The Accumulation of Citrate during Oxidation of Pyruvate by Breis and Slices of Pigeon Brain

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A previous communication (Coxon, Liébecq & Peters, 1949) reported the formation of citrate, together with α-ketoglutarate, in the course of the oxidation of pyruvate by dialysed dispersions of pigeon brain, and the findings were discussed in relation to the tricarboxylic acid cycle (Krebs & Johnson, 1937). The present paper is mainly concerned with studies of citrate formation under similar conditions by breis and slices from the same organ, but some further experiments bearing upon the comparative behaviour of dispersions are also included. Appreciable formation of citrate has been shown to accompany the oxidation of pyruvate by both breis and slices, but the quantities found were much less than in the earlier studies on dispersions of brain.

In an endeavour to clarify the relationship between this citrate formation and the overall pathway of pyruvate oxidation in pigeon brain, some experiments were also performed on the interconversion by this tissue of citrate and the other two tricarboxylic components of the Krebs's cycle. So that any possible complications arising from permeability factors might be minimized, the latter experiments were carried out with dispersions, in which considerable aconitase activity was demonstrated. This is in agreement with the findings of Johnson (1939) for rat brain.

EXPERIMENTAL

Reagents. Sodium pyruvate, racemic *iso*citric acid and *cis*-aconitic anhydride were prepared by Mr R. Wakelin of the Department of Biochemistry, Oxford. The sodium citrate employed was a sample from Kahlbaum A. G. Other reagents used in chemical analyses were of A.R. grade and supplied by British Drug Houses Ltd.

Analytical methods. Citrate was estimated by the method of Pucher, Sherman & Vickery (1936) as modified by Buffa & Peters (1949). Light petroleum for this procedure was purified by washing successively with conc. H_2SO_4, 0·5 M-$KMnO_4$ and water, and all tissue extracts were subjected to a preliminary boiling with H_2SO_4 as recommended by Breusch & Tulus (1947) to reduce interference by chromogenic substances other than citrate. *iso*Citrate was determined enzymically using *iso*citric dehydrogenase. The reaction was followed in a Beckman spectrophotometer, model DU, according to the method of Ochoa (1948) by observing the coupled reduction of triphosphopyridine nucleotide (TPN). The enzyme used in this determination was prepared from pig's heart by Dr W. D. Lotspeich, who generously made it available (see Lotspeich & Peters, 1951). The TPN was prepared from horse liver by the writer in collaboration with Dr Lotspeich and was about 14% pure as judged by the extinction coefficient at 340 mμ. The pyruvate determinations quoted in Table 4 were carried out according to Long (1942).

Tissue preparations. Breis were prepared by mashing on a warm plate as described by Peters, Rydin & Thompson (1935) and slices were cut free-hand. In both instances the tissues were introduced into tared flasks and their weight found by difference. Dispersions were prepared and dialysed as described by Banga, Ochoa & Peters (1939).

RESULTS

Formation of citrate in breis and slices. Table 1 gives the results of citrate determinations upon a series of breis after incubation with pyruvate, and

Table 1. *Accumulation of citrate in pigeon-brain brei after incubation with pyruvate*

(Each flask contained approx. 400 mg. of tissue suspended in 4 ml. of Krebs-Ringer bicarbonate solution. Temp. 37°. Incubation for 2 hr. in 5% CO_2 + 95% O_2.)

Expt. no.	Pyruvate added (μmoles/flask)	Citrate found (μmoles/g. tissue) Initial	Final
149	45	0·40	1·30
150	45	0·70	2·20
151	0	—	0·42
	45	—	2·10
147	45	0·50	1·40
	45 + Ba*	0·50	2·60

* This flask contained 0·05 M-$BaCl_2$.

Table 2 gives similar data in respect of slices. It has also been found that citrate formed in slices does not diffuse freely into the suspending medium (Coxon, 1950). The extent of citrate formation in breis and slices is roughly the same, amounting to about 1 μmole/g. tissue in 2 hr. The demonstration that citrate is formed by breis and slices respiring in pyruvate, despite the fact that the quantities produced are small compared with those arising in dispersions, is good evidence in favour of the thesis that in breis and slices, as in dispersions (Coxon *et al.* 1949), the metabolism of pyruvate proceeds through a tricarboxylic stage. A quantitative comparison of the amounts of citrate found in relation to the

accompanying oxygen uptake in slices on the one hand, and dispersions on the other, makes it clear that the molar citrate/oxygen ratio is vastly greater

Table 2. *Accumulation of citrate in pigeon-brain slices after incubation with pyruvate*

(Each flask contained approx. 400 mg. of tissue in 4 ml. of Krebs-Ringer phosphate solution. Temp. 37°. Incubation was for 100 min. in O_2.)

Expt. no.	Pyruvate added (μmoles/flask)	Citrate found (μmoles/g. tissue) Initial	Final
158	0	0·55	1·00
	90	0·75	1·70
160	0	0·40	0·50
	90	1·20	2·30
186	0	—	0·60
	90	0·60	1·70
187	0	—	0·35
	90	—	1·30

Table 3. *Formation of citrate relative to the uptake of oxygen in dispersions and slices*

(Dispersions were incubated for 40 min. in buffered saline,* pH 7·2, at 37° in air; slices were incubated for 100 min. in Krebs-Ringer phosphate solution at 37° in O_2. Values are based on four experiments in each instance and expressed as means with S.D. Pyruvate was present as substrate in all experiments.)

	Dispersions	Slices
Citrate formed (μmoles/g. tissue)	12± 1·1	1·1±0·15
Oxygen uptake (μmoles/g. tissue)	91±10·5	198 ±14
Molar ratio (citrate/oxygen)	1/7·5	1/180

* Buffered saline contained Na_2HPO_4, $2H_2O$ (12·460 g.), KH_2PO_4 (4·083 g.), KCl (8·0 g.), and $MgCl_2$ (0·122 g.) in 1 l.

Table 4. *Proportion of added pyruvate converted to citrate by pigeon-brain brei*

(Medium was Krebs-Ringer bicarbonate solution. Temp. 37°. Incubation for 2 hr. in 5% CO_2+95% O_2. The values given for pyruvate and citrate are based on four experiments in each instance and expressed as means with S.D.)

Pyruvate disappearing (μmoles/g. tissue)	Citrate formed (μmoles/g. tissue)	Conversion of pyruvate to citrate (%)
29·0±2·1	1·2±0·3	4·1

in dispersions. Table 3 presents such a comparison and was constructed from the results of the same experiments as those quoted in Table 2, with the addition of certain other experiments carried out in the course of related studies. The relative smallness of the amounts of citrate found in slices and breis would be compatible with the idea that their oxidative processes are far better co-ordinated than in dispersions, so that there is far less tendency in them for the 'stranding', as it were, of intermediates and by-products.

Table 4 summarizes data from a number of experiments (including those recorded in Table 1) on which can be based an estimate of the proportion of the pyruvate metabolized by a brei which appears as citrate. This is seen to be about 4%.

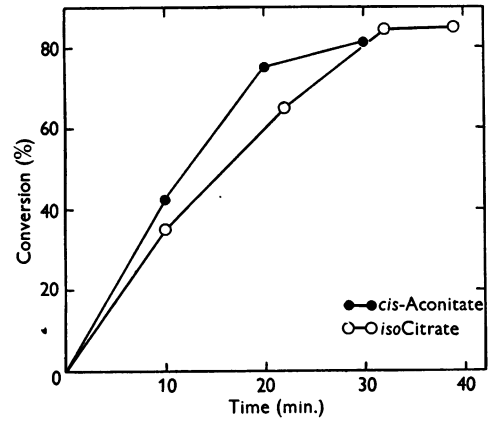

Fig. 1. Conversion of *cis*-aconitate and D-*iso*citrate into citrate by pigeon-brain dispersions. The initial concentration of substrate was 0·002 M in each case.

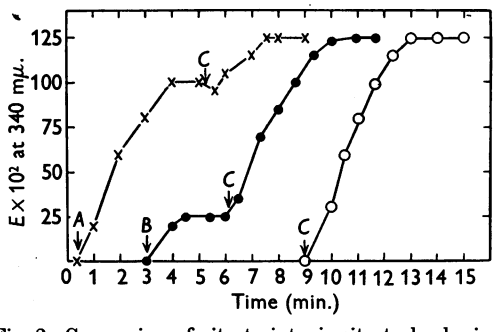

Fig. 2. Conversion of citrate into *iso*citrate by brain dispersion. At zero time all cuvettes contained TPN (approx. 0·1 μmole) + $MnCl_2$ (1·0 μmole) + *iso*citric dehydrogenase (equivalent to 0·8 mg. pig-heart powder) in 0·01 M-phosphate buffer, pH 7·2. Where indicated by the arrows the following were added: at *A*, filtrate from flask in which dispersion had been incubated with citrate; at *B*, filtrate from flask in which dispersion had been incubated without citrate; at *C*, 0·5 μmole of DL-*iso*citrate.

Spectrophotometric examination of the 2:4-dinitrophenylhydrazone on which the estimation of pyruvate in these experiments depended revealed no admixture of α-ketoglutarate; slight traces of the latter compound were noticed when paper chromatography according to Cavallini, Frontali & Toschi (1949) was applied to the products of incubation, but these were insufficient to affect the pyruvate determinations.

Interconversion of tricarboxylic acids in dispersions. Figs. 1 and 2 show the results of some experiments on

the interconversion of the three tricarboxylic acids of the Krebs's cycle in pigeon brain. It is clear from Fig. 1 that when either *iso*citrate or *cis*-aconitate is added, equilibrium is reached after some 85% of the substrate has been converted into citrate and that the establishment of this condition requires approximately 30 min.

Fig. 2 provides qualitative evidence that, when citrate is added, a proportion of it is converted into *iso*citrate. The combined data in the two figures thus demonstrate that citrate and *iso*citrate are interconvertible in pigeon brain and that *cis*-aconitate is a likely intermediate in the process since it can be shown to give rise to citrate in the system. Although for technical reasons no attempt was made to demonstrate directly the transformation of citrate or *iso*citrate to *cis*-aconitate, these reactions can reasonably be inferred from the other data taken in conjunction with the known characteristics of aconitase. Both transformations were, in fact, demonstrated by Johnson (1939) in rat brain.

State of division of the tissue in breis. It is generally found that at the end of 2 hr. incubation the fluid in a flask containing brei has become markedly turbid, implying that tissue constituents have been liberated into it; to a much lesser extent the same applies to slices. Thus, when citrate accumulation is observed in a brei, the question arises as to whether this has been formed by the larger tissue masses characteristic of a brei or by the more freely dispersed tissue elements. Breis were accordingly prepared and shaken vigorously at 4° until disintegration was well advanced; at this stage the coarser pieces of tissue were separated from the finer by straining through muslin as is customarily done when preparing dispersions. The larger particles were then resuspended in fresh medium and incubated at 37° while the filtered fluid was incubated in another flask.

Table 5. *Formation of citrate in fractionated brei*

(Brei from 1400 mg. of pigeon brain was fractionated by straining through muslin after preliminary shaking at 4°. The fractions were then incubated for 2 hr. in Krebs-Ringer phosphate solution containing 0·012 M-pyruvate, in air.)

	Aggregated fraction	Dispersed fraction
Dry weight (mg.)	114	102
Citrate found (μmoles/g. dry weight)	3·3	9·5

The results in Table 5 show that citrate formation occurred in both moieties, the amount found being greater per unit of dry weight in the case of the finely divided suspension as might be expected from its greater similarity to a dispersion. None the less, appreciable formation also occurred in the flask containing the larger particles.

DISCUSSION

Acceptance of a tricarboxylic acid cycle operating in breis of pigeon brain calls for some attempt at a reconciliation of such a process with certain apparently contradictory observations reported in earlier communications. In particular the finding of McGowan & Peters (1937) that the stimulant effect of thiamine on the respiration of vitamin-deficient brei incubated in pyruvate was not modified by the simultaneous metabolism of succinate, appeared to indicate an independence of the oxidative pathways for these two substances. However, evidence that has accumulated since their experiments were carried out suggests that the added succinate could be metabolized at a site different from that at which the tricarboxylic acid cycle was in operation, for it is now well known that an enzyme can be found in more than one locus in a tissue preparation, as exemplified by the case of *iso*citrate dehydrogenase which has been recovered from both soluble and mitochondrial fractions of liver 'homogenates' (Hogeboom & Schneider, 1950). Moreover, it is known (Potter, Recknagel & Hurlbert, 1951) that cells frequently contain quantities of enzymes which are in excess of their requirements for normal co-ordinated metabolism) so that a surplus of exogenous succinate could presumably be handled without necessarily depressing the cyclical mechanism in experiments such as those of McGowan & Peters. The postulate of at least two spatially distinct systems for oxidizing succinate in brain breis is now advocated by Peters (1953) and is consistent with the finding (Long, Ochoa & Peters, 1939) that exogenous succinate undergoes only a one-stage conversion into fumarate in such preparations. The important question as to whether the functional separation of these two succinoxidase systems is or is not an artifact resulting from the method of preparing breis must for the present remain open.

Turning to the results recorded in Table 4 showing the proportion of pyruvate metabolized by breis which appears as citrate, it is satisfactory to find that the estimated 4·1% is in good agreement with expectation from the careful pyruvate balance figures of Long (1938). This observer found that of the pyruvate which was metabolized by breis, some 20% underwent only oxidative decarboxylation to acetate, a further 10% entered into an anaerobic dismutation to lactate, while 67% was completely oxidized to carbon dioxide and water. He was thus left with 3% unaccounted for, and this may now be said to be traceable as citrate. Both Long's and the present value also tally well with that of 2–4%

which was the estimated yield of citrate from pyruvate in some large-scale experiments on bovine brain carried out by Simola & Alapeuso (1943).

As shown by the results in Table 5, a classification of tissue preparations into slices, breis and dispersions is somewhat arbitrary and in certain respects misleading. This should not, however, obscure the fact that the metabolism of the more organized preparations is better systematized to the extent that intermediate reactants accumulate to a lesser degree in them, although the appearance of such intermediates at all in recognizable form is in a sense a measure of the artificiality of the experimental conditions.

SUMMARY

1. The accumulation of citrate in association with the oxidation of pyruvate by breis and slices of pigeon brain is reported.

2. The extent of citrate formation in such preparations is compared with that previously found in dispersions, and the implications of this comparison are discussed.

3. Some reactions between *cis*-aconitate and D-*iso*citrate and citrate in dispersions of pigeon brain have been studied.

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A Third Unsaturated Amino Acid in Groundnut Plants: Evidence for the Occurrence of γ-Amino-α-methylenebutyric Acid

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In recent years several new amino acids have been isolated from plant materials, whilst the presence of many other compounds of similar type, as yet lacking definite characterization, has been indicated by the methods of paper chromatography. Two amino acids isolated from groundnut plants (*Arachis hypogaea*) and provisionally identified as γ-methyleneglutamine and γ-methyleneglutamic acid, have proved unusual in that they are unsaturated compounds (Done & Fowden, 1952). These compounds are subsequently referred to as I and II respectively. No general survey of their distribution within the plant kingdom was then undertaken, but evidence for their presence in the leaves of tulips and certain other members of the Liliaceae has been obtained more recently. Prof. F. C. Steward of Cornell University has informed us that he and his co-workers are investigating two compounds in tulips which appear to be identical with those found in groundnut plants. It would