normal rats in the same way.

The results obtained after malononitrile injection are collected in Table 6. In the first five rats the values were within the normal variation. Though these animals showed no obvious difference in behaviour and appearance from the rest, the effects of malononitrile had presumably largely subsided when they were killed. The other ten experiments show a significant increase in lactic

Fig. 1. Effect of varying concentrations of malononitrile on the respiration and aerobic glycolysis of rat-brain slices.

Slices suspended in medium III, modified; incubated in O_2 for 1 hr. All points are the means of three to four experiments. Curve A: inhibition of respiration. Curve B: aerobic glycolysis in percentage of anaerobic glycolysis. c , Concentrations of malononitrile and cyanide (M).

Fig. 2. Effect of varying concentrations of cyanide on the respiration and aerobic glycolysis of rat-brain slices.

acid concentration. It is particularly marked in the last three rats which had received a second injection.

Table 6. *Concentration of lactic acid in brains of rats previously injected with malononitrile*

(All rats received an injection (14 mg./kg.) 2 hr. before sacrifice; rats 13, 14 and 15 received a second injection 1 hr. after the first.)

No.	Lactic acid (mg./100 g.)	
	Found	Excess over mean of control series
1	20.4	-9.0
2	24.9	-4.5
3	29.7	0.3
4	31.0	1.6
5	31.4	2.0
6	47.8	18.4
7	49.4	20.0
8	54.2	24.8
9	72.0	42.6
10	82.5	53.1
11	98.0	68.6
12	122.0	92.6
13	103.0	73.6
14	107.0	77.6
15	112.0	82.6

In fifteen control experiments the mean value was 29.4 mg./100 g., s.d. 7.0, s.e. 1.81. Deviation from mean at $P \leq 0.05$, ≥ 15.0 .

These experiments show that the increase of aerobic glycolysis observed with brain slices also occurs in the living brain after an injection of malononitrile.

The concentration of cozymase in rat brain was not significantly changed after malononitrile injection. The mean value found in twelve control rats was 26.74 ± 1.78 mg./100 g. of fresh brain, that in seventeen injected rats, 24.57 ± 1.91 mg. The probability of the difference being due to chance variation is 0.44. The cozymase concentration found in these experiments is midway between the figures reported by Gore, Ibbott & McIlwain (1950) and by Axelrod & Elvehjem (1939).

The effects of tissues on malononitrile

If malononitrile, together with sodium thio-sulphate, is added to a tissue homogenate, a formation of SCN^- is observed which increases linearly with time (Fig. 3). The activities of homogenates of liver, kidney and brain decrease in that order; in the rat they are in the proportion of 34:14:1. Rhodanese shows a very similar distribution; according to Himwich & Saunders (1948), rhodanese activities of rat liver, kidney and brain cortex are as 20-40:15:1. The amounts of SCN^- formed from cyanide by kidney and liver preparations are however 2500-3000 times higher, per unit of weight, than those formed from malononitrile. This comparison is based on our own estimations of rhodanese activity at 30° which, for extracts of rat kidney and liver, gave figures three to four times higher than those reported by Himwich & Saunders. Rhodanese is therefore present in vast excess. Since rhodanese activity is not inhibited by malononitrile in concen-

trations up to 0.02 M (Table 10) it cannot be a limiting factor in the formation of SCN^- from malononitrile.

The system responsible for the formation of SCN^- from malononitrile by rat-liver homogenate in the presence of 0.05 M-sodium thiosulphate is saturated

Fig. 3. Formation of thiocyanate from malononitrile (0.005 M) and thiosulphate (0.05 M) by rat tissue homogenates. Final dilution of liver and kidney homogenates 1:33, of brain homogenate 1:9. The medium contained 0.04 M-glycine buffer, pH 8.2. All points were corrected for thiocyanate content of control experiments containing boiled homogenates.

by 0.0033 M-malononitrile. The low concentration level at which saturation occurs is in favour of a specific catalyst.

The pH optimum for the formation of SCN^- from malononitrile by liver extract is near 7.0 (Fig. 4). The activity of the system is higher in glycine than in phosphate buffer. Although phosphate buffer was used in the earlier experiments it was later replaced by a glycine buffer of pH 8.2. The final pH of the reaction mixture which was repeatedly checked with a glass electrode was 7.2–7.3.

Saunders & Himwich (1950) noted that rhodanese is strongly inhibited by cysteine. The same applies to SCN^- formation from malononitrile. Inhibition of a less severe degree was also observed in the presence of reduced glutathione and of adenosinetriphosphate. Sodium fluoride, on the other hand, is without effect (Table 7).

No trace of SCN^- formation was found when any of the following substances, in 0.005 M-concentration, was incubated with rat-liver homogenate (1:33) for 2 hr. at 30°: acetonitrile, propionitrile,

butyronitrile, isovaleronitrile, succinonitrile, glutaronitrile, dimethylmalononitrile and ethyl cyanoacetate.

The effect of thiosulphate. The action of tissue preparations on malononitrile was further studied in experiments in which CN^- and SCN^- were estimated in separate samples of the same solution

Fig. 4. Effect of pH on thiocyanate formation from malononitrile (0.005 M) by rat-liver extract (1:33). The medium contained $\text{Na}_2\text{S}_2\text{O}_3$ 0.05 M, buffers 0.05 M. Incubated at 30° for 2 hr. Curve A: phosphate buffers. Curve B: glycine buffers. The values of pH were determined after the incubation with a glass electrode. All points were corrected for the thiocyanate content of control experiments with boiled extract.

Table 7. *Inhibition of thiocyanate formation from malononitrile by rat-liver extract*

(5 ml. solution contained 0.005 M-malononitrile, 0.05 M- $\text{Na}_2\text{S}_2\text{O}_3$, 0.05 M-phosphate buffer pH 7.4, extract 1:16.67. Incubated for 2 hr. at 30°.)

Addition	Rate of SCN^- formation as percentage of control experiment
0.01 M-Cysteine	10.1
0.01 M-Glutathione (GSH)	38.5
0.005 M-Adenosinetriphosphate	68.5
0.03 M-NaF	92

(Table 8). It was usually found that the sum of the molar concentration of the two ions was larger when the incubation was performed in the presence of sodium thiosulphate. The results were corrected for non-enzymic effects by control experiments with boiled tissue preparations. Some non-enzymic formation of CN^- took place; the boiled preparations also seemed to possess a residue of rhodanese activity. The non-enzymic reaction is, however, small compared with the enzymic reaction, especi-

ally in presence of $S_2O_3^{--}$. The results obtained with kidney and brain, though on a smaller scale, were similar to those observed with liver. Liver homogenate was slightly more active than liver extract, but no significant difference was found between homogenate and extract in the case of kidney or brain.

Similar experiments were performed with methyl and ethyl thiocyanate (Table 9). The results are complicated by a considerable non-enzymic formation of cyanide, especially from methyl thiocyanate. $S_2O_3^{--}$ has little effect in presence of a denatured tissue preparation, but it does increase the sum total of $CN^- + SCN^-$ in presence of an enzymically active

Table 8. *Effect of tissue homogenates and extracts on malononitrile (0.01 M) in presence and absence of thiosulphate*

(5 ml. solution contained 0.04 M-glycine buffer, pH 8.2. Incubation for 2 hr. at 30°. H, homogenate. E, extract.)

No.	Tissue (rat)	Final dilution	Preparation	$Na_2S_2O_3$ (M)	μ moles								
					(1) Preparation enzymically active			(2) Preparation boiled			Difference (1) - (2)		
					CN ⁻	SCN ⁻	Total	CN ⁻	SCN ⁻	Total	CN ⁻	SCN ⁻	Total
1	Liver	1:33	H	0	0.91	0.46	1.37	0.37	0.26	0.63	0.54	0.20	0.74
				0.05	0.40	2.42	2.82	0.27	0.41	0.68	0.13	2.01	2.14
				0	0.96	0.36	1.32	0.37	0.31	0.68	0.59	0.05	0.64
			E	0.05	0.40	1.75	2.15	0.28	0.41	0.69	0.12	1.34	1.46
				0	0.56	0.31	0.87	0.58	0.31	0.89	-0.02	0	-0.02
				0.05	0.15	2.12	2.27	0.47	0.41	0.88	-0.32	1.71	1.39
2	Liver	1:33	H	0	0.56	0.31	0.87	0.58	0.31	0.89	-0.02	0	-0.02
				0.05	0.15	2.12	2.27	0.47	0.41	0.88	-0.32	1.71	1.39
			E	0	0.54	0.31	0.85	0.50	0.31	0.81	0.04	0	0.04
				0.05	0.19	1.65	1.84	0.56	0.41	0.97	-0.37	1.24	0.87
			H	0	0.35	0.26	0.61	0.28	0.26	0.54	0.07	0	0.07
				0.05	0.20	1.03	1.23	0.23	0.31	0.54	-0.03	0.72	0.69
3	Kidney	1:33	H	0	0.35	0.26	0.61	0.28	0.26	0.54	0.07	0	0.07
				0.05	0.20	1.03	1.23	0.23	0.31	0.54	-0.03	0.72	0.69
			E	0	0.38	0.26	0.64	0.26	0.21	0.47	0.12	0.05	0.17
				0.05	0.28	0.98	1.26	0.21	0.26	0.47	0.07	0.72	0.79
			H	0	0.12	0.29	0.41	0.09	0.19	0.28	0.03	0.10	0.13
				0.05	0.03	0.52	0.55	0.08	0.20	0.28	-0.05	0.32	0.27
4	Brain	1:16.7	H	0	0.12	0.29	0.41	0.09	0.19	0.28	0.03	0.10	0.13
				0.05	0.03	0.52	0.55	0.08	0.20	0.28	-0.05	0.32	0.27
			E	0	0.14	0.32	0.46	0.08	0.15	0.23	0.06	0.17	0.23
				0.05	0.03	0.57	0.60	0.09	0.19	0.28	-0.06	0.38	0.32

Table 9. *Effect of liver homogenates and extracts on methyl and ethyl thiocyanate*

Arrangement as described in Table 8

(Controls. Exp. 1: control contained boiled tissue preparation and was incubated for 2 hr. at 30°. Exp. 2 and 3: 5 ml. 10% trichloroacetic acid were added to control sample before addition of tissue preparation. The sample was analysed without previous incubation. Me-SCN, methyl thiocyanate; Et-SCN, ethyl thiocyanate; H, homogenate; E, extract.)

No.	Preparation	Drug added	Na ₂ S ₂ O ₃ (M)	μmoles											
				(1) Preparation enzymically active			(2) Control			(3) Drug incubated without tissue			Difference (1) - (2)		
				CN ⁻	SCN ⁻	Total	CN ⁻	SCN ⁻	Total	CN ⁻	SCN ⁻	Total	CN ⁻	SCN ⁻	Total
1	H	Me-SCN (0.01 M)	0	1.50	0.57	2.07	2.44	0.26	2.70	—	—	—	-0.94	0.31	-0.63
			0.05	1.50	1.68	3.18	2.84	0.46	3.30	—	—	—	-1.34	1.22	-0.12
	E	0	1.31	0.41	1.72	1.31	0.21	1.52	—	—	—	0	0.20	0.20	
		0.05	1.50	1.50	3.00	1.89	0.37	2.26	—	—	—	-0.39	1.13	0.74	
2	H	Me-SCN (0.005 M)	0	0.56	0.21	0.77	1.35	0	1.35	1.04	0	1.04	-0.79	0.21	-0.58
			0.05	0.60	1.01	1.61	1.35	0.04	1.39	1.53	0	1.53	-0.75	0.97	0.22
	E	0	0.77	0.10	0.87	1.48	0	1.48	—	—	—	-0.71	0.10	-0.61	
		0.05	0.70	0.83	1.53	1.30	0.04	1.34	—	—	—	-0.60	0.79	0.19	
3	H	Et-SCN (0.005 M)	0	0.23	0.52	0.75	0.47	0	0.47	0.21	0	0.21	-0.24	0.52	0.28
			0.05	0.35	0.81	1.16	0.58	0	0.58	0.62	0	0.62	-0.23	0.81	0.58
	E	0	0.54	0.46	1.00	0.40	0	0.40	—	—	—	0.14	0.46	0.60	
		0.05	0.35	0.77	1.12	0.36	0	0.36	—	—	—	-0.01	0.77	0.76	

preparation, and even without any tissue at all. The evidence for an enzymic effect is less ambiguous in the experiment with ethyl thiocyanate; whether SCN^- is formed by direct hydrolysis or by the secondary reaction of the CN group with an S compound cannot be decided, but the increase of SCN^- formation in presence of $\text{S}_2\text{O}_3^{--}$ is in favour of the second mechanism. A surprising feature, especially of the experiments with methyl thiocyanate, is the relatively high concentration of CN^- which is observed even in the presence of $\text{S}_2\text{O}_3^{--}$ and

with and one without $\text{S}_2\text{O}_3^{--}$; to a third $\text{S}_2\text{O}_3^{--}$ was added 10 min. before termination of the experiment. Owing to the large excess of rhodanese activity, it can be expected that 10 min. are sufficient to allow the transformation of any CN^- present into SCN^- after adding $\text{S}_2\text{O}_3^{--}$. The formation of SCN^- was larger when $\text{S}_2\text{O}_3^{--}$ was present throughout the experiment than when its addition was delayed. The result was similar with each of the three compounds tested, but it was most marked in the case of malononitrile.

Table 10. *Effect of malononitrile and alkyl thiocyanates on rhodanese activity*

(5 ml. solution contained 0.05 M-KCN, neutralized, 0.05 M- $\text{Na}_2\text{S}_2\text{O}_3$, 0.04 M-glycine buffer pH 8.2, 0.02 M-malononitrile or alkyl thiocyanate. Incubated for 10 min. at 30°.)

Tissue extract (rat)	Final dilution	Activity as percentage of control experiment in presence of		
		Malononitrile	Methyl thiocyanate	Ethyl thiocyanate
Liver	1:3333	100	60.6	67.2
Kidney	1:1667	100	60.0	63.5
Brain	1:250	100	66.0	74.6

an active rhodanese. This may to some extent be accounted for by an inhibition of rhodanese activity in presence of methyl or ethyl thiocyanate (Table 10).

Table 11. *Effect of delayed addition of thiosulphate on thiocyanate formation from malononitrile, methyl and ethyl thiocyanate by rat-liver homogenate*

(5 ml. solution contained 0.04 M-glycine buffer pH 8.2, malononitrile or alkyl thiocyanate 0.005 M, homogenate 1:33. Incubated for 2 hr. at 30°.)

Addition of 0.05 M- $\text{Na}_2\text{S}_2\text{O}_3$	Formation of SCN^- (μ moles) from		
	Malono- nitrile	Methyl thiocyanate	Ethyl thiocyanate
None	0.25	0.26	0.36
At start	1.44	0.75	0.58
After 110 min.	0.55	0.39	0.48

The effect of $\text{S}_2\text{O}_3^{--}$ was further demonstrated by the following experiment (Table 11): of three samples of liver homogenate one was incubated

For the interpretation of the effect of sodium thiosulphate it is essential to know whether the presence of sodium thiosulphate affects the recovery of the sum total of $\text{CN}^- + \text{SCN}^-$ after the incubation of CN^- with tissues. In experiments with rat-liver homogenate it was found that added CN^- is quantitatively recovered in the form of SCN^- when $\text{S}_2\text{O}_3^{--}$ is present, but in its absence the sum of $\text{CN}^- + \text{SCN}^-$ does not account for the CN^- initially added (Table 12). This result suggests that the effect of $\text{S}_2\text{O}_3^{--}$ is due, at least in part, to the successful competition of rhodanese with other systems capable of removing CN^- .

Effects of purified rhodanese. Several observations suggest a connexion between rhodanese and the system responsible for the SCN^- formation from malononitrile and alkyl thiocyanates, e.g. the distribution of activity, the dependence on $\text{S}_2\text{O}_3^{--}$ and the inhibition of cysteine. The action of purified liver rhodanese was therefore investigated. The enzyme was prepared from rabbit liver by the

Table 12. *Recovery of cyanide and thiocyanate after incubation of rat-liver homogenate with cyanide in presence and absence of thiosulphate*

(5 ml. solution contained 150 mg. homogenized rat liver and 0.04 M-glycine buffer, pH 8.2. Final pH adjusted to 7.3. Incubated for 2 hr. at 30°.)

Exp. no.	$\text{Na}_2\text{S}_2\text{O}_3$ (M)	CN^- added	μ moles								
			Initial recovery (1)			Final recovery (2)			Difference (2) - (1)		
			CN^-	SCN^-	Total	CN^-	SCN^-	Total	CN^-	SCN^-	Total
1	0	3.85	3.77	0	3.77	2.62	0	2.62	-1.15	0	-1.15
	0.05		3.85	0	3.85	0.03	3.81	3.84	-3.82	+3.81	-0.01
2	0	3.70	3.46	0	3.46	2.85	0.07	2.92	-0.61	+0.07	-0.54
	0.05		3.62	0	3.62	0	3.87	3.87	-3.62	+3.87	+0.25
3	0	3.54	3.39	0	3.39	2.54	0	2.54	-0.85	0	-0.85
	0.05		3.54	0.07	3.62	0	3.87	3.87	-3.54	+3.80	+0.25

method of Cosby & Sumner (1945). The final product, assayed according to these authors, formed $4.5 \mu\text{moles SCN}^-/\text{mg. protein in 5 min. at } 20^\circ$. The effect of this preparation on malononitrile, methyl and ethyl thiocyanate was tested in an experiment in which these substances were incubated for 3 hr. at 30° with an amount containing 38 mg. protein. Samples containing boiled enzyme solution served as controls. Ten minutes before the termination of the experiment, active enzyme solution containing 4.2 mg. protein was added to both experimental and control flasks in order to convert any CN^- formed in the control samples to SCN^- . The ratio of activities should remain unchanged after purification; a simple calculation shows that, if the effects of the crude extracts were due to the activity of rhodanese, malononitrile would yield at least $2 \mu\text{moles SCN}^-$ (at 20°). Instead, purified rhodanese was without effect on the formation of SCN^- from malononitrile (Table 13). Ethyl and particularly

of respiratory inhibitors, such as nicotine (Baker, Fazekas & Himwich, 1938), barbiturates (Webb & Elliott, 1951), maleic (Weil-Malherbe, 1938) or cinnamic acid (Weinbach, Lowe, Frisell & Hellerman, 1951). On the other hand, out of a series of nitriles, including dimethylmalononitrile, none except malononitrile showed either cyanide-like effects on tissue metabolism or gave rise to SCN^- formation in presence of tissue preparations. The only substances which in both these respects resembled malononitrile were methyl and ethyl thiocyanate. It is therefore highly probable that the effects on tissue metabolism are correlated with the transformations induced by tissue preparations.

Compared with its antidotal activity *in vivo* the antagonistic effect of sodium thiosulphate against malononitrile is slight and transient in tissue-slice experiments. The discrepancy also applies to cyanide, though to a lesser extent. Rhodanese is known to be localized mainly in the mitochondrial fraction of liver cells (Ludewig & Chanutin, 1950; Archibald, Meyer & Greenough, 1951) and is thus accessible only to intracellular substances; as Himwich & Saunders (1948) have shown, the rhodanese activity of rabbit liver or brain is about sixteen times larger when the tissue is homogenized than when it is sliced. Permeability factors may thus account for the results *in vitro*, though it is obscure why they seem less important *in vivo*.

Whereas a formation of SCN^- could be detected *in vitro* only from malononitrile, the reaction is known to occur with most other nitriles *in vivo*. This difference is probably quantitative rather than qualitative. Next to cyanogen, malononitrile has a more rapid toxic action than other dinitriles in the body (Heymans & Masoin, 1897). Toxic symptoms and SCN^- excretion are slow to appear and to disappear with most nitriles, and it may be assumed that their rate of breakdown is too slow to be demonstrable in a short-term experiment with tissue preparations.

The increase of lactic acid concentration in the brains of injected rats shows that the aerobic glycolysis observed with brain slices also occurs in the intact animal. The results are in line with those obtained in studies of cyanide poisoning (Albaum, Tepperman & Bodansky, 1946; Olsen & Klein, 1947). On the other hand, the cozymase content of rat brain was found to be unaltered after malononitrile injection. The microspectrographic observations of Hydén & Hartelius (1948) are thus not reflected in concentration changes of this particular nucleotide which might be expected to be especially sensitive to changes in respiratory activity.

The effect of $\text{S}_2\text{O}_3^{2-}$ on the formation of SCN^- from malononitrile seems to be adequately explained by the prevention of side reactions in which part of the cyanide liberated from the nitrile may dis-

Table 13. *Effects of purified rhodanese*

(7.5 ml. solution contained 38 mg. protein, $0.05 \text{ M Na}_2\text{S}_2\text{O}_3$, 0.04 M -glycine buffer pH 8.2. Incubated at 30° . After 3 hr. 0.5 ml. enzyme solution ($=4.2 \text{ mg. protein}$) were added to all flasks and incubation was continued for 10 min.)

Additions (0.01 M)	Formation of SCN^- (μmoles)	
	Boiled enzyme	Active enzyme
Malononitrile	0.14	0.14
Methyl thiocyanate	2.45	1.07
Ethyl thiocyanate	1.60	0.56

methyl thiocyanate gave rise to SCN^- formation, but the effect was larger in the sample containing denatured protein and was therefore presumably non-enzymic.

DISCUSSION

The observations here recorded are compatible with the supposition that the pharmacological effects of malononitrile are the consequence of a gradual liberation of cyanide in the tissues. The increase and eventual inhibition of the aerobic glycolysis of tissue slices by increasing concentrations of cyanide is of course well known (cf. Warburg, 1949). As our results with rat-brain slices show, low concentrations of cyanide seem to affect the aerobic glycolysis more strongly than the respiration; the stimulation of aerobic glycolysis reached its peak when the respiration was inhibited by about 50%. Similar results were obtained with malononitrile, though in concentrations about 100 times higher than those of cyanide. The similarity does not necessarily indicate the identity of the inhibitory mechanism, since results of this kind may be observed with a variety

appear, but the possibility cannot be excluded that $S_2O_3^{--}$ in some way activates the decomposition of malononitrile. Saunders & Himwich (1950) showed that rhodanese, unless protected by $S_2O_3^{--}$, is inhibited by cyanide. Though the concentration of CN^- present in our experiments is too small to make rhodanese activity limiting, the activity of some other enzyme taking part in the reaction may be similarly affected. There is further the possibility that $S_2O_3^{--}$ may be involved in the reaction as a specific electron donor. In favour of such an assumption is the catalytic effect of $S_2O_3^{--}$ on the formation of CN^- from methyl and ethyl thiocyanates in absence of a tissue preparation. The action of tissue preparations on the alkyl thiocyanates may be largely due to an unspecific effect of SH groups, since it persists after denaturation of protein. It is interesting to note that Aldridge (1951) has proposed a mechanism for the dissociation of cyanogen chloride involving complex formation with reduced glutathione. As far as nitriles are concerned it is known that $S_2O_3^{--}$ accelerates the dissociation of the labile lactonitrile (Meurice, 1900). On the other hand, no effect of $S_2O_3^{--}$ on the spontaneous dissociation of malononitrile could be detected at 30° in short-term experiments. Moreover, malononitrile was only attacked by enzymically active, but not by denatured, tissue preparations and this reaction was inhibited by SH compounds. It thus seems that two mechanisms must be distinguished: an unspecific effect which is predominant in the case of alkyl thiocyanates and which may be mediated by SH-groups and a specific, enzymic effect which is predominant in the case of malononitrile.

SUMMARY

1. Out of a series of compounds, including eleven nitriles and ethyl cyanoacetate only malononitrile, methyl thiocyanate and ethyl thiocyanate were found to have any marked effect on tissue slices and to give rise to the formation of cyanide and thio-

cyanate in presence of tissue homogenates or extracts.

2. In experiments with tissue slices, malononitrile caused an inhibition of respiration, progressive with time, and an increase of aerobic glycolysis. Its action resembled that of cyanide at a molar concentration 100 times smaller. Thiosulphate antagonizes the effects of cyanide to some extent while it alleviates those of malononitrile only in the first, not in the second, hour of the experiment.

3. Subcutaneous injection of malononitrile (14 mg./kg.) increased the lactic acid content of rat brain after 1–2 hr., but did not change the concentration of cozymase.

4. The formation of thiocyanate from malononitrile and thiosulphate is highest in presence of liver tissue, lowest with brain and intermediate with kidney. The liver enzyme system is saturated at a concentration of 0.0033 M-malononitrile and has a pH optimum of 7.0. It is inhibited by cysteine and glutathione and inactivated by boiling.

5. The sum total of cyanide and thiocyanate formed from malononitrile by enzymically active tissue preparations was larger in presence than in absence of thiosulphate. The effect appears to be mainly due to the conversion of cyanide into thiocyanate thus preventing its disappearance in secondary reactions.

6. The formation of cyanide and thiocyanate from methyl and ethyl thiocyanate seems to be largely due to unspecific, non-enzymic factors. Both substances, at 0.02 M-concentration, cause a 40 % inhibition of rhodanese activity.

7. A purified preparation of rhodanese did not induce any formation of thiocyanate from malononitrile and thiosulphate.

A specimen of malononitrile has been put at our disposal by Dr A. Roberts, Medical Department, Messrs Boots Pure Drug Co. Ltd. J. Stern has been working under an assistance grant from the Medical Research Council. We wish to express our gratitude for the help received.

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The Oxidation of Manganese by Enzyme Systems

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(Received 21 December 1951)

Kenten & Mann (1949, 1950) found that certain plant extracts catalysed the oxidation of bivalent manganese (Mn^{++}) by added hydrogen peroxide and showed that this catalysis was due to peroxidase systems in the plant extracts. It was suggested that *in vivo* hydrogen peroxide formed by flavoprotein enzyme systems could be used for the oxidation of Mn^{++} by peroxidase systems and colorimetric evidence of such oxidation was obtained using the xanthine-oxidase-hypoxanthine system as a source of hydrogen peroxide. In later work, Kenten & Mann (1951) demonstrated that many plant extracts, in the absence of added hydrogen peroxide, oxidized Mn^{++} and that with pea-seedling extracts the oxidation depended on the formation of hydrogen peroxide by an amine oxidase present in the extracts.

In the present work it is shown that manganese oxidation can be brought about by coupling a number of enzyme systems producing hydrogen peroxide with peroxidase systems.

MATERIALS AND METHODS

Peroxidase. This was prepared from horse-radish extracts by precipitation with $(NH_4)_2SO_4$ followed by fractional precipitation with ethanol according to the method of

Keilin & Mann (1937). The 'Purpurogallinzahl' (P.Z.) of the preparation used in the present work estimated by the method of Keilin & Mann (1937) was 250.

Catalase. This was prepared from horse liver by a method based on that of Agner (1938). The activity of the preparation was estimated by the method of Sumner & Somers (1943). The decomposition of 0.01N- H_2O_2 in 0.0067M-orthophosphate at pH 7 and 0°, by a suitable amount of catalase, was followed by iodine titration on samples taken at 0, 3, 6, 9 and 12 min. From the equation

$$k = (1/t) \log_{10} \{a/(a-x)\},$$

where a is the initial H_2O_2 concentration and $a-x$ is the concentration at time t , k values for the time intervals were calculated and the k value at zero time obtained by extrapolation. Using this value for k the catalase activity = k/g . enzyme of the preparations used varied from 5000 to 6000.

Plant amine oxidase. This was prepared from pea seedlings by the method of Kenten & Mann (1951). The dry preparation was suspended in 0.2M-pyrophosphate buffer at pH 7.5 or 0.2M-orthophosphate at pH 7 and centrifuged clear before use.

Xanthine oxidase. This was prepared from milk by the method of Dixon & Kodama (1926). The 'whey preparation' was suspended in 0.2M-pyrophosphate at pH 7.5 and centrifuged before use.

D-Amino-acid oxidase. This was prepared from sheep kidney. The method of Negelein & Brömel (1939) was followed as far as the first precipitation with $(NH_4)_2SO_4$. This precipitate was redissolved in 0.0033M-pyrophosphate