CXXVI. *ISO*CITRIC DEHYDROGENASE AND GLUTAMIC ACID SYNTHESIS IN ANIMAL TISSUES

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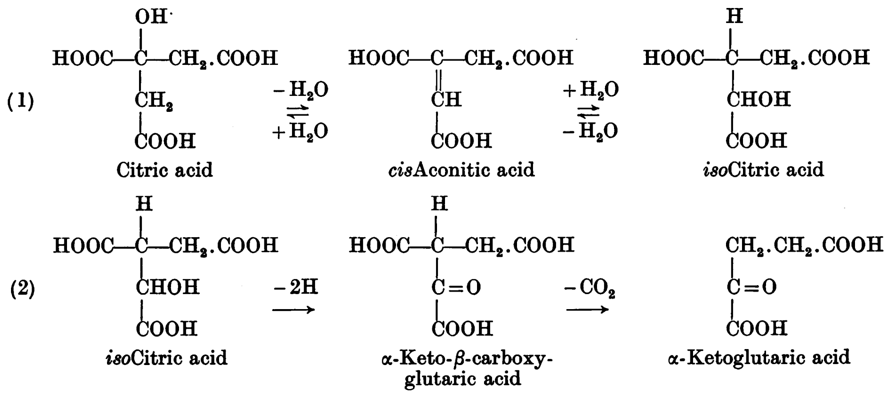
Martius & Knoop [1937; Martius, 1937; 1939] have recently shown that the biological breakdown of citric acid proceeds according to the following scheme:



Citric acid first undergoes transformation into *iso*citric acid by the action of the enzyme "aconitase". *iso*Citric acid is then dehydrogenated to the corresponding keto-acid, which is unstable and splits off CO_2 spontaneously to form α-ketoglutaric acid. Thus the old term "citric dehydrogenase" designates a mixture of enzymes and the actual dehydrogenation is brought about by an "*iso*citric dehydrogenase".

In connexion with our work on glutamic dehydrogenase and the enzymic synthesis of glutamic acid, i.e. reductive amination of α-ketoglutaric acid [Euler *et al.* 1938], we were interested in the nature of *iso*citric dehydrogenase, because it catalyses the formation of α-ketoglutaric acid and thus forms a link between carbohydrate breakdown and protein synthesis in the cells.

The history of "citric dehydrogenase", especially the work of Thunberg, Batelli & Stern, and Bernheim, is given in the monograph of Franke [1934]. More recently Andersson [1933] found that a crude cozymase preparation accelerated the reduction of methylene blue by citric acid and a plant extract, and Wagner-Jauregg & Rauen [1935, 1] described an activation of a similar system by a crude coenzyme prepared according to Warburg & Christian [1935] from red blood cells. Wagner-Jauregg & Rauen [1935, 2] also found that *iso*citric acid was a hydrogen donator for the plant "citric dehydrogenase" system.

From the point of view of our present knowledge of dehydrogenase systems it was of fundamental interest to find out what coenzyme had been active in

Andersson's and Wagner-Jauregg's experiments. It seems likely that the coenzyme preparations used by these authors contained both codehydrogenase I (cozymase, diphosphopyridinenucleotide, Co I) and codehydrogenase II (Warburg's coenzyme, triphosphopyridinenucleotide, Co II). Using pure preparations of these coenzymes we have found that Co II is the specific coenzyme for *iso*citric dehydrogenase from animal tissues, higher plants and yeast,[1] while Co I is completely inactive. The bearing of this fact on the problems connected with the importance of the *iso*citric dehydrogenase in carbohydrate breakdown and amino-acid synthesis is discussed in a later section.

On the basis of the Co II-specificity we were able to study the distribution of the *iso*citric apodehydrogenase in biological material, to purify the enzyme and to study the dehydrogenating system. The apodehydrogenase was found to be present in all animal tissues so far examined, which makes it probable that citric acid breakdown is part of a general cell reaction. It was possible to obtain the *iso*citric dehydrogenase free from "aconitase" and thus enzyme preparations were obtained which used *iso*citric but not citric acid as H donator. It was further shown that in the isolated *iso*citric dehydrogenase system CO_2 and α-ketoglutaric acid were formed as the end products, thus confirming Martius and Knoop's scheme.

In experiments with purified apodehydrogenase the system showed a typical "dilution effect", i.e. the rate of the reaction was not proportional to the enzyme concentration, but disappeared more or less completely when the enzyme was diluted to a certain degree. The analysis of this observation led us to the surprising fact that Mn^{++} was necessary for the full action of the dehydrogenase; Mg^{++} could replace the Mn^{++}, but was less active and its optimal concentration was higher than that of Mn^{++}. As yet no other dehydrogenase could be found in which Mn or Mg salts had an effect, whilst a number of other enzymic reactions, e.g. transphosphorylation [Ohlmeyer & Ochoa, 1937] and pyruvic acid decarboxylation [Euler *et al.* 1937] by yeast enzymes, are known to be activated by Mn^{++}. Concerning the mode of action of these ions in the case of *iso*citric dehydrogenase it may be possible, that they "link up" the substrate with the apodehydrogenase by salt formation. If the Mn^{++} effect involved the reaction between the codehydrogenase and the apodehydrogenase, one would expect that other dehydrogenases would be similarly activated.

According to the results described in this paper reaction (2) of the scheme given above can be written as follows:

(2*a*) $\textit{iso}\text{Citric acid} + \text{Co II} \xrightarrow[+\text{Mn}^{++} \text{ or } \text{Mg}^{++}]{\textit{iso}\text{citric apodehydrogenase}} \alpha\text{-keto-}\beta\text{-carboxyglutaric acid} + \text{CoH}_2\ \text{II}.$

(2*b*) $\alpha\text{-Keto-}\beta\text{-carboxyglutaric acid} \longrightarrow \alpha\text{-ketoglutaric acid} + CO_2.$

Reaction (2*b*) goes spontaneously and is very fast and therefore the whole reaction (2*a*+2*b*) must go to completion in the direction given by the arrows. That means that in presence of an excess of *iso*citric acid the total amount of Co II is hydrogenated, and on the other hand, in presence of an excess of Co II, or if the CoH_2 II formed is continuously reoxidized, the total amount of *iso*citric acid is converted into ketoglutaric acid. Whether reaction (2*a*) is reversible in analogy with other hydroxy-acid $\rightleftharpoons$ keto-acid reactions cannot be said, because it was not possible to study this reaction independently of reaction (2*b*).

[1] The experiments with higher plants and yeast have been done by L. Elliot and will be published separately.

Experimental

The components of the system

(*a*) *Enzyme preparations*. Acetone-dried heart muscle was used as starting material. Pig heart was freed from fat and ground in a mincer. The pulp was stirred up 3 times with twice the volume of ice-cold acetone, pressed out through muslin each time and finally dried in air by spreading out on filter paper. From the resulting stable preparation enzymes were prepared in different ways.

Enzyme A. Acetone-dried tissue was ground in a mortar with sand and 6 times its wt. of water and filtered through muslin on a Büchner funnel. The extract was dialysed for 12 hr. and centrifuged. The enzyme attacks *iso*citric acid, but not at all or only slowly citric acid.

Enzyme B. Fractionation with ammonium sulphate: 70 g. acetone-dried heart muscle were ground twice with 420 ml. 0·1 M Na_2HPO_4 and sand and pressed out through muslin. The pH of the crude extract (700 ml.) was adjusted to 6·5 by addition of 150 ml. 0·5 M KH_2PO_4 and 1700 ml. sat. ammonium sulphate solution were added (degree of saturation is 0·66). The protein precipitate was filtered through a thin layer of kieselguhr on a Büchner funnel and redissolved by rubbing up the filter cake with 0·1 M Na_2HPO_4. The resulting solution was centrifuged and the supernatant was neutralized with 50 ml. 0·5 M KH_2PO_4; 175 ml. sat. ammonium sulphate were then added and the precipitate formed was discarded. The filtrate was precipitated once more with an equal volume of ammonium sulphate, and the proteins remaining on kieselguhr after filtration were redissolved in 70 ml. water and centrifuged. The reddish, clear solution contains aconitase and a very active *iso*citric dehydrogenase.

Enzyme C. Fractionation with acetone: 50 g. acetone-dried heart muscle were thoroughly ground with 400 ml. water and sand. The extract (250 ml.) was precipitated at 0° with 750 ml. cold acetone; the precipitate was dissolved in 130 ml. water; the centrifuged solution (125 ml.) was precipitated at 0° with 65 ml. acetone and after centrifuging off the precipitate formed, another 65 ml. acetone were added. Both precipitates were dried with cold acetone and ether. The second fraction was used in most of the experiments and is called "enzyme C". The dry powders are nearly completely soluble in water; both fractions are rich in *iso*citric dehydrogenase but free from aconitase.

(*b*) *Coenzyme*. Codehydrogenase II was prepared by enzymic phosphorylation of cozymase according to the principle previously described [Euler & Adler, 1938]. The details of this will be published separately.

(*c*) *Substrate*. *iso*Citric acid was a synthetic preparation for which we wish to express our thanks to Prof. P. Karrer, Zürich. The Na salt was used in the experiments.

Citric acid as a substrate[1]

Methylene blue as acceptor. Table I shows that codehydrogenase II and flavinenzyme are necessary for methylene blue reduction when citrate is used as substrate for "enzyme B".

Exps. 2 and 3 show that the methylene blue decoloration with 40 μg. Co II was about 25 times faster than that with 250 μg. of a cozymase preparation of highest purity. It is difficult to say whether cozymase has an action in this system which is 150 times weaker than that of codehydrogenase II or if cozymase is actually inactive and the low rate obtained in Exp. 3 is due to traces of codehydrogenase II present in the cozymase preparation. At any rate the great difference in the degree of action indicates that *iso*citric dehydrogenase is practically specific for codehydrogenase II.

[1] For a preliminary report see Adler *et al.* [1938, 3].

Table I. *The "citric acid dehydrogenase" system*

0·5 *M* Na-citrate; "enzyme B"; cozymase (Co I) of highest purity, 1 mg./ml.; codehydrogenase II (Co II), 200 μg./ml.; flavinenzyme from yeast, 2·5 μg. bound lactoflavin per ml. Each Thunberg tube contained 0·25 ml. 0·5 *M* phosphate buffer, *p*H 7·6, and in a small inner tube 0·5 ml. 0·02% methylene blue, which is mixed with the other components after evacuation. Temp. 30°.

In Exp. 1 the citrate was added to the other components immediately before the experiment was started; in all other experiments citrate + enzyme + buffer + water were incubated in the open tube for 15 min. at 30°, then the other components were added and the experiment was started.

Exp. no.	Citrate ml.	Enzyme ml.	Co I ml.	Co II ml.	Flavin-enzyme ml.	Decoloration time min.
1	0·25	0·25	—	0·20	0·25	8
2	0·25	0·25	—	0·20	0·25	2
3	0·25	0·25	0·25	—	0·25	53
4	—	0·25	—	0·25	0·25	240
5	—	0·25	0·25	—	0·25	205
6	0·25	0·25	—	0·25	—	79
7	0·25	0·25*	—	0·25	0·25	240
8	0·25	0·25	—	0·25	0·25†	78
9	0·25	0·25	—	0·25‡	0·25	240

* Boiled after incubation with citrate. † Boiled flavinenzyme.
‡ Alkali-heated Co II.

A comparison between Exps. 1 and 2 shows that the decoloration time is remarkably shorter when the citrate is incubated with the enzyme for some time before the other components are added and the dehydrogenation is started. This effect is explained by the Martius-Knoop scheme: during incubation the transformation citric acid ⇌ *iso*citric acid takes place and possibly the equilibrium is reached within the incubation time, whilst in the experiment without incubation there is not enough *iso*citric acid formed to reach the optimal concentration for the dehydrogenase reaction.

Oxygen as acceptor. Fig. 1 shows that the system citrate + enzyme B + codehydrogenase II takes up O_2 when it is completed by flavinenzyme and methylene blue.

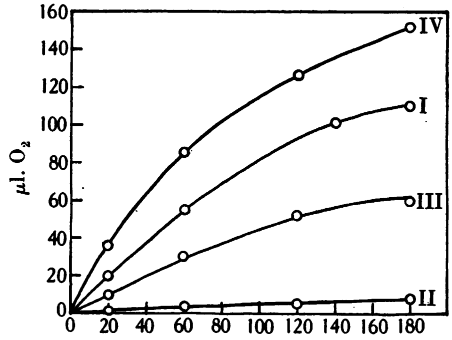


Fig. 1. Aerobic breakdown of citric acid. Curve I: 0·25 ml. *M*/2 citrate, 1 ml. "enzyme B", 0·5 ml. flavinenzyme, 0·25 ml. veronal buffer *p*H 7·66, 0·5 ml. methylene blue 1:5000; after 15 min. incubation at 30°, 0·25 ml. (=25 μg.) Co II were added from the side bulb of the manometer flask. Curve II: with heated flavinenzyme. Curve III: without methylene blue. Curve IV: with double amount of flavinenzyme. No O_2 uptake was obtained in controls without substrate, without Co II, with heated enzyme and with Co I instead of Co II. The centre cup of the vessels contained 0·2 ml. 10% KOH.

In the absence of methylene blue the O_2 uptake is slower, because then the rate is limited by the autoxidizable fraction of our flavinenzyme preparation, i.e. the flavinphosphate protein fraction, whilst the flavin-adenine-dinucleotide protein fraction [Warburg & Christian, 1938; Haas, 1938) will react rapidly only if methylene blue is added. In absence of flavinenzyme the reaction is extremely slow; this means that "enzyme B" does not contain appreciable amounts of "diaphorase II" [Adler *et al.* 1939], a flavoprotein of animal tissues, which transports hydrogen from CoH_2 II to acceptors like methylene blue. This conclusion was confirmed by direct spectrophotometric determination in the system CoH_2 II + "enzyme B" + O_2; with the same technique it was shown that "enzyme B" was relatively rich in "diaphorase I", the CoH_2 I-specific hydrogen transporting enzyme.

Separation of aconitase and iso*citric dehydrogenase*

When "enzyme B" was dialysed in a cellophane tube against running water for 20 hr., the activity towards citric acid was practically abolished; the activity towards *iso*citric acid was decreased too, but could be restored to the level of the non-dialysed enzyme by addition of Mn^{++} (cf. p. 1037), whilst citrate was not attacked even in presence of Mn^{++} or of boiled undialysed enzyme; but when citrate was incubated with non-dialysed "enzyme B" for 15 min., the mixture deproteinized by heating and used as a substrate in a Thunberg experiment containing the dialysed "enzyme B", the MB was rapidly decolorized. Therefore, the aconitase must have been inactivated by dialysis.

Another way of separating aconitase is to precipitate the crude enzyme with acetone. Thus "enzyme C" which is a stable acetone powder, is completely inactive with citrate though highly active with *iso*citrate, especially after addition of Mn^{++}. Sensitivity to acetone treatment is also characteristic of fumarase [Clutterbuck, 1928], but according to Martius [1939] aconitase and fumarase are different enzymes.

The iso*citric dehydrogenase system*

(*a*) *Methylene blue as acceptor.*

The dehydrogenation of *iso*citric acid by one of the enzymes A, B, or C with methylene blue as H acceptor needs the addition of the same components as if citric acid is used as a substrate, namely codehydrogenase II and flavinenzyme. Codehydrogenase II cannot be replaced by cozymase.

Fig. 2 shows the dependence of the rate of methylene blue reduction on the codehydrogenase II concentration and in Fig. 3 the influence of increasing amounts of yeast flavinenzyme is shown. The relation between the rate of *iso*citric acid dehydrogenation and the amount of apodehydrogenase is described in a later section in connexion with the experiments on Mn^{++} activation. Here it may be mentioned that proportionality between rate and apodehydrogenase concentration exists only if the system contains an optimal amount of Mn^{++}.

Substrate affinity. Experiments on the influence of *iso*citric acid concentration showed that the rate was still optimal when the concentration of the substrate was as low as $5 \times 10^{-5}\,M$, assuming that the *iso*citric acid preparation used contained 50% of the natural form. Thus, in a Thunberg experiment with 2·0 ml. total volume, 19 μg. *iso*citric acid (equivalent to 37 μg. methylene blue) were sufficient to decolorize the methylene blue (20 μg.) at the optimal rate. Since a further decrease in the amount of methylene blue would have introduced considerable error in the measurement of the decoloration time, experiments with still lower amounts of *iso*citric acid could not be done. In spectrophotometric experiments (cf. p. 1036), the *iso*citric acid concentration could be lowered to $1{\cdot}25 \times 10^{-5}$ *M*, without a distinct decrease in the rate of dehydrogenation. Thus, the Michaelis constant K_m must have a value $< 1{\cdot}25 \times 10^{-5}$ *M*, which means that the affinity of *iso*citric acid for the apodehydrogenase is extremely high.

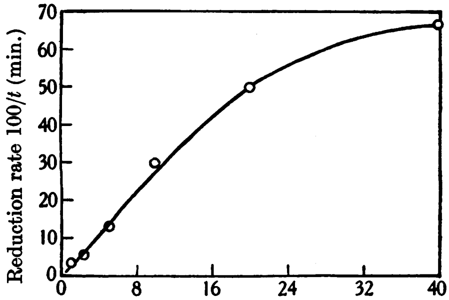


Fig. 2.

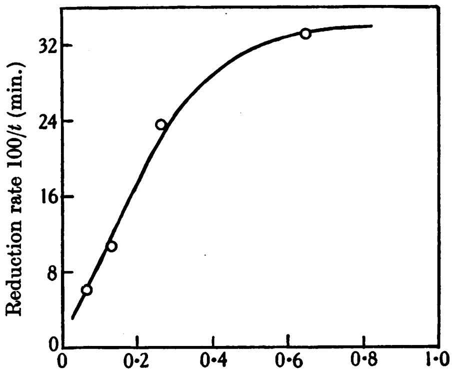


Fig. 3.

Fig. 2. *iso*Citric acid dehydrogenation; effect of coenzyme concentration. Thunberg technique. 0·1 ml. "enzyme B" was used in each experiment.

Fig. 3. *iso*Citric acid dehydrogenation; effect of flavinenzyme concentration. Thunberg technique. 0·1 ml. "enzyme B" was used in each exp.

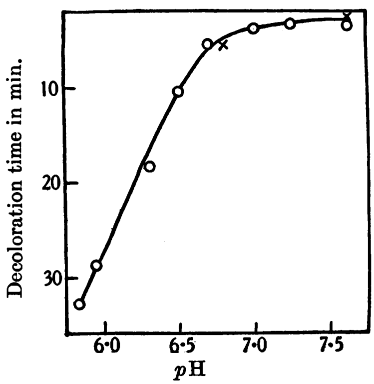


Fig. 4. *iso*Citric acid dehydrogenation; effect of *p*H. Thunberg technique. 0·1 ml. "enzyme C", 20 µg. Co II, 0·25 ml. flavinenzyme and 2 mg. *iso*citric acid were used in each exp. ○ = veronal buffer; × = glycine buffer.

Influence of p*H*. Fig. 4 shows the influence of the *p*H of the solution on the rate of *iso*citric acid dehydrogenation, determined by the Thunberg technique. It is important to say that phosphate buffer could not be used in these experiments as it was found that phosphate ions inhibit the dehydrogenase reaction (cf. p. 1040) and that the % inhibition varies considerably with the *p*H. Therefore, the *p*H curve with phosphate buffer showed a maximum between *p*H 6 and 6·5, where the phosphate inhibition is relatively small, and a minimum between *p*H 7 and 8, where phosphate exerts a strong inhibiting action. By the use of veronal and glycine buffer this difficulty was overcome and a normal *p*H curve, analogous to other animal dehydrogenases was found. The rate is high at *p*H 7–7·5 and falls off rapidly below *p*H 6·5; the values at alkaline reaction, where the curve also falls off, are not shown in the figure, because in this region the rate may be decreased by destruction of the codehydrogenase.

(*b*) *Oxygen as acceptor.*

The components of the system. A mixture of *iso*citric acid, apodehydrogenase and codehydrogenase II takes up O_2 if the transfer of H from the reduced coenzyme to the O_2 is made possible by the addition of flavinenzyme. A further addition of methylene blue increases the rate of O_2 uptake very much, because in presence of this dye even the "new", i.e. not readily autoxidizable, flavoprotein, present in the flavinenzyme preparation, is utilized (Table II).

Table II. *Aerobic dehydrogenation of* iso*citric acid*

The total system contained 0·25 ml. apodehydrogenase solution (corresponding to 4 mg. acetone powder "enzyme C"); 0·20 ml. (=20 μg.) codehydrogenase II; 0·25 ml. flavinenzyme from yeast (corresponding to 0·8 μg. bound lactoflavin); 0·50 ml. methylene blue 1:1000; 0·25 ml. veronal-acetate buffer (Michaelis), *p*H 7·66, and 0·25 ml. Na *iso*citrate (corresponding to 5 mg. *iso*citric acid); the total volume was 2·25 ml. The centre cup of the Warburg vessels contained 0·3 ml. 7 % KOH, absorbed on filter paper. The substrate was added from the side bulb.

	μl. O_2 in 20 min.
Total system	49
No substrate	0
With heated enzyme	0
No coenzyme	0
No flavinenzyme	3
No methylene blue	8
With Co I instead of Co II	0

The oxygen equivalence. The amount of O_2 taken up by a certain amount of our *iso*citric acid preparation agrees rather well with the theory: one pair of H atoms is taken away from the substrate, dihydrocoenzyme is formed and the 2H transported to the O_2, giving H_2O_2; the latter is split by catalase, the presence of which in the apodehydrogenase preparation was shown in separate experiments, and 1/2 mol. O_2 is liberated again. Thus an actual uptake of 1/2 mol. O_2 per mol. *iso*citric acid would be expected.

We found in several experiments with "enzyme C" an O_2 uptake a little higher than calculated, e.g. for 1·9 mg. *iso*citric acid an O_2 uptake of 65 μl. (calculated 56 μl.) and for 3·8 mg. *iso*citric acid an oxygen uptake of 125 μl. (calculated 112 μl.) was found. The small discrepancy between the theoretical and the experimental values can have several reasons; it can be at least partially explained by the fact that our *iso*citric acid preparation contained an unknown amount of the corresponding lactone which has a lower mol. wt. and so will give higher values for the O_2 uptake than the same amount of free acid. With crude enzyme preparations, e.g. "enzyme A", the O_2 uptake was usually lower than calculated, which possibly indicates that part of the reduced coenzyme is used up in an anaerobic reaction; one could assume that *cis*aconitic acid which, in presence of crude enzymes, is in equilibrium with *iso*citric acid, might act as H acceptor, forming tricarballylic acid. However, no direct proof of the existence of such a reaction has been found as yet.

CO_2 as a reaction product. If in the aerobic experiment no KOH is used in the centre cup, the negative pressure is replaced by a positive one, showing that CO_2

is formed during the reaction. The respiratory quotient was determined according to Warburg & Yabusoe [1924]. In experiments in which the reaction mixture was the same as that given in Table I, but with 4 mg. substrate, after 100 min., when the reaction was finished, 140 μl. O_2 had been taken up and 278 μl. CO_2 had been formed. Thus, the quotient mol. CO_2/mol. $O_2=2$, i.e. for each mol. *iso*citric acid 1/2 mol. O_2 is taken up and 1 mol. CO_2 is formed. This result is in complete agreement with Martius and Knoop's scheme.

α-Ketoglutaric acid as a reaction product. Definite proof of the mechanism of the isolated dehydrogenase reaction as given by scheme (2) consists in the isolation of α-ketoglutaric acid.

The collected reaction mixtures of five aerobic experiments, corresponding to those given in Table II, were deproteinized with 2% trichloroacetic acid and centrifuged. The supernatant solution was freed from methylene blue by slow filtration through a layer of kieselguhr and concentrated *in vacuo* to 5 ml. After addition of 3 ml. sat. 2:4-dinitrophenylhydrazine in 2*N* HCl, crystals of a 2:4-dinitrophenylhydrazone settled out. They were collected after standing overnight and washed with 2*N* HCl. For purification the hydrazone was taken up in diluted Na_2CO_3, in which it was completely soluble, and reprecipitated with HCl. The substance was identified as the 2:4-dinitrophenylhydrazone of α-ketoglutaric acid by M.P. (218°) and mixed M.P. (218°); the 2:4-dinitrophenylhydrazone of the pure acid melted at 219°. The yield was 12 mg. of the crude substance.

Summarizing the preceding qualitative and quantitative results, it has been shown that the aerobic dehydrogenation of *iso*citric acid by the isolated system proceeds in the following way:

(2*a*) *iso*Citric acid + Co II $\xrightarrow[\text{dehydrogenase}]{\text{apo-}}$ α-keto-β-carboxyglutaric acid + COH_2 II.

(2*b*) α-Keto-β-carboxyglutaric acid → α-ketoglutaric acid + CO_2.

(3) CoH_2 II + O_2 $\xrightarrow{\text{flavinenzyme + MB}}$ Co II + H_2O_2.

(4) $H_2O_2 \xrightarrow{\text{catalase}} H_2O + \frac{1}{2}O_2$.

The sum of these reactions will be

(5) *iso*Citric acid + 1/2 O_2 → α-ketoglutaric acid + CO_2 + H_2O.

The catalytic components of the system which brings about the primary anaerobic step of the dehydrogenation, i.e. *iso*citric apodehydrogenase and codehydrogenase II, are present probably in all animal cells. But for the transport of H from CoH_2 II to O_2 we used flavinenzyme from yeast, because our apodehydrogenase preparation did not contain a carrier enzyme capable of reacting with CoH_2 II. However, as Adler *et al.* [1939] have shown, animal tissues do in fact contain an enzyme, "diaphorase II", which transfers H from CoH_2 II to acceptors like methylene blue and probably also to cytochrome; hence the possibility of aerobic dehydrogenation of *iso*citric acid in animal tissues is evident. Actually, citric acid has been shown to increase the O_2 uptake of various animal tissues [Batelli & Stern, 1911; Krebs & Eggleston, 1938].

Besides the aerobic way there is another possibility for continuous dehydrogenation of *iso*citric acid in the cells, namely the anaerobic reoxidation of the CoH_2 II by iminoglutaric acid, i.e. by α-ketoglutaric acid + NH_3, catalysed by glutamic apodehydrogenase. This reaction will be discussed in a later section.

(*c*) *Spectrophotometric experiments.*

The primary reaction (2*a*) between *iso*citric acid and codehydrogenase II in presence of the apodehydrogenase can be easily studied by spectrophotometric determination of the characteristic absorption band with maximum at 340 mμ of the dihydro-codehydrogenase II.

In Fig. 5, Curve I shows the hydrogenation of a certain amount of codehydrogenase II by an excess of *iso*citric acid, catalysed by "enzyme C". In the parallel experiment (Curve II), the same amount of Co II was used, but the hydrogenation was brought about by hexosemonophosphate in presence of hexosemonophosphate apodehydrogenase from yeast. This reaction is known to be irreversible, i.e. if an excess of substrate is used, the total amount of coenzyme is hydrogenated. The end extinctions in Exps. I and II are equal; thus the hydrogenation of the Co II was complete in the *iso*citric system.

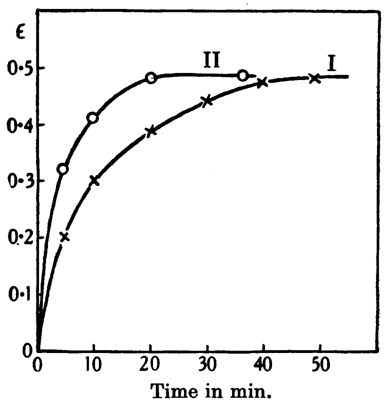


Fig. 5.

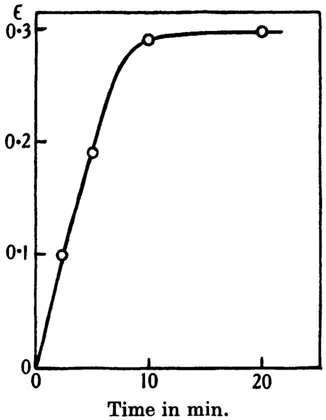


Fig. 6.

Fig. 5. Hydrogenation of codehydrogenase II by excess of *iso*citric acid (Curve I) and hexosemonophosphate (Curve II). Curve I: 0·19 mg. ($=10^{-3}$ m*M*) *iso*citric acid, 0·1 ml. "enzyme C", 0·5 ml. ($=0{\cdot}17\times10^{-3}$ m*M*) Co II, 0·5 ml. veronal buffer. Curve II: 0·2 ml. *M*/10 hexosemonophosphate, 0·1 ml. hexosemonophosphate dehydrogenase, 0·5 ml. Co II, 0·3 ml. *M*/2 phosphate buffer. Total volume 4 ml. The extinction (ϵ) at $\lambda=334$ mμ, indicating the formation of CoH_2 II, was measured photoelectrically.

Fig. 6. Hydrogenation of Co II by less than the equivalent amount of *iso*citric acid. $0{\cdot}2\times10^{-3}$ m*M* *dl-iso*citric acid and $0{\cdot}13\times10^{-3}$ m*M*. Co II were used. CoH_2 II found: $0{\cdot}11\times10^{-3}$ m*M* (calc. $0{\cdot}10\times10^{-3}$).

When an excess of codehydrogenase was used, the dihydro-compound formed was equivalent to the *iso*citric acid (Fig. 6).

These experiments show that the reaction between *iso*citric acid and Co II goes to completion. This can be explained by the following alternative assumptions: (1) the primary reaction (2*a*) is irreversible; (2) reaction (2*a*) is reversible and gives an equilibrium, which is rapidly disturbed by the decomposition of the keto-acid formed (2*a*+2*b*). The observation that in aerobic experiments CO_2 output begins immediately shows that the α-keto-β-carboxyglutaric acid actually is rapidly decarboxylated. Thus, the conditions for a reaction according to the second assumption seem to be given. Then, concerning the rate of the spontaneous decarboxylation, it could be said that it must be rather high, because in spectrophotometric as well as in methylene blue experiments the rate of dehydrogenation seemed to be dependent solely on the enzyme concentration, even when this was relatively high. If the decarboxylation were a slow reaction, and if assumption (2) were correct, it should have limited the rate of the dehydrogenation. A more detailed study of the kinetics of this reaction would be of interest.

Fig. 7 represents a spectrophotometric proof of the Co II-specificity of *iso*citric apodehydrogenase. Pure cozymase (Co I) gave no reduction band, but after addition of Co II the extinction was raised to a value corresponding to complete transformation into CoH_2 II. When pyruvate was added at the end of the reaction, no change occurred. This rules out the possibility that the reaction might involve a dephosphorylation of CoH_2 II to CoH_2 I, because the latter compound would have been reoxidized by the pyruvate and lactic apodehydrogenase which was present in the enzyme preparation.

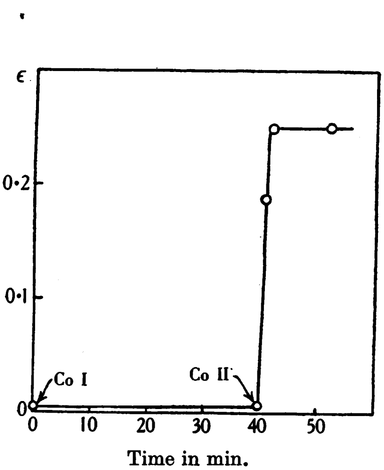


Fig. 7.

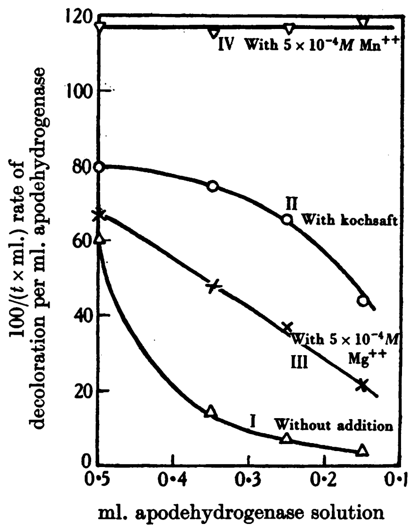


Fig. 8.

Fig. 7. Codehydrogenase II-specificity of *iso*citric dehydrogenase.

Fig. 8. Effect of enzyme dilution; activation by kochsaft, Mg^{++} and Mn^{++}. The curves refer to the Thunberg exp. of Table III.

Mn^{++} and Mg^{++} as complements of the iso*citric dehydrogenase*

Methylene blue experiments. As mentioned before, it was observed that the *iso*citric dehydrogenase system showed a typical "dilution effect", especially if purified enzymes were used. This may be illustrated by the experiments given in Table III and by Curve I in Fig. 8. The table contains the original figures for the decoloration times obtained with different amounts of apodehydrogenase, whilst in the curves of Fig. 8 the reciprocal values of the decoloration times, calculated for 1 ml. apodehydrogenase solution, are plotted against the amounts of apodehydrogenase solution used. It is seen that Curve I falls off sharply with decreasing amounts of apodehydrogenase; if there had been no dilution effect the curve would have remained horizontal.

From this observation one must conclude that the apodehydrogenase solution contained besides the enzyme another substance, essential for the reaction and present in a suboptimal concentration. If this substance were thermostable, then kochsaft of the apodehydrogenase solution, added to the system, should be able to remove the dilution effect. In fact, kochsaft caused a great activation, especially at low enzyme concentrations, i.e. it partially removed the dilution effect. Further, it was shown that the ash of the apodehydrogenase preparation still activated the reaction; the activating substance must therefore have been inorganic. Mn salt was then found to activate enormously and to bring about a complete proportionality between rate and apodehydrogenase concentration. This is demonstrated in Table III and by Curve IV in Fig. 8. Mg^{++} had a similar action but the activation was less and a dilution effect was still found.

Table III. *Effect of* Mn^{++} *and* Mg^{++} *on the* iso*citric dehydrogenase*

The apodehydrogenase solution used contained 1·8 mg. "enzyme C" per ml. The reaction mixture contained 0·1 ml. (=0·19 mg.) *iso*citric acid as Na salt, apodehydrogenase solution in the varying amounts given in the first column, 0·20 ml. (=0·20 μg.) Co II, 0·25 ml. flavinenzyme, 0·25 ml. veronal buffer, *p*H 7·66, 0·5 ml. methylene blue 1:5000, plus various additions as indicated. Total volume 2·0 ml.

Apodehydro-genase ml.	Decoloration time: No addition min. sec.		Kochsaft from 3·6 mg. "enzyme C" min. sec.		Ash from 1·5 mg. "enzyme C" min.	$MgSO_4$ $5\times10^{-4}M$ min. sec.		$MnSO_4$ $5\times10^{-4}M$ min. sec.	
0·50	3	20	2	30	—	3	0	1	45
0·35	20	0	3	50	—	6	0	2	30
0·25	52	0	6	0	11	10	50	3	20
0·15	140	0	16	0	—	30	30	5	30

Controls with Mg^{++} and Mn^{++} and without substrate or without Co II or without flavinenzyme were negative.

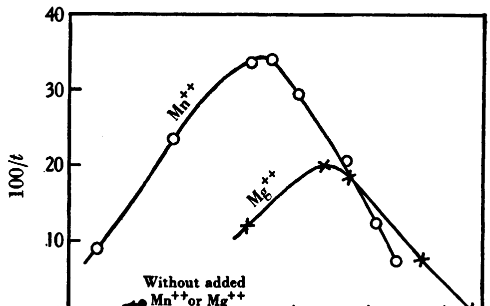


Fig. 9.

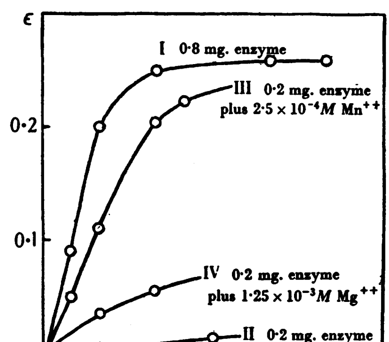


Fig. 10.

Fig. 9. Effect of Mg^{++} and Mn^{++} concentration. Thunberg experiments with 0·25 ml. "enzyme C" (=0·45 mg. dry powder) and the other components as given in Table III.

Fig. 10. Effect of enzyme dilution and of Mn^{++} and Mg^{++} on the rate of CoH_2 II-formation. 0·2 mg. *iso*citric acid and 70 μg. Co II were used in all experiments. Amounts of "enzyme C" and additions of $MnSO_4$ and $MgSO_4$ as indicated.

Fig. 9 shows that the activating action of Mn and Mg salts is dependent on their concentration. For both ions distinct concentration optima exist, which were found to be about 5×10^{-4} *M* for Mn^{++} and about $2{\cdot}5 \times 10^{-3}$ *M* for Mg^{++}. The maximal activity brought about by Mg^{++} is only 59% of that caused by Mn^{++}.

If suboptimal amounts of Mn^{++} and Mg^{++} are added simultaneously, the activity was only very slightly higher than the activity found with Mn^{++} alone. This seems to indicate that Mn^{++} and Mg^{++} are involved in the same reaction and their action is in principle the same, but Mn^{++} may have a greater affinity for the enzyme. $CaCl_2$, $ZnSO_4$ and $CdSO_4$ had no effect.

As is known, Mg and Mn ions are normal constituents of tissues, and therefore it is likely that these ions actually represent the natural complements of the *iso*citric dehydrogenase system.

Spectrophotometric experiments. The spectrophotometric determination of the rate of CoH_2 II-formation shows that the Mn^{++} and Mg^{++} effect is involved in the primary step of the dehydrogenation and has nothing to do with the transport of H from CoH_2 II to the acceptor. Curve I in Fig. 10 gives the rate of CoH_2 II-formation with 0·8 mg. of the acetone powder "enzyme C". When 1/4 of this amount was used (Curve II), the rate was much less than 1/4 of the rate given in Curve I: within 10 min. only about 1/15 as much CoH_2 II was formed. Thus, the "dilution effect", shown with the Thunberg technique, is confirmed by the spectrophotometric experiment. Curve III shows the effect of Mn^{++} and Curve IV that of Mg^{++} on the rate of CoH_2 II-formation with the lower apodehydrogenase concentration.

Concerning the mechanism of Mn^{++} and Mg^{++} action it has been already mentioned that the most probable assumption may be that the ions favour the combination of apodehydrogenase and substrate. We have tested a number of other dehydrogenase systems, including lactic, malic, glutamic and hexosemonophosphate dehydrogenases, without finding any Mn or Mg effect. Therefore, the effect probably does not involve the equilibrium apodehydrogenase + codehydrogenase $\rightleftharpoons$ holodehydrogenase, which occurs in all these systems. Another possibility to be taken into consideration is an acceleration of the decarboxylation of the primary reaction product, α-keto-β-carboxyglutaric acid, by Mn^{++} and Mg^{++}. According to the assumption discussed on p. 1037, the primary dehydrogenation reaction (2*a*) is reversible and proceeds to completion only if the keto-acid decomposes. Therefore, dependence of the decarboxylation on Mn^{++} would result in the formation of only a small amount of CoH_2 II in systems deprived of Mn^{++}. There is, however, experimental evidence against the view that Mn^{++} affects the decarboxylation. If that were the case, the dehydrogenation should proceed, even in the absence of Mn^{++}, if a ketone fixative is added to combine with the keto-acid and so disturb the assumed equilibrium (2*a*). With the spectrophotometric technique it was, however, shown that dimedon does not accelerate the CoH_2 II-formation in a Mn^{++}-poor system, but that addition of Mn^{++} then had the known effect.

Inhibitors

Iodoacetic acid. The rate of methylene blue decoloration in the complete *iso*citric dehydrogenase system was inhibited 97% by *M*/100 and 75% by *M*/1000 iodoacetic acid. This effect was confirmed with the spectrophotometric technique, although here higher concentrations of iodoacetic acid seemed to be necessary to bring about a similar inhibition.

Thus *iso*citric dehydrogenase belongs to the group of apodehydrogenases which are inhibited by iodoacetic acid: triosephosphate and alcohol apodehydrogenase from yeast [Adler *et al.* 1938, 2], triose dehydrogenase of animal tissues [Green *et al.* 1937], succinic dehydrogenase [Hopkins *et al.* 1938] and, to a less extent, alcohol dehydrogenase from liver [Adler *et al.* 1938, 2] and lactic dehydrogenase from yeast (unpublished experiment). All the other dehydrogenases were found to be resistant to iodoacetic acid. According to our present knowledge, sensitivity to this substance indicates the presence in the enzyme molecule of SH-groups which are essential for activity.

Pyrophosphate. The *iso*citric acid dehydrogenation is strongly inhibited by pyrophosphate; addition of Mn^{++} can, however, abolish this inhibition (Table IV).

Table IV. *Pyrophosphate inhibition*

Thunberg experiments with 0·2 mg. *iso*citric acid, 4 mg. "enzyme C", 20 μg. Co II, flavin-enzyme, veronal buffer. Total volume 2·0 ml.

Addition	Decoloration time min.	sec.
—	2	30
0·2 ml. M/10 $Na_4P_2O_7$ (neutralized)	20	0
5×10^{-4} M Mn^{++}	2	0
$Na_4P_2O_7$ plus Mn^{++}	2	0

From these experiments it becomes clear that the pyrophosphate inhibition is due to combination with the Mn^{++} or Mg^{++} present in the enzyme preparation.

As shown by Leloir & Dixon [1937], pyrophosphate also inhibits succinic dehydrogenase and it is suggested by these authors that pyrophosphate may compete with the substrate for the affinity centres of the enzyme, just as is assumed for malonate. Our experience with *iso*citric dehydrogenase indicated that the pyrophosphate inhibition of succinic dehydrogenase might be due to Mn or Mg removal. However, in connexion with other experiments, it was found in this institute [Euler & Hellström, 1939], that Mn did not influence the succinic dehydrogenase. Thus, the mechanism of pyrophosphate inhibition actually seems to be different in the two systems.

Phosphate. As mentioned before (cf. p. 1033), phosphate exerts a distinct inhibition, which is higher in the alkaline than in the acid region (Table V).

Table V. *Phosphate inhibition*

0·1 ml. "enzyme B" and different amounts of phosphate buffer were used; the other components were the same as given in Table IV. The *p*H was determined after the experiment.

*p*H	Phosphate concentration (M)	Rate of MB-decoloration (100/t)	Inhibition %
7·56	$1{\cdot}25 \times 10^{-2}$	16	—
7·54	$1{\cdot}88 \times 10^{-2}$	10	37·5
7·58	$2{\cdot}50 \times 10^{-2}$	5	56
7·56	$6{\cdot}25 \times 10^{-2}$	2·8	82
6·64	$1{\cdot}25 \times 10^{-2}$	11·8	—
6·63	$2{\cdot}50 \times 10^{-2}$	8·7	26
6·63	$6{\cdot}25 \times 10^{-2}$	7·7	34

Phosphate inhibition was observed by Theorell [1935] in the complete hexosemonophosphate system and confirmed by Adler & Günther [1938] by the spectrophotometric method, thus showing that phosphate affects the primary reaction between substrate, apo- and co-dehydrogenase. A competition by the phosphate with either the substrate or the Co II for the apoenzyme may be the reason for the inhibition. Similarly, in the *iso*citric system, phosphate may compete with the Co II. Furthermore, as manganese phosphate is only slightly soluble, the phosphate inhibition could be explained by precipitation of Mn^{++}.

No inhibition was found with cyanide, malonate, fluoride and oxalate.

Distribution

*iso*Citric apodehydrogenase has been found in all animal tissues so far examined. The tissues were minced and dried with acetone, the acetone preparations were extracted by grinding with 5 times the weight of water and sand and the extracts were dialysed for 5–20 hr. In the cases of brain and spleen the apodehydrogenase was not found in extracts prepared in this way, but its presence could be shown in extracts from fresh tissue. Table VI gives the relative amounts of the apodehydrogenase in various tissues as determined by Thunberg experiments. Mn salt was added to the reaction mixture.

Table VI. *Distribution of* iso*citric apodehydrogenase*

Tissue	Relative concentration
Heart	+ + +
Liver	+ + +
Kidney	+ + +
Adrenal gland	+ + +
Ovary	+ +
Intestine	+ +
Muscle	+
Brain	+
Lung	+
Testis	+
Jensen sarcoma [cf. Euler *et al.* 1939]	+
Spleen	(+)

The ratio of the activities of *iso*citric and glutamic apodehydrogenases is different for different tissues. Furthermore, the activities of two purified enzyme solutions, the one prepared from liver and the other from heart muscle, were determined spectrophotometrically in the systems (*a*) *iso*citric acid + Co II, and (*b*) CoH_2 II + ketoglutaric acid + NH_3. When the two enzymes were diluted in such a way that they had the same activity in system (*a*), the ratio of their activities in system (*b*) was 1:4, Therefore *iso*citric and glutamic apodehydrogenases are not identical.

The formation of glutamic acid from iso*citric acid*

Recently it was shown that the specific glutamic dehydrogenase catalyses the reductive amination of α-ketoglutaric acid [Euler *et al.* 1938]. Both Co I and Co II were found to act as coenzymes of this apodehydrogenase. Therefore, it follows that *iso*citric acid can be converted to glutamic acid if *iso*citric apodehydrogenase, Co II, glutamic apodehydrogenase and NH_3 are present. The following reactions occur:

(2*a*) *iso*Citric acid + Co II $\xrightarrow{\textit{iso}\text{citric apodehydrogenase}}$ α-keto-β-carboxyglutaric acid + CoH_2 II.

(2*b*) α-Keto-β-carboxyglutaric acid → α-ketoglutaric acid + CO_2.

(6) α-Ketoglutaric acid + NH_3 → α-iminoglutaric acid + H_2O.

(7) α-Iminoglutaric acid + CoH_2 II $\xrightarrow{\text{glutamic apodehydrogenase}}$ glutamic acid + Co II.

The sum of these reactions is

(8) *iso*Citric acid + $NH_3 \rightarrow$ glutamic acid + $H_2O + CO_2$.

Thus, in the *iso*citric system, not only the substrate for the glutamic acid synthesis but also the H in the form of CoH_2 II is available for the reductive amination. The reaction represents an oxidoreduction between *iso*citric acid and α-iminoglutaric acid, catalysed by Co II, which oscillates between the two specific apodehydrogenases. This is a new example of the validity of the "two enzyme scheme" for coenzyme-dependent oxidoreductions, which was proposed previously for the oxidoreductions between triosephosphate and acetaldehyde or pyruvic acid and similar reactions [cf. Euler *et al.* 1936; Adler *et al.* 1938, 1].

The experimental evidence for the mechanism given above is based on the observation that not only *iso*citric but also glutamic dehydrogenase uses Co II as coenzyme. It has been shown [Euler *et al.* 1938] that glutamic apodehydrogenase from liver catalyses the dehydrogenation of both CoH_2 I and CoH_2 II by iminoglutaric acid. Since then we have been able to confirm this fact with glutamic apodehydrogenase from other animal tissues. Thus, it becomes clear that the non-specificity of glutamic apodehydrogenase with respect to the codehydrogenases is common for animal tissues and that reaction (8) can be a general cell reaction for the animal body. These facts are demonstrated by the following spectrophotometric experiments.

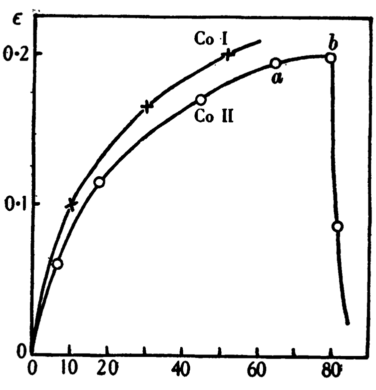


Fig. 11.

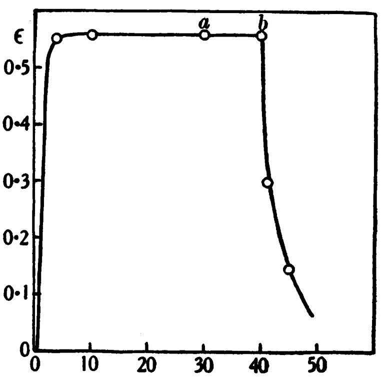


Fig. 12.

Fig. 11. Codehydrogenases I and II as coenzymes of glutamic dehydrogenase. 0·5 ml. $M/2$ glutamate, 0·1 ml. dialysed aqueous extract from acetone-liver, 0·2 ml. Co I or Co II. In the experiment with Co II were added: at (*a*) 0·1 ml. $M/2$ acetaldehyde, at (*b*) 0·1 ml. $M/10$ ketoglutarate plus 0·1 ml. $M/2$ NH_4Cl.

Fig. 12. Hydrogenation of Co II by *iso*citric acid and dehydrogenation of the CoH_2 II by α-iminoglutaric acid. 0·1 ml. $M/10$ *iso*citrate, 0·1 ml. dialysed aqueous extract of acetone kidney, 145 μg. Co II. At (*a*) 0·1 ml. $M/10$ ketoglutarate, at (*b*) 0·1 ml. $M/2$ NH_4Cl were added.

Dewan [1938], in his paper on glutamic dehydrogenase, says that this enzyme is Co I-specific. The exps. of Fig. 11, however, make it clear, in addition to the previous results [Euler *et al.* 1938], that this is not the case. The curves show the hydrogenation of Co I by glutamic acid and apodehydrogenase from liver, and the hydrogenation of Co II by the same system. When the equilibrium was nearly reached in the experiment with Co II, acetaldehyde was added, but had no effect; if, during the reaction, a conversion of Co II into CoH_2 I had occurred, the extinction would have disappeared because the enzyme contained alcohol apodehydrogenase. However, after addition of ketoglutaric acid and NH_3 the equilibrium was pushed back and the CoH_2 II formed was reoxidized.

Fig. 12 demonstrates the oxidoreduction between *iso*citric acid and iminoglutaric acid. In this experiment Co II was hydrogenated by *iso*citrate in presence of a dialysed aqueous extract from acetone-dried kidney, which contains highly active *iso*citric as well as glutamic dehydrogenase. Addition of α-ketoglutaric acid alone had no effect, but when NH_3 was added, the extinction fell off instantaneously, showing that the CoH_2 II formed in the *iso*citric system was reoxidized by the iminoglutaric acid.

If no extra ketoglutaric acid had been added in this experiment the oxidoreduction would have occurred as well, because then the ketoglutaric acid formed from *iso*citric acid would have been aminated. But the concentration of iminoglutaric acid would have been so small that the rate of CoH_2 II-disappearance would probably have been less than the rate of re-hydrogenation of the Co II by the excess of *iso*citric acid, and no decrease of extinction would have been observed until the whole of the *iso*citric acid had been used up. It is, however, possible to demonstrate the direct transformation of *iso*citric acid into glutamic acid (Fig. 13) if an excess of Co II is used instead of an excess of *iso*citric acid. Then, in the first phase of the experiment, the total amount of *iso*citric acid is

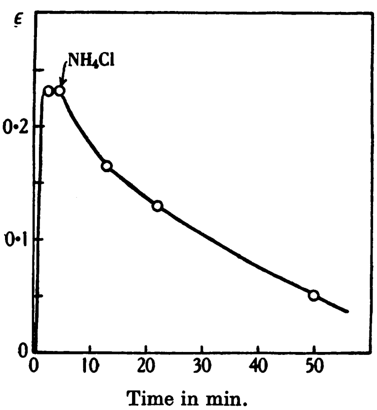
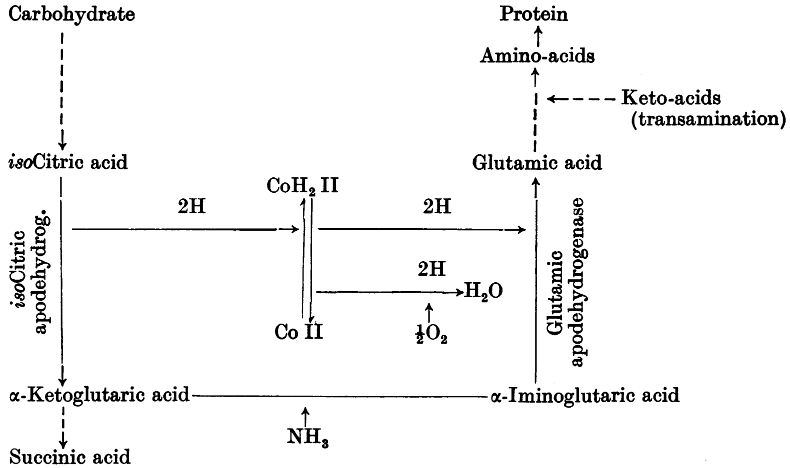


Fig. 13. Oxidoreduction between *iso*citric acid and iminoglutaric acid. $1{\cdot}8 \times 10^{-4}$ mM *dl-iso*citric acid, $0{\cdot}95 \times 10^{-4}$ mM Co II, 0·1 ml. "enzyme C", which contains glutamic besides *iso*citric apodehydrogenase.

converted into ketoglutaric acid and an equivalent amount of CoH_2 II is formed. If now NH_4Cl is added, iminoglutaric acid is formed, which dehydrogenates the CoH_2 II. "Enzyme C", which contains both apodehydrogenases, was used in this experiment.

Discussion

The mechanism of the transformation of *iso*citric acid and the function of the *iso*citric dehydrogenase system as a link between carbohydrate breakdown and protein synthesis, can, according to the results presented in this paper, be symbolized as follows:



The products of *iso*citric acid dehydrogenation, ketoglutaric acid and CoH_2 II, can react further in two directions. Either they can be used in the glutamic dehydrogenase system for the fixation of NH_3 (glutamic acid synthesis), or H can be transferred from the CoH_2 II over diaphorase II and the cytochrome system to O_2, and the ketoglutaric acid can be broken down to succinic acid. The H necessary for the reductive amination of ketoglutaric acid can come, via CoH_2 I or CoH_2 II, from other dehydrogenase systems too, but it appears likely that the direct coupling of the *iso*citric and glutamic acid systems will be the most effective way. Ketoglutaric acid is regenerated from glutamic acid by transamination [Braunstein & Kritzmann, 1937].

According to the "citric acid cycle" theory of Krebs [cf. Krebs & Eggleston, 1938 and preceding papers] the system *iso*citric acid—ketoglutaric acid is part of a catalytic system in the oxidative breakdown of carbohydrate. If this theory is correct, codehydrogenase II is an indispensable part of the complex of cell respiration, especially the pyruvic acid oxidation, whilst codehydrogenase I is known to be necessary for the removal of the first pair of H atoms from the carbohydrate, i.e. from the triosephosphate molecule. Mn^{++}, as a complement of *iso*citric dehydrogenase, would also be involved in that part of cell respiration which concerns the breakdown of pyruvic acid. It seems possible that one could find support for the citric acid cycle theory by studying the action of Co II and Co I separately as well as of Mn^{++} on cell respiration.

Summary

1. *iso*Citric apodehydrogenase was prepared from heart muscle. This enzyme catalyses specifically the dehydrogenation of *iso*citric acid by codehydrogenase II. The substrate affinity of the enzyme is extremely high. Citric acid is used only if aconitase is present in the enzyme preparation.

2. When the system was completed with flavinenzyme, 1/2 mol. O_2 was taken up and 1 mol. CO_2 was formed per mol. *iso*citric acid, and α-ketoglutaric acid was isolated as the reaction product.

3. The system *iso*citric acid + Co II + apodehydrogenase does not react unless Mn^{++} or Mg^{++} are present; Mn^{++} is more active than Mg^{++}.

4. Iodoacetic acid and pyrophosphate are strong inhibitors, the first reacting with the apodehydrogenase, the second binding Mn and Mg ions.

5. *iso*Citric apodehydrogenase was found in all animal tissues so far examined; the highest concentrations are present in heart, liver, kidney and adrenal gland.

6. The mechanism of the conversion of *iso*citric acid into glutamic acid was demonstrated and the biological importance of this reaction discussed.

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