photoelectrically; (c) that this difference might be missed using old (photographic) methods, for the changes, involving a shift of the band maximum only 1–2 $m\mu$. and a small decrease in the slope on the short wave side of the maximum, are nearing the limits of detection; (d) that the curve of the β -carotene incubated alone is little different qualitatively from that obtained after incubation with iodinated casein.

Although experiments using more refined techniques have failed to substantiate earlier claims that thyroactive compounds catalyse the *in vitro* conversion of β -carotene into vitamin A, it is not to be assumed that the thyroid has no action on the *in vivo* metabolism and vitamin A. In fact, it has recently been shown in this laboratory that desiccated thyroid

enhances and thiouracil reduces the absorption of β -carotene from the intestinal tract (Cama & Goodwin, 1949).

SUMMARY

1. It has not been possible to substantiate the claim that iodinated casein and desiccated thyroid are active in converting β -carotene into vitamin A *in vitro*.

2. The most likely error leading to this conclusion is the failure to distinguish the *cis* peak of isomerized β -carotene from the 326 $m\mu$. absorption band of vitamin A.

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The Pyruvate-oxidase System in Brain and the Tricarboxylic Acid Cycle*

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The term pyruvate-oxidase system has been used in this laboratory to denote the complex of enzymes in brain which induce oxidation of added pyruvate. In earlier work (Long, Ochoa & Peters, 1939; Banga, Ochoa & Peters, 1939) the idea that this system in pigeon brain involved a tricarboxylic acid cycle (Krebs & Johnson, 1937; Krebs & Eggleston, 1940) was not supported. One of the main reasons was that, in contrast with kidney preparations, the increased oxygen uptake observed on addition of fumarate as well as pyruvate was not observed with citrate, which could not therefore be an intermediate; but, since then, the role of citrate has been changed to that of a side product rather than that of an

intermediate (Wood, Werkman, Hemingway & Nier, 1941). It has always been difficult to account for the action of fumarate upon any other view than that of a tricarboxylic acid cycle, and we now think that the pyruvate-oxidase system in our homogenates does in fact include much of this cycle when fumarate is present; we are giving our evidence for this, though all details are not yet clear. The most important new point is that citrate and a substance, which is estimated as α -ketoglutarate, accumulate during the oxidation of pyruvate in the presence of fumarate and when malonate is also present.

METHODS

Estimations

Pyruvic acid. The Neuberg-Case method (Case, 1932) has been used as modified by Peters & Thompson (1934), the final colour of the hydrazone in ethanolic KOH being

* A preliminary account was given to the Biochemical Society on 22 January 1949 (Coxon, Liébecq & Peters, 1949).

† With the technical assistance of R. W. Wakelin.

measured in a Hilger absorptiometer with filter no. 7; the modified procedure described by Long (1942) has also been used. It has, however, been realized during this work that these methods give misleading results since they do not distinguish between pyruvate and α -ketoglutarate in a mixture, though the extinction of the latter is less for a given concentration.

The Friedemann & Haugen (1943) and the Straub (1936) methods have been employed as described by them, with the exception that in the case of the former a Beckman spectrophotometer was used for the final reading as explained below in connexion with α -ketoglutarate.

α -Ketoglutarate has been estimated most often by the method of Friedemann & Haugen (1943), employing the 420 and the 540 μ m. bands on the Beckman spectrophotometer instead of the simple photometer with different filters recommended by them. The enzymic method of Krebs (1938) was also used on a few occasions (as an independent check on the identity of the α -ketoglutarate). It is conceivable, however, as will be discussed later, that the substance which was determined by either method as α -ketoglutarate was originally present in the tissue suspension as oxalosuccinate and was converted to α -ketoglutarate by the subsequent analytical operations. Provided that the mixture contains more than 10% of α -ketoglutarate, the acids can be determined individually by the Friedemann & Haugen technique to an accuracy of $\pm 5\%$ in known mixtures. Duplicates agree to within $\pm 2\%$.

Citric acid has been determined by the usual colorimetric method of Pucher, Sherman & Vickery (1936) and acetic acid by Long's (1938) modification of the method of Weil-Malherbe (1937). The details are given by Liébecq & Peters (1949). Latterly, preparatory to the citric acid estimation, all samples were boiled in presence of acid; the oxidation was carried out with permanganate, added drop by drop, and at 0.5N instead of 1.5N.

Preparations

Homogenates ('dispersions') from pigeon brain were made by grinding in a mortar according to the description of Banga *et al.* (1939); they were either dialysed as described by these authors or, more recently, some of the homogenates have been centrifuged for 30 min. in the cold room at 4000g, the centrifuged solid particles being then stirred into 1% KCl

solution after separation from the supernatant fluid, to give a 'centrifuged homogenate'. It should be emphasized that the most satisfactory 'ground' homogenates are made by adding the cold KCl solution to the sticky pounded brain tissue drop by drop in the initial stages. Weights quoted in tables are of fresh tissue.

Adenosinetriphosphate (ATP) was prepared from the Ba salt obtained commercially (Boots Pure Drug Co. Ltd.) and was 80% pure.

The following acids were prepared by Mr R. W. Wakelin in this laboratory: α -ketoglutaric acid, m.p. 114.5 (corr.); *cis*-aconitic acid from *cis*-aconitic anhydride, m.p. 77°; *iso*-citric acid by hydrolysis of *isocitric* acid lactone, m.p. 138° after several recrystallizations from ethyl acetate; this last m.p. is lower than that given by Krebs & Eggleston (1944), but the compound behaved normally with aconitase.

RESULTS

It is convenient to give first the new facts and then to consider these in relation to previous work.

Accumulation of citrate

The experiments given in Table 1 are representative of several, and show that with dialysed

Table 1. Citrate formed in absence and presence of fumarate by dialysed homogenate of pigeon brain

(Period of incubation 30 min., following equilibration for 10 min. Temp. 38°. Tissue, approx. 200 mg.; fumarate, 0 or 10 μ mol.; pyruvate, 30 μ mol.; ATP, 1 μ mol.; $MgCl_2$, 4 μ mol. in 2.5 ml. in each bottle with 0.08M-phosphate buffer (pH 7.3). Addition of fumarate without pyruvate in control experiments produced somewhat less citrate than did pyruvate without fumarate.)

Exp. no.	Citrate formed (μ mol./bottle)		Difference due to fumarate
	Without fumarate	With fumarate	
1	0.36	2.43	+ 2.07
2	0.71	2.31	+ 1.60
3*	0.05	2.70	+ 2.65

* Centrifuged.

Table 2. Effect of *cis*-aconitate and citrate upon oxygen uptake of centrifuged and dialysed brain dispersions

(Tissue: approx. 200 mg./bottle; temp. 38°. Period of exp. 30 min. Each bottle contained 0 or 30 μ mol. pyruvate; 0 or 10 μ mol. fumarate; 1 μ mol. ATP, 4 μ mol. $MgCl_2$ and 0.08M-phosphate buffer pH 7.3 in 2.5 ml.)

Oxygen uptake (μ mol./bottle)											
Exp. no.	Concentration (μ mol./bottle)		Fumarate absent						Fumarate present		
			No other additions	Pyruvate		Pyruvate + <i>cis</i> -aconitate	Pyruvate + Citrate	No other additions	Pyruvate	Pyruvate	
	<i>cis</i> -Aconitate	Citrate		<i>cis</i> -Aconitate	Citrate					+ <i>cis</i> -aconitate	+ citrate + <i>cis</i> -aconitate
1	45	0	—	—	—	5.0	6.7	—	2.5	9.8	—
2	40	9.6	—	—	—	—	—	3.0	12.8	13.0	10.0
3	15	11	—	—	—	—	—	—	12.7	—	12.5
4	20	20	2.8	2.5	2.3	4.8	5.6	4.5	3.9	7.3	—
5*	10	0	—	1.4	—	4.5	7.7	—	—	—	—
6*	10	0	—	2.3	—	4.2	8.0	—	8.9	—	—

* Dialysed and contained only 10 μ mol. of pyruvate.

Table 3. α -Ketoglutarate estimated as formed in absence and presence of fumarate in dialysed homogenates of pigeon brain

(Tissue: approx. 200 mg./bottle; temp. 38°. Each bottle contained ATP, 1 μ mol.; $MgCl_2$, 4 μ mol. with 0.08M-phosphate buffer at pH 7.3; 0 or 10 μ mol. fumarate, and pyruvate as indicated, in 3 ml.)

Exp. no.	Pyruvate added (μ mol.)	Period of incubation (min.)	α -Ketoglutarate found (μ mol./bottle)		Method of estimation
			Fumarate absent	Fumarate present	
1	30	40	1	3.7	Friedemann & Haugen (1943)
2	18	40	Nil	2	
3	9	40	1	2.8	
4	30	10	Nil	2.5	
		40	Nil	3.5	
5	9	10	0.5	1.5	
		40	1	2.8	Krebs (1938)
6	9	40	2.4	4.5	
7	9	40	Nil	5.0	Friedemann & Haugen (1943)
8	0	40	Nil	0.35	
9	0	40	Nil	0.25	

brain homogenates respiring in pyruvate, citrate is formed, and that upon addition of fumarate much more citrate accumulates than in its absence. It was shown previously (Banga *et al.* 1939) that addition of citrate does not produce a 'fumarate' effect in these homogenates; hence any two-carbon fragment from pyruvate, after condensation with oxaloacetate and formation of citrate via *cis*-aconitate, cannot be oxidized further. Addition of *cis*-aconitate, together with pyruvate, gives some extra oxygen uptake in the absence of fumarate, tending to be less than that produced by fumarate; with fumarate and pyruvate there is no further increase. Citrate is inhibitory under some conditions (Table 2). Of other intermediates in the cycle, α -ketoglutarate is known from previous work in this laboratory to be oxidized by the brain preparation (see p. 323). The simplest interpretation of these facts is that in the brain homogenates some form of tricarboxylic acid cycle is operating; and that the oxidation sequence tends to be choked between *cis*-aconitate and α -ketoglutarate so leading to the accumulation of citrate.

Formation of α -ketoglutarate

During the respiration period of a brain homogenate, the concentration of a substance estimated as α -ketoglutarate is greatly increased if fumarate is present (Table 3). In the presence of pyruvate this can be demonstrated only by Friedemann's spectrophotometric method in the mixed 2:4-dinitrophenylhydrazones in alkaline solution. It cannot be demonstrated with the various modifications of the Neuberg-Case method (Case, 1932), though its presence can be deduced by estimating the total hydrazone in this way and subtracting the value for pyruvate obtained by the more specific

procedure of Straub (1936). The enzymic method of Krebs (1938) provides an independent means of assay.

Acetic acid

The formation of acetate ion has not been so thoroughly investigated, but Table 4 shows that its behaviour in presence and absence of fumarate is the converse of that occurring with citrate and α -ketoglutarate. This indication that, in absence of fumarate, the pyruvate is largely degraded to acetate is consistent with the hypothesis advanced by Long & Peters (1939).

Table 4. Formation of acetate in absence and presence of fumarate by dialysed homogenate of pigeon brain

(Each bottle contained ATP, 1 μ mol.; $MgCl_2$, 4 μ mol.; 0.08M-phosphate buffer (pH 7.3); pyruvate, 30 μ mol.; either 0 or 10 μ mol. fumarate and tissue, approx. 200 mg. in 2.5 ml. Period of experiment: 30 min. + 10 min. equilibration.)

Exp. no.	Acetate found (μ mol./bottle)	
	Fumarate absent	Fumarate present
1	—	3.16
2	—	2.70
3	13.25	4.42
4	10.9	—

The effect of malonate and the origin of α -ketoglutarate

One of the important facts used in the proof of a tricarboxylic acid cycle in other tissues has been that there is formation of succinate from fumarate in the presence of excess of malonate (Krebs & Eggleston, 1940). Since excess of malonate inhibits the action of succinic dehydrogenase, it should also stop any possible formation of α -ketoglutarate from fumarate via succinate. That the α -ketoglutarate

Table 5. *Effect of malonate on oxygen uptake, disappearance of pyruvate and formation of citrate and α -ketoglutarate*

(Each vessel contained ATP, 1 μ mol.; $MgCl_2$, 4 μ mol.; 0.08M-phosphate buffer (pH 7.3); 10 μ mol. pyruvate and dialysed brain homogenate equivalent to 150 mg. tissue. Malonate (where added), 0.025M. Time of incubation was 30 min. + 10 min. equilibration. Temp. 38°. All figures given are in μ mol./bottle, but the O_2 values have not been corrected for the equilibration period.)

	Exp. 1				Exp. 2		
	Pyruvate alone	With fumarate (10 μ mol.)	With malonate	With fumarate + malonate	Alone (10 μ mol.)	With fumarate (10 μ mol.)	With fumarate + malonate
Pyruvate disappearance	3.5	8.1	3.0	6.7	5.5	7.5	6.5
O_2 uptake	2.2	6.9	1.4	5.0	1.6	6.7	4.7
Citrate formation	—	2.5	0.15	2.4	—	—	—
α -Ketoglutarate formation	0.37	2.7	0.6	2.3	0.20	1.5	1.2

found in our experiments cannot be formed in this way is shown by Table 5; citrate and α -ketoglutarate are produced in presence of malonate.

It seems certain that the substance estimated in this work is α -ketoglutarate 2:4-dinitrophenyl-hydrazone. Not only does it behave spectrophotometrically in the same way as an authentic specimen, but also the substance can be converted into succinate and estimated enzymically as such. Nevertheless, there are some facts which make it uncertain at first sight whether this substance is present in the bottles as α -ketoglutarate; its amount is not appreciably increased by *isocitrate* (Table 6) and in

Table 6. *Comparison of α -ketoglutarate formation in presence of cis-aconitate and iso-citrate with that in presence of fumarate*

(Each bottle contained 10 μ mol. pyruvate; 1.0 μ mol. ATP; 4.0 μ mol. $MgCl_2$ and 0.08M-phosphate buffer at pH 7.3. Final volume 3 ml./bottle. Temp. 38°.)

Exp. no.	cis-Aconitate added (μ mol.)	isoCitrate added (racemic) (μ mol.)	Fumarate added (μ mol.)	α -Keto-glutarate found (μ mol.)
1	10	0	0	0.75
	0	0	10	3.75
2	10	0	0	0.5
	0	0	10	2.5
3	0	20	0	0.1
	0	0	10	2.0
4	0	20	0	0.3
	0	0	10	2.0
5*	0	20	0	0.6
	0	0	10	1.8

* $MnSO_4$, 4 μ mol. and coenzyme II, approx. 1 μ mol. also added.

presence of malonate no extra α -ketoglutarate appears; α -ketoglutarate itself is known to be oxidized by the brain tissue. In view of this, we have considered the possibility that the ' α -ketoglutarate' is originally present as oxalosuccinate (Ochoa, 1948); however, both the statements of Ochoa and our observations suggest that this is so

unstable that it would not long remain unchanged. The experiments with comparatively low concentrations of α -ketoglutarate given in Table 7 show

Table 7. *Fate of α -ketoglutarate added in vitro*

(Each bottle contained 1 μ mol. ATP; 4 μ mol. $MgCl_2$; 0.08M-phosphate buffer at pH 7.3, together with dialysed brain dispersion equivalent to 200 mg. tissue. Volume/bottle 2.5 ml. Preliminary equilibration for 10 min. Temp. 38°.)

Exp. no.	Malonate added (μ mol.)	α -Ketoglutarate (μ mol.)		
		Initial	After 10 min.	After 20 min.
1	0	3.0	—	0.8
	0	6.0	—	1.7
	70	6.0	—	2.2
2	0	3.0	1.5	1.1
	0	6.0	2.9	2.1
	70	3.0	1.5	1.4

that, though larger amounts are readily oxidized, smaller amounts tend to remain as such and their rate of disappearance is not greatly influenced by malonate. Hence the behaviour resembles that in our experiments, and indicates that the α -ketoglutarate found can be simply that accumulating in the system during the oxidation.

Oxygen uptake and the balance sheet

In the past, it has been thought possible to draw up a balance sheet indicating that 1.5 mol. oxygen was used per mol. pyruvate (Banga *et al.* 1939), whilst the oxygen:pyruvate ratio for the extra metabolism produced by adenylic acid in the presence of fumarate has been found to approach more nearly the theoretical ratio for complete oxidation, viz. 2.5 (Long, 1946). In absence of adenylic acid or fumarate the ratio is considerably lower. Such calculations have been based on the assumption that the action of fumarate was a simple catalysis. Since we now consider that the fumarate enters into a complex series of oxidations such a balance sheet is unsatisfactory; less oxygen is used than would be necessary for complete oxidation of pyruvate and

we are forced to believe that incompletely oxidized products are left at various stages in the cycle. No balance sheet can be made until full information is available of the amounts present of all partially oxidized products.

DISCUSSION

Our experiments have led us to the belief that, as shown in other tissues, fumarate increases the oxygen uptake with pyruvate in brain because it supplies oxaloacetate, and that therefore the total pyruvate oxidase system in brain, as understood in this laboratory, includes some form of 'tricarboxylic acid' cycle. Though there are still discrepancies to be explained, these do not justify any longer a rejection of the idea of oxidation via a cycle, which is consistent with so much other evidence. At the same time, it is not yet clear just how citrate is formed and there are other incongruities which should be mentioned. Unlike kidney preparations, the brain homogenates do not oxidize citrate; they oxidize *cis*-aconitate only to a small extent. Further, as mentioned, added *cis*-aconitate gave rise to much smaller amounts of ' α -ketoglutarate' than did fumarate in a few experiments made by us (Table 6). We do not feel that the latter fact necessarily means that the α -ketoglutarate formed was arising by a separate path, because the conditions may be different when *cis*-aconitate is formed as an intermediate in presence of plentiful fumarate. The simplest view is that in the preparations used here the cycle is partially blocked between the stages *cis*-aconitate and α -ketoglutarate and also between α -ketoglutarate and succinate, but this interpretation still lacks the decisive proof that addition of some factor can remove the block.

Considering the oxidation in presence of fumarate, Banga *et al.* (1939) found that both adenine nucleotides and fumarate (as well as other acids in this series) were involved in the more complete oxidation of pyruvate. In further careful work, Long (1946) concluded that the same amount of pyruvate disappeared in presence and absence of fumarate, but that with fumarate, there was a more complete oxidation of the pyruvate disappearing. This led him to consider that the idea advanced by Banga *et al.* (1939), that fumarate increased the utilization of pyruvate, was erroneous, no allowance having been made for pyruvate formed during the experiment by oxidation of fumarate to pyruvate. It is shown here that part of the α -keto acid estimated by

Neuberg-Case methods with fumarate present is α -ketoglutarate, which interferes only to an exceedingly small extent in the Straub (1936) method used by Banga *et al.* (1939). Hence the discrepancy might be explained by the failure of specificity in the Neuberg-Case methods. Nevertheless, the idea that the function of the fumarate is not simply catalytic is correct. Doubtless an exact knowledge of the amounts of various components of the cycle left in a partially oxidized state would lead to a quantitative balance sheet in these brain preparations, but, since so much will depend upon uncertain factors in the fragmentation, this investigation seems of secondary importance at the present time when various features of the cycle are more readily studied in other tissues (cf. Green, Loomis & Auerbach, 1948).

In absence of fumarate, the oxidation of pyruvate takes a different course and seems to go to acetate rather than *cis*-aconitate; as mentioned, this is consistent with the hypothesis of Long & Peters (1939), with the observations of Banga *et al.* (1939) in absence of fumarate, and with later work by Long (1943).

It is hoped to deal with this reaction later, and to leave to a later communication also the explanation of the apparent discrepancy between these results and some earlier reports from this laboratory based upon the assumption that succinic dehydrogenase and the pyruvate oxidase system were acting independently in *brei*. Some of the implications of these experiments for pharmacology have been discussed elsewhere (Peters, 1948).

SUMMARY

1. An accumulation of citrate and of α -ketoglutarate has been found to accompany the oxidation of pyruvate by dialysed 'dispersions' (i.e. finely ground homogenates) of pigeon brain in a medium containing fumarate and reinforced with magnesium ions and adenine nucleotide.

2. This evidence, despite the previous observation in this laboratory that citrate cannot replace fumarate in its effect on the oxygen uptake of these preparations, is taken to favour the operation in them of a tricarboxylic acid cycle when fumarate is present.

3. When fumarate is absent, the oxidation of pyruvate is associated with the formation of acetate.

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Acceleration of Bacterial Glutamic Decarboxylase and Glutaminase by Cetyltrimethylammonium Bromide

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It has been shown in a previous paper from this laboratory (Krebs, 1948) that cetyltrimethylammonium bromide ('cetavlon') can accelerate the decarboxylation of glutamate and the deamidation of glutamine by washed suspensions of *Clostridium welchii*. The present paper is concerned with the mechanism of this effect. As it is known that detergents may alter the permeability of cells (Gale & Taylor, 1947; Hotchkiss, 1946), it was thought that cetavlon might facilitate the entry of the substrates into the cells. To test this hypothesis, the effect of cetavlon on the rate of decarboxylation of glutamate and glutamine by cell-free extracts has been examined, but contrary to expectation the detergent gave the same effect as with intact cells. Thus the effect cannot be due to changes in the permeability of the cell wall.

METHODS

Organisms. *Clostridium welchii* S.R. 12 (National Collection of Type Cultures (N.C.T.C.) no. 6784) was grown for 16 hr. at 40° and a strain of *Escherichia coli* isolated locally and *Proteus morgani* (N.C.T.C. no. 2818) for 24 hr. at 30°, on the medium described by Krebs (1948). *Bacterium cadaveris* (N.C.T.C. no. 6788), *Cl. welchii*, (N.C.T.C. no. 6785), and *Streptococcus faecalis* (N.C.T.C. no. 6783), were grown as described by Gale (1947), but 'Pronutrin' (a commercial casein hydrolysate marketed by Herts Pharmaceuticals Ltd., Welwyn Garden City) was used instead of tryptic casein.

Clostridium septicum (N.C.T.C. no. 547) served as a source of ornithine decarboxylase. The activity of the ornithine decarboxylase was increased 25-fold by reinforcing Gale's (1947) medium with extra ornithine (1 mg./ml. medium).

After growth, the cultures were centrifuged, the cells washed twice with 0.9% NaCl, and suspended in the appropriate buffer (Table 6). Phosphate buffer was used for the pH range from 7.0 to 5.5, acetate buffer for the range 5.5 to 4.0 and lactate buffer for the pH range from 4.0 to 2.5. The final concentration of the buffer was 0.10 M.

Chemicals. The preparations of L-glutamic acid, L-glutamine and cetavlon used in this work were those described by Krebs (1948). Ornithine was prepared from male herring roe, by the following method. The roe (250 g.) was heated on the water bath in a mixture of 150 ml. of conc. HCl and 50 ml. of TiCl₃ (20%). During the heating the roe was broken up with a glass rod and when fairly uniform it was autoclaved at 120° for 45 min. Water and HCl were removed by distillation *in vacuo* on a steam bath. The residual gum was dissolved in 100 ml. of hot water and 40% NaOH added until the pH was 6. The solution was filtered and, after adjustment of the pH to 7.6, an arginase preparation (15 ml. of 10% homogenized fresh rat liver) was added, and the mixture was incubated overnight. Urea was estimated manometrically and the ornithine content calculated from the urea formed.

Chemical determinations. The decarboxylation of glutamate and of glutamine was followed by measuring the evolution of CO₂ in a Warburg manometric apparatus. Buffer and substrate solutions were usually placed in the main compartment of the cup and the cells or extracts (see p. 326) containing the bacterial decarboxylase in the side arm. The gas space