stance of 5:10-dihydromepacrine) may of course have a substantially different spectrum. It is possible that such a compound would absorb at higher wavelengths than both mepacrine and its degradation products do. The possible formation of a diphenylamine derivative retaining the mepacrine side chain was suggested in the first paper.

The results of the solubility experiments must also be considered in conjunction with the low stability of these compounds. A solution containing about 50 mg./l., although much too dilute for preparative work, would suffice for spectroscopy and, given stability, the correct spectrum in the ultraviolet region could be obtained. The result with acridane, however, suggests that the slowly dissolving compound is simultaneously oxidized into the stable and soluble acridine, with some unchanged acridane present at the time of the solubility determination, possibly as the hydrochloride of the molecular compound between acridine and acridane suggested by Blout & Corley (1947). The unexplained result obtained with the chloromethoxy compound is presumably connected with the additional change observed in the spectrum of its acid solution.

SUMMARY

1. The stability, acid-solubility and ultraviolet absorption spectra of acridane and of 2-chloro-7-methoxyacridane have been measured under selected conditions.

2. The instability of these compounds, especially in acid media, makes measurements of solubility and absorption spectra of doubtful accuracy, and sometimes impossible.

3. Previous findings on the nature of mepacrine degradation products have been compared with those of the present work. The conclusions formerly reached still hold, but acridanes unsubstituted in the 5:10-positions can now be ruled out as excretion products from subjects on mepacrine therapy.

I should like to thank Prof. E. J. King for suggesting this investigation and for his continued interest in the work; Dr J. Kenyon, F.R.S. (Battersea Polytechnic, London, S.W. 11), for the hospitality of his department during one stage of this work; the Directors of Messrs May and Baker Ltd., for laboratory facilities given at another stage; Mr S. Bance, for the semimicro-analyses; and Mr W. R. Weedon for determining the absorption spectra.

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The Effect of Oxygen and of Cocarboxylase on the Formation of Citrate and α-Ketoglutarate by Pigeon Brain Homogenates

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The fact that vitamin B_1 is necessary for the relief of deficiency signs in the pigeon and for the disappearance of added or accumulated pyruvate *in vitro* and *in vivo* has always been an indirect argument for the decarboxylation of pyruvate to some C_2 fragment before its further oxidation, since aneurin pyrophosphate is concerned essentially with decarboxylation; thus in the animal an oxidative decarboxylation has appeared to be obligatory. In spite of this cogent evidence there has been no proof that, in fact, such a C_2 fragment is formed, which is clearly an unsatisfactory position. In a recent communication (Coxon, Liébecq & Peters, 1949) it was shown that citrate and α-ketoglutarate are produced during the oxidation of pyruvate by dialysed homogenates of pigeon brain, and that the yields of these compounds are increased greatly when fumarate is added. Though all difficulties in the application of the tricarboxylic acid cycle to brain tissue were not resolved, these findings pointed to the condensation of a derivative of pyruvate, or of pyruvate itself, with a four-carbon dicarboxylic acid along the lines

* With the technical assistance of Mr R. W. Wakelin.

postulated in that scheme, and, in agreement with this, oxaloacetate has since been shown to have an effect similar to that of fumarate. The formation of citrate and α-ketoglutarate through such a condensation should involve at some stage an oxidative decarboxylation and therefore oxygen should increase the yields of these substances. Evidence for the formation of a C_2 intermediate has been obtained by the demonstration that cocarboxylase as well as oxygen is necessary for a maximum production of these substances by brain tissue obtained from the vitamin B_1-deficient pigeon.

A preliminary account of this work was given to the Biochemical Society on 7 May 1949 (Coxon & Peters, 1949).

EXPERIMENTAL

Reagents. Oxaloacetic acid was kept as a solid which was dissolved in water and neutralized with NaOH immediately before use. Both Na pyruvate and oxaloacetic acid were prepared in this laboratory by Mr R. W. Wakelin. Adenosinetriphosphate (ATP) was obtained from Boots Pure Drug Co. as the Ba salt and was converted to the K or Na salt before use. Its purity was approximately 80%. Cocarboxylase was a commercial sample kindly supplied by Hoffman La Roche, to whom our best thanks are due. The buffer solution in all experiments was a mixture of KH_2PO_4 and Na_2HPO_4, the final concentration being 0·1 M and the pH 7·3.

Animals. The normal pigeons were maintained on a diet of equal parts of wheat and maize. The deficient birds were fed on polished rice until opisthotonus developed. As has been customary in this laboratory, on the first appearance of signs they received 200 mg. of glucose in 8 ml. of water. If the opisthotonus persisted after this treatment the bird was used immediately; otherwise it was allowed to survive until a relapse occurred. By this procedure more constant results are achieved when testing the effect of cocarboxylase *in vitro*.

Analytical methods. Citrate was determined by the method of Pucher, Sherman & Vickery (1936) as modified in this laboratory (Buffa & Peters, 1949) with the safeguards for specificity recommended by Breusch & Tulus (1947).

α-Ketoglutarate was determined by the method of Friedemann & Haugen (1943). In addition to the straightforward determination carried out according to their description, a second determination involving a modification of their procedure (Coxon, 1949, unpublished) was also performed. This modification minimized interference by the oxaloacetate and pyruvate present and consisted in the successive extraction of the mixture of 2:4-dinitrophenylhydrazones first with toluene and then with ethyl acetate. The second extract made in this way contained the greater part of the α-ketoglutarate compound with relatively little of the others and its composition provided a useful check on the figures obtained by the single-solvent technique. All readings were made with the Beckman spectrophotometer at 420 and 540 mμ.

Preparation of homogenates. The homogenates used throughout were 'dispersions' made by grinding the tissue in an ice-cold mortar with 0·9% KCl according to the technique of Banga, Ochoa & Peters (1939*b*) and were in all cases dialysed against 0·45% KCl at 2° for about 4 hr. Similar results could be obtained by centrifuging in the cold instead of dialysing.

Incubation of tissue and substrates. Solutions containing the requisite amounts of pyruvate, $MgCl_2$ and ATP were introduced into the main compartments of Warburg flasks while oxaloacetate (or fumarate) was placed in the side bulbs. The flasks were kept in ice until the homogenate, which was mixed with buffer after removal from the dialyser, had been added, and while CO_2-absorption papers were placed in the centre wells. Where it was desired to induce O_2 lack, the flasks were connected, while still surrounded with ice, to manometers which were then flushed with N_2 for 5–10 min. After this period they were transferred to the water bath (38°) for temperature equilibration and the passage of N_2 was continued for 5 min. more. Temperature equilibrium was assumed to have been established after a further 5 min., at the end of which time the taps were closed and the contents of the side bulbs were tipped into the main compartments of the flasks. The flasks containing air were treated similarly as regards conditions of temperature. Incubation (in the thermostat) was carried out for 30 min. in most of the experiments; trichloroacetic acid to a final concentration of 5% was then added, and, after allowing 15 min. for the protein precipitate to coagulate, this was separated and the filtrate stored in a refrigerator to await analysis.

RESULTS

Optimal concentrations of substrates

Owing to the limitations of the analytical method it was necessary to restrict the amount of pyruvate and oxaloacetate added so that their total concentrations in the final filtrate would not mask the determination of the α-ketoglutarate. These restrictions resulted in the production of submaximal quantities of citrate (and presumably of α-ketoglutarate), but the pattern of behaviour in the different conditions under test was the same whether large or small total concentrations were employed, as may be seen from Table 1. It was also found in preliminary experiments that large amounts of oxaloacetate tended to produce inhibition of oxygen uptake instead of the stimulation which was constantly observed with the smaller amounts. This point is illustrated in Table 2. A similar effect was described by Potter, Pardee & Lyle (1948) using

Table 1. *Effect of differing amounts of pyruvate and oxaloacetate*

(Each bottle contained in 3·0 ml. of 0·1 M-phosphate buffer, pH 7·3, Mg^{++}, 4·0 μmol., ATP, 1·0 μmol., together with pyruvate and oxaloacetate as stated and approx. 200 mg. of tissue. Incubation 30 min. at 38°.)

Substrate added (μmol.)		Citrate found (μmol.)	
Pyruvate	Oxaloacetate	In N_2	In air
10	10	0·67	1·31
30	20	1·75	3·04

Table 2. *Effects on oxygen uptake of differing concentrations of oxaloacetate*

(Each bottle contained in 3·0 ml. of 0·1 M-phosphate buffer, pH 7·3, Mg^{++}, 4·0 μmol.; ATP, 1 μmol.; together with pyruvate and oxaloacetate as stated and approx. 200 mg. of tissue. Temp., 38°.)

	O_2 uptake (μl. in 30 min.)			
Exp.	Pyruvate alone (10 μmol.)	Pyruvate (10 μmol.) + oxaloacetate (10 μmol.)	Pyruvate alone (30 μmol.)	Pyruvate (30 μmol.) + oxaloacetate (30 μmol.)
1	87	112	—	—
2	—	—	135	105
3	—	—	191	178

kidney homogenates. As a result of these findings we used 10 μmol./bottle of both pyruvate and oxaloacetate in most of our experiments.

Effect of oxygen on normal brain

The starting point of the studies on avitaminous brains was the observation that the production of citrate and α-ketoglutarate was very markedly reduced in the absence of oxygen. Some data illustrating this phenomenon are given in Table 3.

Table 3. *Effect of oxygen on production of citrate and α-ketoglutarate by brain homogenate from normal pigeons*

(Each bottle contained in 3·0 ml. of 0·1 M-phosphate buffer, pH 7·3, Mg^{++}, 4·0 μmol.; ATP, 1 μmol.; together with pyruvate and oxaloacetate as stated and approx. 200 mg. of tissue. Incubation 30 min. at 38°.)

	Citrate found (μmol./bottle)		α-Ketoglutarate found (μmol./bottle)	
	In N_2	In air	In N_2	In air
After equilibration	0·5	1·3	Nil	About 0·2
30 min. later	0·6	1·9	About 0·2	1·4

Effects of oxygen and of cocarboxylase on avitaminous brain

The effects of oxygen and of cocarboxylase are set out together in Tables 4 and 5, since in a number of instances they were tested simultaneously on the same brain. Tables 4 and 5 show that these two factors both increased the production of citrate and α-ketoglutarate; where the experiments are similarly numbered in the two tables this means that the same filtrates were analysed for the two products.

Table 4. *Effects of oxygen and of cocarboxylase on the production of citrate by dialysed homogenates of pigeon brain*

(Each bottle contained pyruvate, 10 μmol.; $MgCl_2$, 4 μmol.; ATP, 1·0 μmol.; homogenate equivalent to 200 mg. of fresh brain tissue, together with 10 μmol. of oxaloacetate in the side bulb. Bath at 38°.)

	Citrate formed in 40 min. (μmol./bottle)			
Exp. no.	In N_2	In N_2 with cocarboxylase (10 μg.)	In air	In air with cocarboxylase (10 μg.)
1	0·38	0·68	0·40	0·90
2	0·48	0·84	0·41	1·10
3	0·50	0·83	1·20	1·50
4	—	—	0·56	1·00

Table 5. *Effect of oxygen and of cocarboxylase on the production of α-ketoglutarate by dialysed homogenates of pigeon brain*

(Each bottle contained pyruvate, 10 μmol.; $MgCl_2$, 4 μmol.; ATP, 1·0 μmol. and homogenate equivalent to 200 mg. of fresh brain tissue, together with 10 μmol. of oxaloacetate in the side bulb. Incubation 40 min. at 38°.)

	α-Ketoglutarate formed (μmol./bottle)			
Exp. no.	In N_2	In N_2 with cocarboxylase (10 μg.)	In air	In air with cocarboxylase (10 μg.)
1	0·55	0·73	—	—
2	0·0	0·13	0·80	2·20
3	0·0	0·55	1·37	1·78
4	—	—	0·20	0·94

Table 6. *Effect of cocarboxylase on the production of α-ketoglutarate and of citrate by dialysed homogenates of pigeon brain in presence of fumarate*

(Each bottle contained pyruvate, 10 μmol.; $MgCl_2$, 4 μmol.; ATP, 1 μmol. and homogenate equivalent to 200 mg. of fresh brain, together with 10 μmol. of Na fumatate. Incubation 30 min. at 38°.)

Cocarboxylase added (μg./bottle)	Citrate found (μmol./bottle)	α-Ketoglutarate found (μmol./bottle)
0	0·6	0·0
10	1·3	1·5

Effect of cocarboxylase with fumarate replacing oxaloacetate

Since fumarate is presumed, on the basis of previous work, to increase the yield of citrate and α-ketoglutarate by supplying oxaloacetate, and since this action would involve an oxidation, there seemed no reason to try anaerobic experiments using fumarate; however, the favourable effect of

cocarboxylase on the aerobic production of citrate and α-ketoglutarate in one instance is shown in Table 6.

DISCUSSION

Much previous work from this laboratory (Banga, Ochoa & Peters, 1939*a*) has shown that cocarboxylase acts in the animal as coenzyme for the decarboxylating step in the degradation of pyruvate. (Another suggestion by Krebs & Eggleston (1940) that it may catalyse the carboxylation of pyruvate to form oxaloacetate does not appear to have been substantiated.) We therefore consider that the evidence put forward in the present paper, while confirming earlier suggestions (Coxon *et al.* 1949) regarding the connexion between the oxidation of pyruvate and the formation of citrate and α-ketoglutarate in brain homogenates, also demonstrates directly that an oxidative decarboxylation of pyruvate with the production of some two-carbon fragment must precede its condensation with oxaloacetate and subsequent participation in a tricarboxylic acid cycle. This follows from the finding that the addition of cocarboxylase and oxygen to suitable preparations increased the yields of citrate and α-ketoglutarate. For pigeon brain, it appears to dispose of the suggestion (Wood, Werkman, Hemingway & Nier, 1942; Breusch, 1943) that the first product of condensation is a seven-carbon compound, for if such a compound, of the type of pyruvofumarate, were formed (that is, if the oxidative decarboxylation which we have demonstrated occurred after instead of before the condensation) then it would be expected that the hypothetical seven-carbon compound would accumulate in the blood of animals suffering from aneurin deficiency whereas in fact, within the limits of spectrophotometric analysis, it is pyruvate which appears to accumulate in this condition.

The small amounts of citrate and α-ketoglutarate which are formed anaerobically call for some comment. Breusch (1939) reported the anaerobic formation of citrate in small amounts by minced brain (from pigeons and cats) incubated with pyruvate and oxaloacetate. Krebs, Eggleston, Kleinzeller & Smyth (1940) also recorded that considerable amounts of α-ketoglutarate and smaller quantities of citrate could be formed anaerobically from oxaloacetate by minced sheep brain. We have not made determinations of malate in our experiments, but we suggest that such citrate as is formed by our homogenates under anaerobic conditions arises by a process involving an oxidative decarboxylation coupled with a reduction, probably of oxaloacetate, to malate; this would explain the effect of cocarboxylase in augmenting the yield to some extent even in the absence of molecular oxygen.

Since the preliminary report of our findings was given to the Biochemical Society, Stern & Ochoa (1949) have adduced evidence that in liver, at all events, citrate may be formed from acetate plus oxaloacetate without the intervention of *cis*-aconitate or *iso*citrate, while Potter & Heidelberger (1949) have published results implying that much of the reasoning which had been criticized earlier by Ogston (1948), but which had formed the basis for the relegation of citrate to the role of a side product of the tricarboxylic acid cycle, was actually invalid. So far as pigeon-brain homogenates are concerned, the assumption that the main pathway is through *cis*-aconitate with citrate arising by a side reaction affords a readier explanation of the fact that their oxygen uptake is stimulated by the former, but not by the latter (Coxon *et al.* 1949). Nevertheless, in the absence of reliable quantitative data on the readiness with which *cis*-aconitate, *iso*citrate and citrate are interconverted by our system and on the kinetics of the process it would be unjustifiable to propose a rigid concept of the sequence of events at this point in the reaction scheme. Moreover, the synthesis of citrate by Stern & Ochoa's (1949) liver preparation was accomplished anaerobically so that the relationship of their results to the behaviour of our brain dispersion is far from clear. The 'citrogenase' reaction described by Breusch (1943), in which a β-keto acid condenses with oxaloacetate to form citrate, is stated by him to be independent of oxygen tension; to bring pyruvate into that reaction he postulates its conversion to acetate, two molecules of which combine to give one of acetoacetate, a process which would involve an oxidative decarboxylation. However, Breusch (1948) states that 'citrogenase' is not present in great quantity in brain and in the absence of positive evidence in favour of it we see no reason to prefer this scheme to the more usually accepted one.

SUMMARY

1. Both oxygen and aneurin pyrophosphate (cocarboxylase) increase the formation of citrate and α-ketoglutarate from pyruvate and oxaloacetate by homogenates of pigeon brain.

2. It is concluded that in this tissue an oxidative decarboxylation of pyruvate to some C_2 fragment precedes the condensation with oxaloacetate.

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The Reactivity of the Iminazole Ring in Proteins

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The sulphydryl, disulphide (cf. Anson, 1945), phenolic (Crammer & Neuberger, 1943) and amino groups (Porter, 1948) of proteins show varying degrees of reactivity in the native molecule. On denaturation of the protein by any means, these groups, with very few exceptions, become capable of reacting completely with the several reagents examined. This has been interpreted as suggesting the existence, in the native protein, of labile bonds or steric factors which are altered on denaturation. It has now been found, using the reagent 1:2:4-fluorodinitrobenzene (FDNB), that the iminazole rings of the histidine residues of β-lactoglobulin are less reactive in the native than in the denatured protein. Other proteins examined did not show this difference in reactivity. In insulin the iminazole group was reactive in the native molecule, but on heat precipitation, or fibrous insulin formation, almost half the residues became unreactive. Fibrous insulin, superficially resembling denatured protein, is believed to be a long-chain polymer of the insulin molecule (Waugh, 1948), and it would appear that in this case intermolecular association, rather than intramolecular rearrangement is responsible for the change in reactivity of the histidine residues.

MATERIALS

Haemoglobins. These were prepared as described by Porter & Sanger (1948).

Bovine serum albumin. A sample prepared by cold ethanol fractionation was obtained from Armour Ltd., Chicago, U.S.A.

β-Lactoglobulin. This was a crystalline sample given by Prof. Linderstrøm-Lang.

Ovalbumin. This was a five-times recrystallized sample obtained by Prof. A. C. Chibnall from Prof. R. K. Cannan.

Insulin. Crystalline ox insulin from Boots Pure Drug Co. Ltd. was used. Heat-precipitated insulin was prepared by keeping a 3% solution of insulin in 0·1 N-H_2SO_4 at 95° for 30 min. (du Vigneaud, Sifferd & Sealock, 1933). The precipitate was centrifuged and washed with distilled water. Fibrous insulin was prepared as an opalescent gel according to Waugh (1948).

METHODS

The conditions of denaturation and of condensation of FDNB with proteins have been described by Porter (1948).

Estimation of unreactive histidine residues in proteins

It was suggested by Sanger (1945) that FDNB did not form a stable compound with the iminazole ring of histidine, but it has since been found that a hydrolysate of dinitrophenyl-(DNP-) globin contains no free histidine. Dr Sanger has re-examined the reaction of FDNB with *N'*-acetylhistidine and found that a stable crystalline compound, m.p. 221° (decomp.), is in fact formed. After removal of the acetyl group the colourless crystalline DNP derivative, m.p. 280° (decomp.), has a nitrogen content corresponding to mono-DNP-histidine and its probable structure is 1-(2′:4′-dinitrophenyl)-4(5-)iminazole-α-aminopropionic acid (im-DNP-histidine). On acid hydrolysis it did not give rise to histidine (Sanger, unpublished).

The condensation of FDNB with protein is carried out in aqueous ethanol at room temperature; the reaction is so rapid that it precedes and prevents ethanol denaturation of

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