CCV. PYRUVATE OXIDATION IN BRAIN

II. THE OXYGEN/PYRUVATE RATIO AND R.Q.

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IN a previous paper from this laboratory [Peters, 1936], the earlier work on the action of vitamin B₁ on the pigeon's brain was reviewed and correlated. There it was pointed out that the theory which accounts for most of the known facts is that the vitamin is an essential factor in the system responsible for the oxidation of pyruvate. This paper describes the results of an investigation into the oxidation of pyruvate by the brain of the normal and the avitaminous pigeon.

Comparatively little work has been done on the metabolism of pyruvic acid. Toenniessen & Brinkmann [1930] perfused mammalian muscle with pyruvate, and on the results of this and of earlier work on muscle brei by Fischer [1927] and Hahn & Haarmann [1928; 1929] they produced a scheme of oxidation whereby two molecules of pyruvic acid were reduced to form one of succinic acid, and the latter was converted successively into fumaric, malic, oxaloacetic and one molecule of pyruvic acid.

Elliot & Schroeder [1934], working on slices of rabbit kidney cortex, were able to confirm this scheme in some respects, in that they found that an addition of succinate, fumarate, malate and especially oxaloacetate produced an increase in bisulphite-binding substance, which was assumed to be pyruvate. They were able to find no evidence that decarboxylation of pyruvate to form acetaldehyde occurs either aerobically or anaerobically.

Barron & Miller [1932] deduced from measurement of the oxygen uptake that gonococci oxidize pyruvate to form acetic acid and carbon dioxide. Meyer [1933] found that pyruvate was oxidized in alkaline solutions by haemin and certain light-sensitive dyes with the production of oxalic acid in amounts sufficient to account for 70 % of the total oxygen uptake.

Krebs [1936; Krebs & Johnson, 1937] has brought forward another scheme. He suggests that the oxidation of pyruvate is preceded by an anaerobic change whereby two molecules of pyruvate interact to form one molecule of lactic acid, acetic acid and carbon dioxide respectively.

These schemes will be discussed later in connexion with the results of the experiments to be described here. The paper falls into two main parts, recording the results of work on (A) the normal pigeon's brain, and (B) the avitaminous pigeon's brain.

A. THE NORMAL BRAIN

The object of these experiments was to measure the ratio of oxygen uptake to pyruvate disappearance (i.e. the oxygen/pyruvate ratio) and the pyruvate R.Q. of normal brain and to compare these figures with those obtained

on the avitaminous brain. If the vitamin is acting only on pyruvate oxidation, one would expect to find that the presence or absence of the vitamin would not affect these values, but would only affect the total amount of pyruvate oxidation.

Experimental method

For the sake of accuracy all the experiments recorded in this paper were performed on brain tissue which had been washed with Ringer phosphate. The result of this washing is to lower the residual respiration of the tissue without much affecting its ability to take up oxygen in the presence of pyruvate. Table I shows how washing progressively reduces the level of the residual respiration: after two washings there is a large drop in the residual respiration and some drop also in the net pyruvate oxidation: after four washings the residual is still lower, but the pyruvate oxidation has not dropped appreciably further. It is found impossible to reduce the residual much further without grinding up the brain, and this process affects the pyruvate oxidation far more than it does the residual. It is doubtful how far the initial drop in pyruvate oxidation, which

Table I. *Normal brain, progressively washed with Ringer phosphate pH 7.3*

		Oxygen uptake in $\mu\text{l./g./hr.}$						
Period (hr. and min.)	...	0-30	30-1	1.0-1.50	1.50-2.30	2.30-3.0	3.0-4.5	4.5-5.0
Unwashed brain								
Tissue pyr.		2940	2290	1925	1670	1470	1265	940
Tissue alone		1395	920	575	370	250	165	55
Net pyr. oxidation		1545	1370	1350	1300	1220	1100	885
Brain washed twice								
Tissue pyr.		1740	1360	1125	950	820	—	475
Tissue alone		595	390	225	150	115	65	20
Net pyr. oxidation		1145	970	900	800	705	—	455
Period (hr. and min.)	...	0-30	30-1.15	1.15-2.0	2.0-2.45	2.45-3.45	3.45-4.32	
Brain washed 4 times								
Tissue pyr.		1520	1190	850	730	610	450	
Tissue alone		370	210	100	85	45	15	
Net pyr. oxidation		1150	980	750	645	565	435	

pyr. = pyruvic acid.

often occurs after the first washings and not after subsequent ones, is a real one. For, after washing, the tissue always appears more watery than when freshly minced, and the apparent drop in oxygen uptake per g. of tissue is usually associated with an increase in the water content of the brain, which may reach 87%.

Accordingly in these experiments the brain was prepared in the following way. The pigeon was killed by decapitation and the brain dissected out. The membranes and the cerebellum were removed, and the rest of the brain was minced with a bone spatula on an ice-cold plate. The mince was then washed in a tube with 5 ml. of ice-cold Ringer-phosphate and centrifuged; this washing was performed four times in all (five times when two brains were being prepared together) and finally the tissue was well mixed and transferred to the experimental bottles. The whole process of preparation usually took about 1 hr.

The pyruvic acid used had been redistilled twice and crystallized. Periodically a small volume of stock solution was made up to contain about 450 mg. per ml. and was kept in a refrigerator. When required a small portion of this solution, usually 0.2 ml., was taken, diluted with Ringer phosphate and carefully

neutralized with *N* NaOH. The purity of the solution was tested by comparing the titratable acidity with the bisulphite-binding power, and the two were always found to agree within 5%. As a rule 5 mg. of pyruvate were added to each respiration bottle, which contained 3 ml. of Ringer phosphate; the concentration was therefore about 0.02 *M*.

(1) *Estimation of the oxygen/pyruvate ratio.*

The ratio of the oxygen uptake to pyruvate disappearance has been investigated by Elliot & Schroeder [1934] in their work on the rabbit's kidney cortex, and by Braunstein & Severin [1935] who worked on the pigeon's erythrocytes. Both report that the increase in oxygen uptake on the addition of pyruvate is not large enough to account for the complete oxidation of all the pyruvate which is found to have disappeared. In each case the authors explain this result by assuming that the residual respiration is spared when pyruvate is added, and that pyruvate may disappear along some other path which does not involve complete oxidation.

The experiments described in this paper were carried out as follows. Barcroft apparatus were used. The brain was minced, washed and measured into previously weighed bottles. The pyruvate solution was suitably diluted with Ringer-phosphate so that 2 ml. contained about 5 mg., and was measured accurately into the respiration bottles with a 2 ml. Ostwald pipette. For each experiment at least three apparatus were used:

(1) containing pyruvate and tissue, stopped at the end of the equilibration period of 12 min. (i.e. just before the period of measured oxygen uptake);

(2) containing pyruvate and tissue, not stopped until the end of the respiration period;

(3) containing only tissue, not stopped until the end of the respiration period.

The difference in the pyruvate contents of bottles 1 and 2 gives the amount of pyruvate disappearing during the respiration period. The difference in the oxygen uptake of apparatus 2 and 3 is the oxygen uptake associated with the pyruvate oxidation. In this way the oxygen/pyruvate ratio is calculated; it is expressed in terms of μ l. of oxygen and mg. of pyruvate. In most experiments duplicate or triplicate estimations were made.

Before being placed in the bath the apparatus were evacuated and filled with oxygen. The bath was kept at 38° and the apparatus were shaken at a rate of 80 complete rev. per min. The average amount of tissue in each bottle was 400 mg. (wet weight) and the respiration period was usually 4 hr.; these figures are much larger than has been customary in this laboratory; but it was necessary to ensure a substantial oxygen uptake and pyruvate disappearance so that the ratio could be calculated accurately.

The estimation of the pyruvate in the Barcroft bottles was carried out in the following manner. Each bottle was treated with 0.9 ml. of 25% trichloroacetic acid to precipitate the tissue and destroy the enzymes, and the contents were filtered through a Whatman No. 44 filter-paper, smallest size. The bottle was washed out with 1 and 2 ml. of 5% trichloroacetic acid. Some control experiments had shown that pyruvate is adsorbed by filter-paper at an acid reaction: for this reason the finest Whatman papers were used and distilled water (3 successive ml.) was used to wash the filter-paper in the earlier experiments when the estimation was by the bisulphite method and was not affected by the presence of traces of protein carried through by the distilled water. In the later experiments when the hypiodite method was used, it was found that the presence of protein

interfered with the results and therefore the filter-paper was washed with 5% trichloroacetic acid instead; the error thus caused was not large enough to affect the results significantly. The filtrate was neutralized with NaOH and made up to exactly 25 ml. In the earlier experiments four samples of 5 ml. were estimated by the bisulphite method. In the later experiments three samples of 5 ml. were estimated by the bisulphite method and three of 3 ml. by the hypiodite method.

The bisulphite method was carried out in the manner described by Clift & Cook [1932]. The only modifications were in bringing the solution to a rather lower pH (i.e. pink rather than orange in colour to methyl orange) and in using phosphate buffer instead of bicarbonate as recommended by Elliot *et al.* [1935].

The hypiodite method was based on the technique described by Lohmann & Meyerhof [1934]. To 3 ml. of the solution to be tested 1 ml. of *N* NaOH was added and 5 ml. of *N*/50 iodine were run in from an automatic pipette. The mixture was shaken and allowed to stand for at least $\frac{1}{2}$ hr.; it was then acidified with 1.5 ml. of *N* HCl and the free uncombined iodine was titrated against *N*/100 thiosulphate. This figure was subtracted from the average of several blank titrations (i.e. iodine without pyruvate) and the difference, in terms of *N*/100 thiosulphate, was multiplied by 0.146 to give the answer in terms of mg. of

Table II. *Oxygen/pyruvate ratio of normal brain*

Exp.	Duration in hr.	Wt. of tissue in mg.	Total O ₂ uptake in μ l.	Total pyruvate disappearing mg.	O ₂ /pyr.
201	4	310	586	1.43	410
		324	675	1.59	430
202	4	332	545	0.90	(605)
		333	503	0.85	(590)
205	4	482	1125	2.35	480
		639	1550	3.18	490
206	4	513	1025	2.28	450
		609	1030	2.20	465
		462	840	1.82	460
207	4	261	680	1.48	455
		359	850	1.89	450
		496	1165	2.85	410
		531	1440	3.27	445
208	4	331	785	1.56	505
	4	380	720	1.59	450
	5 $\frac{1}{2}$	624	1485	3.21	465
213	4	438	1070	2.66*	400*
		581	1460	3.27	450
				3.03*	480*
		510	1200	2.83	424
				2.63*	455*
		344	825	2.08	400
215	4			1.94*	430*
		402	620	1.42*	435
				1.42*	435*
		207	318	0.55	(580)
				0.65*	490*
222	4	404	1000	2.07	480
				2.01*	495*

Figures marked * estimated by the hypiodite method.

Plain figures estimated by the bisulphite method.

Average of bisulphite results 450, σ 27.

Average of hypiodite results 455, σ 33.

pyruvate. This method was used so that any phosphopyruvic acid formed should be estimated (the bisulphite method does not apparently estimate phosphopyruvate); the two methods give virtually the same results so that presumably phosphopyruvate does not accumulate to any appreciable extent.

The specificity for pyruvic acid of both the bisulphite and the hypiodite methods in these preparations was checked by the hydrazone method, carried out by Prof. Peters. They were found to agree within 5%.

Table II summarizes the results of these experiments and it will be seen that the value of the ratio lies in nearly every case between 400 and 500. In only two experiments, 202 and 215, is the result much wide of these limits and in these experiments alone the disappearance of pyruvate is less than 1 mg. As the bisulphite method was hardly accurate for such small differences, these results have been omitted when calculating the average ratio for the bisulphite method. In the last three experiments duplicate estimations were carried out by the hypiodite method; these results have been averaged separately.

There are nineteen results based on the bisulphite method; they range from 410 to 505, with an average of 450, σ 27. There are only seven results based on the hypiodite method but they give an almost identical result; they range from 400 to 495 with an average of 455, σ 33. The variations are within the experimental error of the method.

The significance of these results will be considered in the discussion. It is only necessary to point out here that for complete oxidation each molecule of pyruvate requires 5 atoms of oxygen

and this gives an oxygen/pyruvate ratio of 635. It is therefore clear that under these conditions all the pyruvate which disappears is not being completely oxidized.

(2) *Determination of the pyruvate R.Q.*

Little work has been published on the R.Q. of pyruvate metabolism in normal tissues. Elliot & Schroeder measured it in their work on the kidney cortex [1934]; they present their results by giving the details of single experiments which they state are typical; according to these the addition of pyruvate to the rabbit's kidney tissue raises the oxygen uptake from 13.1 to 15.75 molecular equivalents and the R.Q. from 0.74 to 1.07; in the rat the oxygen uptake is raised from 18.6 to 27.2 M.E. and the R.Q. from 0.75 to 1.24. These results suggest strongly that the R.Q. for pyruvate oxidation itself is considerably above the theoretical value of 1.2.

All the R.Q. estimations recorded here have been obtained by the method of Dixon & Keilin [1933]. The object was to determine the R.Q. for pyruvate only and this was done in two ways. (1) Two apparatus were used to determine the O_2 uptake and CO_2 output of brain tissue with and without pyruvate; the difference gives the true pyruvate R.Q. (2) One apparatus was used and the pyruvate was placed in a Keilin cup in one bottle while another cup containing only Ringer was placed in the other. When thermal equilibrium had been set up, the Keilin cups were shaken down into the medium containing the tissue, and the difference in respiration on the two sides, caused by the pyruvate, gave the true pyruvate R.Q. directly.

In the use of this apparatus it is of course essential to have very nearly equal amounts of tissue in the two bottles of any one apparatus. This was found to

present difficulties at first because washed minced brain is almost fluid in consistency. Finally good results were obtained with an extemporized micro-syringe composed of a narrow-bore glass tube and a glass rod which acted as a plunger and was suitably graduated; duplicate amounts of tissue were obtained usually within 5%.

The brain was prepared in exactly the same way as described in the determination of the oxygen/pyruvate ratio. Oxygen was passed through the bottles for 10 min. while they were shaking in the bath during the preliminary equilibration period, which lasted 15 min. The apparatus was shaken at a rate of 80 complete rev. per min. Usually 250 mg. of tissue were used in each bottle and the experiment run for 2-3 hr., so that the residual respiration was small compared with the total gaseous exchange. Care was taken to avoid any leakage of the apparatus and, unless constant readings were given for a period of at least 10 min. after adding acid or alkali, the experiment was discarded. The manometer fluid was paraffin.

Eleven R.Q. experiments were carried out. Five of them are recorded in Table III; there are eleven values with an average of 1.30, σ 0.04. The remaining six experiments are not recorded because there was very poor agreement between the O_2 uptakes; the results however are almost identical with those of the other experiments; there were thirteen values with an average of 1.33, σ 0.08.

The R.Q. for the residual respiration, obtained in the course of determining the pyruvate R.Q., averaged 0.68, the mean of eleven values. The results were very scattered, varying from 0.32 to 1.07, and have not been recorded separately. Such variability was not unexpected, for the gaseous exchange was very small and the experimental error therefore large, and variations in the amount of washing may well affect the residual R.Q. considerably. Sinclair [1933] found that the R.Q. for the normal pigeon's brain (unwashed) in Ringer phosphate + lactate was 0.87, σ 0.05. It is unlikely in view of the present work that the R.Q. for lactate alone is less than 1.0, and therefore in his experiments the R.Q. of the residual non-lactate respiration must have been of the order of 0.7, which coincides with the result obtained here.

Table III. R.Q. for pyruvate respiration (washed normal brain)

Exp.	Duration in min.	O ₂ uptake in μ l. per g. of tissue	R.Q.
302	185	2500	1.42
		2250	1.27
304	165	1620	1.25
		1670	1.28
305	150	2730	1.25
		2560	1.28
309	240	2700	1.27
		2620	1.34
311	120	1550	1.26
		1530	1.34
		1640	1.31
Average			1.30, σ 0.04

Discussion

The figure found for the oxygen/pyruvate ratio, i.e. 450, makes it clear that under the conditions of these experiments all the pyruvate which disappears is not being completely oxidized. It should be noted that one explanation given

by Braunstein & Severin, and by Elliot & Schroeder, for similar results—i.e. that the addition of pyruvate spares the residual respiration of the tissues—is quite inadequate here. As a result of washing the tissue the residual oxygen uptake was very small; it was rarely more than a quarter of the net pyruvate oxygen uptake and was often only one-fifth or one-sixth as large. Hence even if the whole of the residual were included in the pyruvate respiration, the oxygen/pyruvate ratio would still be less than the theoretical for complete oxidation.

The results of the R.Q. determinations tend to confirm this view. The figure is found to be 1.30, significantly higher than the theoretical for complete oxidation, i.e. 1.20, just as the oxygen/pyruvate ratio is significantly lower.

Some scheme must ultimately be found to account for these values. The most likely explanations are: (1) that the pyruvate is being incompletely oxidized and that some intermediary metabolite is accumulating; (2) that part of the pyruvate is being completely oxidized, while the rest is disappearing along some anaerobic route. The first hypothesis cannot be discussed in detail at this stage, because the path of pyruvate oxidation in the pigeon's brain is not known. In a current paper [McGowan & Peters, 1937] evidence is produced against both the Toennissen-Brinkmann scheme (via succinic acid) and the simple direct oxidation through acetic acid. With regard to the second hypothesis the scheme recently put forward by Krebs provides a possible route for anaerobic disappearance of pyruvate. A single experiment was carried out to test the applicability of this scheme to the pigeon's brain, the lactate estimations being carried out by Mr J. R. P. O'Brien; this indicated that under anaerobic conditions pyruvate did disappear with the formation of about half as much lactate and with evolution of CO_2 . Now if it is assumed that under aerobic conditions two-thirds of the pyruvate which disappears is being completely oxidized whilst the remaining third is being converted anaerobically according to the Krebs scheme, the combined reactions give a theoretical oxygen/pyruvate ratio of 450 and an R.Q. of 1.29, i.e. values very close to the figures actually found. However, preliminary experiments indicate that, although lactate is actually produced under aerobic conditions, the amount produced is about one-half that required by the hypothesis suggested above (about 1/13 of the pyruvate disappearing instead of 1/6). One can only say that lactate formation will provide a partial explanation of the low oxygen/pyruvate ratio observed. There is, however, no evidence as yet that the Krebs reaction is involved, though the high R.Q. value obtained makes such a reaction not improbable. Elliot & Schroeder [1934] also found an increase in the lactic acid fraction in their experiments on the oxidation of pyruvate by the kidney cortex.

B. THE AVITAMINOUS BRAIN

A further series of experiments was carried out to measure the oxygen/pyruvate ratio in the avitaminous brain (in the absence of added vitamin B_1). As in all previous experiments from this laboratory only those birds were used which showed the typical terminal nervous symptoms of vitamin B_1 avitaminosis and which would no longer respond to glucose treatment. The experiments were carried out in exactly the same way as those on the normal brain, but in addition the oxygen uptake of the avitaminous brain in the presence of added vitamin B_1 was measured, to ensure that the brain was capable of giving a true vitamin effect.

The results of these experiments are set out in Table IV. There are eleven results based on the hypoidite method of estimating pyruvate; they range from

245 to 445, having a mean of 350, σ 50. There are nine results based on the bisulphite method ranging from 250 to 410 with a mean of 325, σ 70. (It must be remembered that both sets of estimations were on the same set of experiments.)

Table IV. *Oxygen/pyruvate ratio of avitaminous brain*

Exp.	Duration hr.	mg. of tissue	Total O ₂ uptake μ l.	Pyr. dis- appearance mg.	O ₂ /pyr.
214	4	420	19	0.19*	—
				0.10	—
		401	72	0.24*	—
219	5	458	155	0.14	—
				0.59*	265*
		367	101	0.49	315
				0.40*	250*
220	5	477	150	0.49	205
				0.41*	365*
		376	115	0.46	325
				0.35*	330*
221	4	400	148	0.28	410
				0.36*	410*
		538	252	0.64*	395*
224	4	461	249	0.68*	365*
				0.78	320
		462	252	1.03*	245*
				1.11	225
228	4½	517	320	0.72*	445*
				0.78	410
		437	227	0.56*	405*
				0.58	390
		448	231	0.67*	345*
				0.69	335

Figures marked * estimated by the hypoiodite method.

Plain figures estimated by the bisulphite method.

Average of hypoiodite results 350, σ 50.

Average of bisulphite results 325, σ 70.

These figures are not very satisfactory, for they show a large scatter. There is, however, a good reason why this result is so much inferior to that of the experiments on the normal brain. In the first place there was rarely more than one avitaminous brain available at any one time, and therefore the experiments had to be on a comparatively small scale. In the second place the oxygen uptake of the avitaminous brain in the presence of pyruvate is very much lower than that of the normal brain. Thus both of these factors tend to reduce the total oxygen uptake and the total pyruvate disappearance, and therefore the effect of any error in the method is multiplied.

Discussion

The oxygen/pyruvate ratio of the avitaminous brain is significantly lower than that of the normal. The probable error (i.e. P.E. = $0.6745 \sqrt{(\sigma_1^2/n_1 + \sigma_2^2/n_2)}$ where n is the number of observations and σ is the standard deviation) of the hypoiodite results of the normal and the avitaminous brains is 13.2, whilst the difference between the two means is 105, i.e. eight times as big as the P.E. Similarly the P.E. for the bisulphite results is only 16.4 whilst the difference between the means is 125 or 7½ times as large.

It is not yet possible to say what this difference signifies. There are various possible explanations which may be mentioned.

(1) There may be two different routes for pyruvate metabolism, each with a different oxygen/pyruvate ratio; in the absence of the vitamin one may be affected more than the other and the ratio thus altered. If there is some anaerobic disappearance of pyruvate which is unaffected by the presence or absence of the vitamin, then of the total pyruvate disappearing in the avitaminous brain a larger proportion would be disappearing anaerobically than in the normal and the ratio would be lowered. This possibility is supported by the finding of lactate formation in the normal brain. (In fact if it is assumed that there is the same lactate formation in the avitaminous as in the normal brain, i.e. about 0.55 mg./g. of tissue in 4 hr., and correction is made for the pyruvate and oxygen involved, then both the ratios come close to 500, provided that the lactate is formed by simple reduction of pyruvate and the Krebs scheme is not involved.)

(2) It may be that the avitaminous brain is in some way altered so that even when vitamin is added the ratio remains lower than the normal. A few experiments which have been carried out do not exclude this possibility. In some earlier work Peters & Thompson [1934] measured the ratio for the extra respiration when vitamin was added to avitaminous brain in the presence of pyruvate; they found an average of 445 at pH 7.3, but the individual results were too variable to be decisively different from either of the two ratios recorded here.

Both of these explanations are compatible with the theory that the vitamin is acting directly on the oxidation of pyruvate. It must, however, be mentioned that Sinclair [1933] measured the R.Q. of the extra respiration caused by the addition of vitamin to avitaminous brain (unwashed) in the presence of lactate, and obtained the figures of 1.6, σ 0.3 in Ringer phosphate, 1.4, σ 0.2 in Ringer bicarbonate saturated with CO₂, 1.8, σ 0.4 in the same containing phosphate. If the vitamin is acting only on the oxidation of pyruvate, one would expect the R.Q. of the extra respiration to be that of pyruvate oxidation, which has been shown here to be 1.30, σ 0.04. These experiments will have to be repeated in the presence of pyruvate and on unwashed tissue, for Sinclair's results as they stand are hardly compatible with the pyruvic oxidase theory of vitamin B₁ action.

The conclusion seems to be this: the aerobic metabolism of pyruvate by the minced pigeon's brain is not a simple process. Until it is properly understood it will be difficult to determine with certainty what part is played by vitamin B₁. The results of the present work provide a quantitative basis for future work on this subject.

SUMMARY

1. Some experiments on the metabolism of pyruvate by the pigeon's brain are described.

2. The results of the determination of the R.Q. and the oxygen/pyruvate ratio for the normal brain are given. They prove that all the pyruvate which disappears is not being completely oxidized. Some possible routes of pyruvate oxidation are discussed.

3. The result of the determination of the oxygen/pyruvate ratio for the avitaminous brain is given. The bearing of this upon the present conception of the mode of action of vitamin B₁ is considered.

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REFERENCES

- Barron & Miller (1932). *J. biol. Chem.* **97**, 691.
Braunstein & Severin (1935). *Biochem. Z.* **276**, 359.
Clift & Cook (1932). *Biochem. J.* **26**, 1788.
Dixon & Keilin (1933). *Biochem. J.* **27**, 86.
Elliot & Schroeder (1934). *Biochem. J.* **28**, 1920.
—— Benoy & Baker (1935). *Biochem. J.* **29**, 1937.
Fischer (1927). *Ber. dtsh. chem. Ges.* **60**, 2257.
Hahn & Haarmann (1928). *Z. Biol.* **87**, 107.
—— ——— (1929). *Z. Biol.* **88**, 516.
Krebs (1936). *Nature, Lond.*, **138**, 288.
—— & Johnson (1937). *Biochem. J.* **31**, 645.
Lohmann & Meyerhof (1934). *Biochem. Z.* **273**, 60.
McGowan & Peters (1937). *Biochem. J.* **31**, 1637.
Meyer (1933). *J. biol. Chem.* **103**, 39.
Peters (1936). *Biochem. J.* **30**, 2206.
—— & Thompson (1934). *Biochem. J.* **28**, 916.
Sinclair (1933). *Biochem. J.* **27**, 1927.
Toenniessen & Brinkmann (1930). *Hoppe-Seyl. Z.* **187**, 137.