Blood Keto Acid Levels during Intoxication with Inhibitors of Carbohydrate Metabolism

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A number of compounds have come to be recognized as more or less specific inhibitors of those enzymes which depend upon SH groups for their activity. Although in many cases our knowledge of the action of these compounds is well founded, most of it is based largely on *in vitro* studies, and relatively few reports are available from which to compare the pattern of *in vivo* effects on different blood constituents produced by these various compounds in the intact animal.

Interest in this problem was aroused in this laboratory by some earlier observations on pyruvate metabolism in an unselected series of patients with peripheral neuritis (Joiner, McArdle & Thompson, 1950). In the course of this work it was found that the blood-pyruvate level was not infrequently raised in patients in whom there were no grounds for suspecting a thiamine deficiency, and in whom treatment with thiamine did not reduce the abnormal levels of pyruvate in the blood. As it is known that pyruvate metabolism can be influenced in a number of ways, apart from a deficiency of thiamine, it is possible that the lesion in such patients may be associated with the presence of some substance reacting with SH groups in an enzyme system essential for the normal metabolism of pyruvate formed from glucose.

Several reports have been made on the *in vivo* effects on the blood-sugar level of various enzyme inhibitors, including the so-called 'SH inhibitors': thus, intoxication with iodoacetate (Neuss, 1931; Irving, 1932), cyanide (Turner & Hulpfen, 1933), arsenite (Oelkers, 1939), malonate (Stare & Baumann, 1940) and fluoride (Kaplan & Greenberg, 1944) all result in hyperglycaemia. The level of keto acids after intoxication with these enzyme inhibitors has not, however, been described very extensively. Peters, Sinclair & Thompson (1946) reported an increase in the level of blood pyruvate after the poisoning of pigeons, mice and rats with sodium arsenite or lewisite. Handler (1945), using the method of Friedemann & Haugen (1943), studied the changes in blood pyruvate in rabbits poisoned with cyanide, azide, fluoride, iodoacetate and malonate, while Buffa & Peters (1950) showed that poisoning with fluoroacetate had no effect on the level of keto acids in the blood of rats. With the exception of the last-mentioned workers,

however, no attempt has been made to estimate pyruvic and α -oxoglutaric acids separately.

Since chromatographic methods, based on the work of Cavallini, Frontali & Toschi (1949*a, b*), are now available for the separation and estimation of the different keto acids in blood filtrates, it was of interest to compare the acute effects produced by the administration of a series of inhibitors of different types, including a number of SH inhibitors, on the levels of pyruvic and α -oxoglutaric acid in the blood, and also to determine any secondary changes which may take place in the blood-sugar, acetoacetate and acetone levels resulting from these interferences with carbohydrate utilization.

Some of the results obtained with the arsenite- and alloxan-poisoned animals have already been reported in a short note (El Hawary & Thompson, 1954) dealing with the nature of the substances responsible for the 'spots' in chromatograms of keto acid hydrazones extracted from blood.

EXPERIMENTAL

Animals. White rats of either sex weighing 150–200 g. were used throughout.

Sodium pyruvate was prepared by the method of Robertson (1942) from redistilled pyruvic acid (L. Light and Co.). For injection into rats aqueous solutions containing 200 mg. sodium pyruvate/ml. were freshly prepared. The animals were injected intraperitoneally with doses of these solutions so that each received 250 mg. sodium pyruvate/kg. body wt.

Enzyme inhibitors

Arsenite. Sodium arsenite, prepared by dissolving As_2O_3 in $N-NaOH$, was neutralized and injected subcutaneously in 0.2 ml. doses equivalent to 12 mg. As_2O_3 /kg. body wt. The animals were killed 2 hr. later.

Maleate. Sodium maleate, prepared by neutralizing maleic acid (British Drug Houses Ltd.) with $NaOH$, was administered intraperitoneally in amounts of 0.5 ml. M maleate/100 g. body wt. (580 mg./kg.). The animals were killed after 90 min. Smaller doses of between 50 and 200 mg./kg. were without effect on the total keto acid level as determined 90–150 min. after injection.

Malonate. Sodium malonate (British Drug Houses Ltd.) was injected intraperitoneally in (i) doses of 1 ml. M solution/100 g. body wt. (1.04 g. malonic acid/kg.) and killed after 2 hr., (ii) two doses of 1 ml. M solution/100 g.

body wt. at 0 and 60 min., and killed after 120 min., and (iii) a dose of 1 ml. 2M malonate/100 g. and killed after 30 min.

Iodoacetate. Iodoacetic acid (British Drug Houses Ltd.), recrystallized from light petroleum (b.p. 100–120°) and uncontaminated with free iodine, was used. Neutral solutions of the sodium salt were prepared, and volumes varying from 0.1 to 0.2 ml. were injected intraperitoneally, so that the rats received the equivalent of 75 mg. iodoacetic acid/kg. They were killed 90 min. later.

Alloxan. 200 mg. alloxan (L. Light and Co.)/kg. were administered intraperitoneally in volumes varying from 0.4 to 0.5 ml. aqueous solution and the animals killed 48–72 hr. later, i.e. after hyperglycaemia had developed.

Fluoride. Sodium fluoride (British Drug Houses Ltd.) was given in doses (0.2–0.35 ml.) varying from 40 to 75 mg./kg. intraperitoneally, and the rats were killed after 30 min.

With the exception of the alloxan-poisoned rats the intoxication was in every case acute, and the animals were killed at times when they were showing symptoms of intoxication (lying quietly in their cages) but were not yet in a state of collapse. In no case did convulsions or any observable increase in muscular activity develop in these acutely poisoned animals. Blood required for keto acid and acetone estimations was collected directly from the cut neck into the deproteinizing solution.

Analytical methods

Blood sugar. This was estimated by the method of Haslewood & Strookman (1939) in blood samples obtained from the tail.

Keto acids. These were estimated in metaphosphoric acid filtrates of blood by the method of Friedemann & Haugen (1943). Chromatographic separation and estimation of pyruvic and α -oxoglutaric acids was carried out as described by El Hawary & Thompson (1953); the second, more rapidly moving 'spot', which has since been identified as derived from pyruvic acid (LePage, 1950; Altmann, Crook & Datta, 1951; Seligson & Shapiro, 1952; Kulonen, Carpen & Ruokolainen, 1953; Markees, 1954; El Hawary &

Thompson, 1954), was eluted and estimated, and its content added to that of the main pyruvate spot.

Acetone bodies. Acetone and acetoacetic acid were estimated in trichloroacetic acid filtrates of blood by a modification (El Hawary & Thompson, 1954) of the method of Thin & Robertson (1952).

RESULTS

The mean values (and the standard errors of the means) for the levels of blood sugar, total keto acids (determined both chromatographically and by the method of Friedemann & Haugen), pyruvate, α -oxoglutarate and acetone bodies in normal rats and in rats poisoned with the various substances studied are shown in Table 1. In no case were any 'abnormal' spots (i.e. derived from keto acids not found in blood from normal animals) detected on the chromatograms, those derived from α -oxoglutarate, pyruvate and, in some instances, faint spots of the acetone hydrazone formed from the acetoacetate derivative being the only ones visible on the papers.

It will be seen that the levels of both pyruvate and α -oxoglutarate are significantly increased in the animals poisoned with each of the inhibitors studied. In all cases except the arsenite-poisoned animals the α -oxoglutarate level was increased to a greater extent than that of pyruvate; this was particularly so in the poisoning with maleate, which resulted in the amount of α -oxoglutarate in the blood actually exceeding that of pyruvate.

The malonate-poisoned animals referred to in Table 1 had each received the high dosage (1 ml. 2M malonate/100 g.). Doses of 1 ml. M malonate/100 g. resulted in a rise of the α -oxoglutarate level only (mean level of two poisoned animals, 1.50 mg./100 ml.), without any significant effect on the

Table 1. *Level of sugar, keto acids and acetone bodies in normal and poisoned rats*

Mean values, with standard errors of the means, are expressed in mg./100 ml. blood; keto acids are expressed in terms of pyruvic acid, and acetone bodies as acetone.

Compound injected	No. of animals	Blood sugar	Keto acids (Friedemann & Haugen, 1943)	Total keto acids (chromatography)	Pyruvate	α -Oxo-glutarate	Acetone + acetoacetate (as acetone)
None	7	79 $\pm$ 4	1.41 $\pm$ 0.1	1.13 $\pm$ 0.05	0.78 $\pm$ 0.03	0.38 $\pm$ 0.03	0.45 $\pm$ 0.04
Arsenite	8	—	4.27 $\pm$ 0.5	4.25 $\pm$ 0.63	3.05 $\pm$ 0.44	1.53 $\pm$ 0.22	0.77 $\pm$ 0.08
Maleate	12	119 $\pm$ 2.9	4.24 $\pm$ 0.17	4.44 $\pm$ 0.21	2.08 $\pm$ 0.12	3.54 $\pm$ 0.24	0.80 $\pm$ 0.06
Iodoacetate	7	155 $\pm$ 4.1	2.73 $\pm$ 0.02	2.44 $\pm$ 0.07	1.55 $\pm$ 0.08	1.19 $\pm$ 0.05	0.69 $\pm$ 0.03
Alloxan	8	420 $\pm$ 19	3.43 $\pm$ 0.23	2.17 $\pm$ 0.16	1.33 $\pm$ 0.17	1.06 $\pm$ 0.05	9.51 $\pm$ 0.69
Malonate	9	116 $\pm$ 7.1	3.56 $\pm$ 0.4	3.03 $\pm$ 0.26	2.15 $\pm$ 0.22	1.42 $\pm$ 0.07	3.84 $\pm$ 0.23
Fluoride	5	139 $\pm$ 6.6	2.87 $\pm$ 0.21	2.46 $\pm$ 0.13	1.55 $\pm$ 0.08	1.08 $\pm$ 0.06	0.84 $\pm$ 0.09

Ratio of mean value in poisoned animals to mean normal value

Arsenite	—	3.0	3.8	3.9	4.0	1.7
Maleate	1.5	3.0	3.9	2.7	9.3	1.8
Iodoacetate	2.0	1.9	2.2	2.0	3.1	1.5
Alloxan	5.3	2.4	1.9	1.7	2.8	21.1
Malonate	1.5	2.5	2.7	2.8	3.7	8.5
Fluoride	1.8	2.0	2.2	2.0	2.8	1.9

pyruvate level; with the medium dosage pyruvate was slightly raised (mean level, 1.37 mg./100 ml.).

The concentration of acetone bodies in the blood was only slightly, though significantly, elevated in the animals poisoned with arsenite, maleate, iodoacetate or fluoride; in the malonate-poisoned group an eightfold increase occurred, whereas, as was to be expected from earlier work (Lackey, Bunde, Gill & Harris, 1944), in the animals poisoned with alloxan a relatively large accumulation of acetone bodies was present.

Since the blood-pyruvate level may be expected to depend on the rate of formation of pyruvate as well as on its rate of utilization it may be argued that the experiments do not necessarily demonstrate that these SH inhibitors have blocked pyruvate metabolism in the intact animal. To study the effect of these inhibitors on pyruvate utilization more precisely, blood keto acid levels were therefore also determined after raising the

blood-pyruvate level by the injection of sodium pyruvate. It was found that the intraperitoneal injection into normal rats of 250 mg. sodium pyruvate/kg. body wt. resulted in a marked elevation of the blood pyruvate level 5 min. after the injection, followed by a fall so that approximately normal levels were regained after 30 min. From these results in normal animals, which are summarized in Fig. 1, it was decided to determine the blood-pyruvate level at 5, 15 and 30 min. after the injection of this dose of pyruvate into animals that had been poisoned with arsenite, maleate, iodoacetate and alloxan. The results of these experiments, together with the dose of inhibitor and the duration of the poisoning, are shown in Table 2. With each of these poisons it will be seen that the blood-pyruvate level remained high over the period studied. Since pure sodium pyruvate was injected in these experiments the rise in the blood keto acid level was estimated by the method of Friedemann & Haugen, although in a few experiments pyruvate was estimated after separation of the hydrazones by paper chromatography.

DISCUSSION

As stated above, although much work has been done on the *in vitro* effects of different inhibitors of the enzymes concerned in carbohydrate metabolism, few comparisons have been made *in vivo* of the effects of these inhibitors on the levels of different intermediary metabolites in the blood. Further, as is well known, although a number of different compounds, acting by different chemical mechanisms, can inhibit various enzymes by interaction with essential SH groups in their molecules, the pharmacological and toxicological effects produced *in vivo* by these inhibitors may vary widely. Apart from possible differences in the chemical reactivity of the SH groups in different enzymes, physiological factors such as penetrability of the inhibitors to the various enzymes may well be a factor in determining these diverse toxicological reactions. It seemed, however, that a better

Fig. 1. Blood pyruvate levels after the intraperitoneal injection into normal rats of sodium pyruvate (250 mg./kg.); ●, level in individual rats; ○, mean level.

Table 2. Mean blood keto acid levels in normal and poisoned rats at 5, 15 and 30 min. after the intraperitoneal injection of sodium pyruvate (250 mg./kg.)

Expressed as mg. pyruvic acid/100 ml. blood, $\pm$ s.e.m. No. of experiments in brackets.

Inhibitor*	Duration of poisoning (hr.)	Pyruvate levels		
		5 min.	15 min.	30 min.
None	—	6.25 $\pm$ 0.42 (6)	2.54 $\pm$ 0.11 (7)	1.80 $\pm$ 0.12 (6)
Arsenite	2	15.76 $\pm$ 2.13 (4)	11.91 $\pm$ 2.02 (5)	9.96 $\pm$ 1.65 (4)
Maleate	1.5	9.26 $\pm$ 0.85 (4)	9.49 $\pm$ 0.51 (4)	11.48 $\pm$ 1.44 (4)
Iodoacetate	1.5	6.61 $\pm$ 1.1 (4)	8.75 $\pm$ 0.71 (3)	7.76 $\pm$ 1.24 (3)
Alloxan	2	8.59 (2)	8.76 $\pm$ 1.37 (6)	4.78 $\pm$ 1.19 (6)
Alloxan	48	—	5.95 $\pm$ 0.52 (9)	—

* Doses of inhibitors as described in the Experimental section.

understanding of these possibilities might be reached if more were known of the changes in blood composition caused by these compounds.

Although each of the inhibitors which have been studied caused a large increase in the blood levels of both pyruvate and α -oxoglutarate, as well as a significant, though in some cases small, rise in acetoacetate, certain striking differences in the effects on these keto acid levels were noted among the various inhibitors.

The results with arsenite confirm the earlier finding of Peters *et al.* (1946) of a rapid rise in the level of pyruvate in the blood; it is here shown that the level of α -oxoglutarate also rises, and that under the conditions of poisoning used in this work the accumulation of each of these acids is about the same, i.e. to about four times the level found in normal animals. The increase in α -oxoglutarate confirms the *in vitro* effects of arsenite on α -oxoglutarate metabolism observed by Krebs & Johnson (1937), Barron & Singher (1945) and Peters & Wakelin (1952). The rise in the pyruvate level in the iodoacetate-poisoned animals is in agreement with the earlier finding of Handler (1945), and also with the *in vitro* work of Peters & Thompson (1934) and Peters, Rydin & Thompson (1935), showing that pyruvate accumulates in respiring normal brain tissue in the presence of iodoacetate.

Maleate was found by Peters & Wakelin (1946) to inhibit pyruvate oxidation by brain *in vitro*, and these results indicate that pyruvate also accumulates in the intact animal. It will be noticed, however, that with maleate the rise in α -oxoglutarate is more than three times as great as for pyruvate. Although, by analogy with arsenite, maleate might be expected to inhibit the α -oxoglutarate oxidase, it may also affect the level of α -oxoglutarate by a different mechanism. Morgan & Friedemann (1938) demonstrated that when maleic acid reacts with a monothiol, such as cysteine or glutathione, some of the acid is rearranged to give fumaric acid. Moreover, Coxon, Lièbecq & Peters (1949) found that the addition of fumarate to the brain-pyruvate-oxidase system increases the amount of α -oxoglutarate in such a system metabolizing pyruvate. It seems possible, therefore, that part of the injected maleate may be converted into fumarate, the latter giving rise, through the citric acid cycle, to α -oxoglutarate, which cannot then be further metabolized owing to the inhibition of the α -oxoglutarate oxidase also produced by the maleate.

In addition to the usual changes (high blood sugar and acetone bodies) exhibited by alloxan-poisoned animals, the levels of pyruvate and α -oxoglutarate are also raised. It is not possible on present evidence to decide whether this increased

pyruvate level is due to interaction of alloxan with SH groups in some component of the pyruvate-oxidase system or whether it is the outcome of the very high blood-sugar level, since it has been shown in rats (El Hawary, unpublished observations) that force-feeding with a high-carbohydrate diet, or the oral administration of a large dose of glucose, can cause a comparable increase in the blood-pyruvate level. The relatively greater rise in α -oxoglutarate than in pyruvate in the alloxan-poisoned animals suggests, however, that the increased levels of these keto acids are due to a direct action of alloxan on the enzyme systems concerned.

Handler (1945) reported an increase in the blood levels of both pyruvate and acetoacetate in malonate-poisoned rabbits. The present author has confirmed these findings in rats, and has also found a rise in the α -oxoglutarate level, which probably results from the inhibition of the succinic dehydrogenase causing a diminution in the rate of α -oxoglutarate breakdown, since Busch & Potter (1952) have reported that doses of malonate, similar to those used in this work, cause a large increase in the succinate level of the tissues. It is interesting in this connexion to recall that high levels of urinary α -oxoglutarate have repeatedly been described in animals poisoned with this compound (Orten & Smith, 1937; Krebs, Salvin & Johnson, 1938; Krusius, 1940; Lee & Lifson, 1951). The relatively large accumulation of acetoacetate in malonate-poisoned animals supports Lehninger's (1946) view that pyruvic acid has two major degradative pathways in rat-liver homogenates: one through the citric acid cycle, and the other resulting in the synthesis of acetoacetate when the cycle is interrupted by malonate. The cause of this accumulation is not clear, although if there is a lack of oxaloacetate for the condensing reaction, acetylcoenzyme A units would interact to form acetoacetate (Stern, Minor & Del Campillo, 1953). If this were so, it is perhaps surprising that a comparable accumulation of acetoacetate is not found in the fluoride-poisoned animals, since it is known that fluoride inhibits both succinic dehydrogenase and α -oxoglutarate oxidase *in vitro* (Slater & Bonner, 1952; Slater & Holton, 1954). It must be remembered, however, that no estimate has been made in these experiments of the degree to which the various enzymes concerned have been inhibited by the doses of the poisons which we have used.

The experiments with injected sodium pyruvate indicate that the administration of arsenite, maleate, iodoacetate or alloxan to rats results, under the conditions used, in a gross impairment in the rate of removal of the injected pyruvate from the blood stream. It is known from the work of Johnson & Edwards (1937) that when the blood-pyruvate level is increased by exercise there is an

accompanying increase in the rate of excretion of pyruvate by the kidney, so that the return to normal of the blood-pyruvate level after the injection of sodium pyruvate will in part be brought about by increased urinary elimination. The rate at which this decline in the blood concentration occurs makes it unlikely, however, that renal excretion is playing more than a minor part in the disappearance of the injected pyruvate from the blood stream. Although impairment of renal function resulting from the action of these inhibitors may therefore in part account for the maintained high blood-pyruvate levels in the poisoned animals it seems unlikely that this is the whole explanation. These results, taken together with those given in Table 1, are therefore interpreted as providing further evidence that an *in vivo* block in pyruvate metabolism has been produced by these inhibitors.

With the exception of the alloxan-poisoned animals, the intoxication has in each case been acute and severe, so that caution is necessary in comparing the results of these experiments with the elevations of the blood-pyruvate level in human patients suffering from conditions not due to thiamine deficiency. It is possible, of course, that more prolonged exposure of rats to smaller doses of these inhibitors might also have led to raised keto acid levels, but owing to the difficulties involved in the production of such chronic intoxications in small animals, together with the danger of the production of co-existent dietary deficiencies in such long-term experiments, we have limited this phase of the work to acute intoxications.

SUMMARY

1. A study has been made of the levels of pyruvate, α -oxoglutarate and acetoacetate in the blood of rats poisoned with different inhibitors of carbohydrate metabolism, namely arsenite, maleate iodoacetate, alloxan, malonate and fluoride.

2. Pyruvate levels have also been studied in normal and poisoned animals after intraperitoneal injection of a standard dose of sodium pyruvate.

3. Each of the inhibitors caused an increase in the blood level of pyruvate and α -oxoglutarate, the rise in the latter being particularly great in the maleate-poisoned animals.

4. Alloxan and malonate each caused a large rise in the level of acetoacetate, the other poisons studied causing only a small but significant rise in this acid.

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REFERENCES

- Altmann, S. M., Crook, E. M. & Datta, S. P. (1951). *Biochem. J.* **49**, lxiii.
- Barron, E. S. G. & Singher, T. P. (1945). *J. biol. Chem.* **157**, 221.
- Buffa, P. & Peters, R. A. (1950). *J. Physiol.* **110**, 488.
- Busch, H. & Potter, V. R. (1952). *J. biol. Chem.* **198**, 71.
- Cavallini, D., Frontali, N. & Toschi, G. (1949a). *Nature, Lond.*, **163**, 568.
- Cavallini, D., Frontali, N. & Toschi, G. (1949b). *Nature, Lond.*, **164**, 792.
- Coxon, R. V., Lièbecq, C. & Peters, R. A. (1949). *Biochem. J.* **45**, 320.
- El Hawary, M. F. S. & Thompson, R. H. S. (1953). *Biochem. J.* **53**, 340.
- El Hawary, M. F. S. & Thompson, R. H. S. (1954). *Biochem. J.* **58**, 518.
- Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
- Handler, P. (1945). *J. biol. Chem.* **161**, 58.
- Haslewood, G. A. D. & Strookman, T. A. (1939). *Biochem. J.* **33**, 920.
- Irving, J. T. (1932). *J. Physiol.* **74**, P4.
- Johnson, R. E. & Edwards, H. T. (1937). *J. biol. Chem.* **118**, 427.
- Joiner, C. L., McArdle, B. & Thompson, R. H. S. (1950). *Brain*, **73**, 431.
- Kaplan, N. O. & Greenberg, D. M. (1944). *J. biol. Chem.* **156**, 525.
- Krebs, H. A. & Johnson, W. A. (1937). *Enzymologia*, **4**, 148.
- Krebs, H. A., Salvin, E. & Johnson, W. A. (1938). *Biochem. J.* **32**, 113.
- Krusius, F. E. (1940). *Acta physiol. scand.* **2**, Suppl. 3.
- Kulonen, E., Carpen, E. & Ruokolainen, T. (1953). *Scand. J. Clin. Lab. Invest.* **4**, 189.
- Lackey, R. W., Bunde, C. A., Gill, A. J. & Harris, L. C. (1944). *Proc. Soc. exp. Biol., N.Y.*, **57**, 191.
- Lee, J. S. & Lifson, N. (1951). *J. biol. Chem.* **193**, 253.
- Lehninger, A. L. (1946). *J. biol. Chem.* **164**, 291.
- LePage, G. A. (1950). *Cancer Res.* **10**, 393.
- Markees, S. (1954). *Biochem. J.* **56**, 703.
- Morgan, E. J. & Friedemann, T. E. (1938). *Biochem. J.* **32**, 733.
- Neuss, F. (1931). *Arch. exp. Path. Pharmacol.* **160**, 551.
- Oelkers, H. A. (1939). *Arch. exp. Path. Pharmacol.* **191**, 661.
- Orten, J. M. & Smith, A. H. (1937). *J. biol. Chem.* **117**, 555.
- Peters, R. A., Rydin, H. & Thompson, R. H. S. (1935). *Biochem. J.* **29**, 63.
- Peters, R. A., Sinclair, H. M. & Thompson, R. H. S. (1946). *Biochem. J.* **40**, 516.
- Peters, R. A. & Thompson, R. H. S. (1934). *Biochem. J.* **28**, 916.
- Peters, R. A. & Wakelin, R. W. (1946). *Biochem. J.* **40**, 513.
- Peters, R. A. & Wakelin, R. W. (1952). Unpublished results cited by Peters, R. A., in *The Tricarboxylic Acid Cycle Symposium, 2nd Int. Congr. Biochem., Paris*, p. 64.
- Robertson, W. B. (1942). *Science*, **96**, 93.
- Seligson, D. & Shapiro, B. (1952). *Analyt. Chem.* **24**, 754.
- Slater, E. C. & Bonner, W. D. (1952). *Biochem. J.* **52**, 185.
- Slater, E. C. & Holton, F. A. (1954). *Biochem. J.* **56**, 28.
- Stare, F. J. & Baumann, C. A. (1940). *J. biol. Chem.* **133**, 453.
- Stern, J. R., Minor, J. C. & Del Campillo, A. (1953). *Nature, Lond.*, **171**, 28.
- Thin, C. & Robertson, A. (1952). *Biochem. J.* **51**, 218.
- Turner, B. B. & Hulpfen, H. R. (1933). *J. Pharmacol.* **48**, 445.