IN VITRO CARBON DIOXIDE FIXATION INTO SUCCINATE*

by

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In a previous report the data on carbon dioxide fixation *in vivo*[1] did not fit expectations if a dicarboxylic or a tricarboxylic acid cycle were required for the fixation of $^{14}CO_2$ into succinate. The experimental approach involved an analysis of the extent of labelling in the chromatographically separated organic acids, without the use of carrier, when the periods of fixation were less than 45 minutes. Incorporation of labelled carbon into succinate, from $NaH^{14}CO_2$ for periods of fixation of less than 20 minutes by rat liver homogenates provide the basis for this report. When it was found in the experiments reported here that succinate stimulated the incorporation of the label into succinate, under anaerobic conditions, the assumption was made that a carbon dioxide acceptor was involved in an exchange reaction with succinate. With certain carboxylations as a model[2,3], the possibility of an enol form of a 3 carbon molecule as a carbon dioxide acceptor for succinate formation was considered. The role of propionate[3,4] in carboxylation reactions explained the consideration of a 3 carbon compound and, in its simplest aspects, it seemed that the loss of a molecule of water from one of the glyceric acids might provide the hypothetical intermediate for the application of the model reaction to anaerobic succinate formation. 3-phosphoglyceric acid was arbitrarily selected and no other glyceric acid was used. While the data presented below permit no conclusions concerning the chemical structure of a carbon dioxide acceptor, they describe the effect of succinate and 3-phosphoglyceric on the incorporation of carbon dioxide into succinate in rat liver homogenates.

EXPERIMENTAL PART

Methods

Rat liver homogenates were incubated at 37° C in a medium (Table I) containing 0.01 millicurie of radioactive bicarbonate. Each beaker contained 1 gram of liver, 2 ml of the medium and except where indicated, 10 mg of the added substrate. In the experiments with 3-phosphoglyceric acid, 5 mg of adenosinemonophosphate per g liver was added as a phosphate acceptor. Ten mg diphosphopyridine nucleotide, per beaker, was used in the anaerobic experiments so that the medium would be identical to that in the aerobic experiments. At the end of each incubation period the reaction was stopped with acid acetone[5] which was added to form extracts which were dried in air. The dried extracts were transferred to pentanol chloroform (50% v/v) (instead of butanol-chloroform) as described in the ISHERWOOD procedure[6]. After a reduction to a final volume of 1.5 ml, these pentanol-chloroform extracts were assayed for total radioactivity and then chromatographed on silica gel[1]. The pH of the aqueous phase of the chromatographic columns was, by design, sufficiently high to permit the complete decomposition of such labile organic acids as oxalacetic and oxalosuccinic

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when the effluent residues were dried in air at approximately 40° C. Radioactive assay, chemical measurement and identification of the acids have been described[1]. Where indicated the *p*-bromphenacyl ester of succinic acid was formed by refluxing the acid and *p*-bromphenacyl bromide for 2 hours. The fumarate from the succinate in one experiment was degraded by acid permanganate[7].

TABLE I

COMPOSITION OF INCUBATION MEDIUM

Component	*Concentration g per 100 ml solution*
NaCl	0.72
$NaHCO_3$	0.10*
KH_2PO_4	0.10
$MgSO_4 \cdot 7H_2O$	0.06
Glucose	0.10
Sodium Citrate	0.03

* Containing 0.01 millicurie as labeled bicarbonate per volume (2 ml) of medium added to each g liver.

RESULTS

In Table II, the data for succinate incubated with the homogenate in air and nitrogen, for periods of from 2–30 minutes, indicate that the specific activity of the succinate increased with time and more than 50% of the radioactivity of the total extract resided in the succinate in every case. The data for succinate in air do not exclude the possibility that compounds labeled at other points on a normal oxidative cycle were carried to and blocked with succinate. Under nitrogen the data allow the possibility of a labeling of succinate by a reversed cycle (*i.e.* malate to fumarate to succinate) but do not rule out the possibility of an equilibrium between succinate and a carbon dioxide acceptor outside the Krebs cycle which combines with the labeled CO_2.

TABLE II

INCORPORATION OF $^{14}CO_2$ INTO SUCCINIC ACID BY LIVER HOMOGENATES INCUBATED WITH $NaH^{14}CO_3$ IN THE PRESENCE OF ADDED SUCCINATE*

Conditions	*Incubation time*	*Per cent. total activity of extract***	*Specific activity****
Aerobic	2	74.4	400
	5	66.5	980
	15	67.0	1960
Anaerobic	10	54.0	1860
	20	60.1	1613
	30	84.7	2887

* Nitrogen atmosphere. Composition of medium and radioactive dose appear in Table I.

** Per cent. of total activity of extract $= \frac{\text{Total activity in succinate}}{\text{Radioactivity of total effluent}} \times 100$.

*** cts/Min/Mg.

The extent to which 3-phosphoglyceric acid anaerobically stimulated the incorporation of the ^{14}C label into the total organic acids in the extracts of the homogenate as compared to this incorporation in homogenates incubated without and with succinate and pyruvate is shown in Table II.

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TABLE II

RADIOACTIVITY OF COMPARABLE ALIQUOTS* OF PENTANOL-CHLOROFORM (50% N/N) EXTRACTS OF LIVER HOMOGENATES INCUBATED WITH ADDED SUBSTRATE**

The time of incubation was 10 minutes

Added substrate	*Radioactivity, cts per min*
3-Phosphoglyceric acid and AMP	3360
None and AMP	348
3-Phosphoglyceric acid AMP and DPN***	5265
None, AMP and DPN	576
3-Phosphoglyceric acid (2 Mg and AMP)	2592
3-Phosphoglyceric acid (8 Mg) and AMP	4868
3-Phosphoglyceric acid (14 Mg) and AMP	3621
Succinate	2700
Succinate and ATP	1587
Succinate and ATP	1590
Succinate and DPN	2622
Succinate, DPN and ATP	2220
Pyruvate, AMP and DPN	120
Pyruvate (30 mg)	852
Control, AMP and DPN	120

* An aliquot is equivalent to 66.6 mg liver. For example, the ratio between this aliquot and that chromatograph in Fig. 1 is 1 to 6. (*i.e.* sum of all cts/min per fraction × 2, because alternate fractions were counted, equals 31,682 or approximately 6 times the value, 5265, in the above table).

** Except where indicated, 10 mg substrate, 5 mg AMP and mg DPN were added per g liver.

*** AMP = Adenosine monophosphate
DPN = Diphosphopyridine nucleotide.

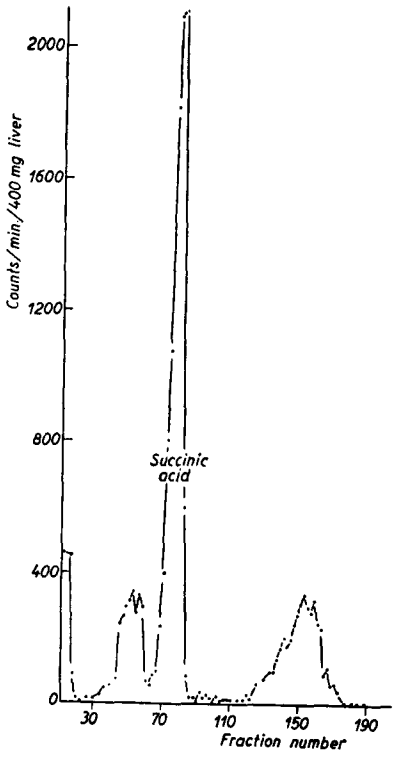

Fig. 1. Chromatogram of pentanol-chloroform (50% v/v) extract of rat liver homogenate incubated for 10 minutes in a modified (See Table I) Krebs Ringer solution in the presence of 10 mg 3-phosphoglyceric acid and 0.01 mc $NaH^{14}CO_3$ per g liver. The atmosphere was nitrogen. Specific activity of the fumarate (peak, fraction 51), succinate, and malate (peak, 151) was $1.3 \cdot 10^2$, $1.7 \cdot 10^3$, $3.1 \cdot 10^2$ cts/min/micromol respectively for the above dose. Fifty-eight per cent of the total activity of the extract was due to succinate.

A typical chromatogram showing the effects of incubation of 3-phosphoglyceric acid with the homogenates in nitrogen is shown in Fig. 1. The fraction at the peak of this curve, when distributed between 0.1 *N* HCl and ether, showed a distribution coefficient of 0.153 as compared with 0.154 for the authentic acid. The fractions on either side of this peak were mixed with inert succinate so that the specific activity of the acid was 1040 cts/min/millimol and esterified with *p*-bromphenacyl bromide. The ester, melting at 209° (m.p., authentic acid, 210°) had a specific activity of 960 cts/min/millimol.

Figs. 2 and 3 are typical of the results when pyruvate and 3-phosphoglyceric acid are incubated with the liver homogenates in the presence of oxygen. The chromatographic curves illustrate two points of difference, namely, the distribution of radioactivity within the acids of Fig. 2 differs from that of Fig. 3 and the incorporation of the label into the organic acids between fractions 110 and 150, in the chromatogram

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for the incubation with pyruvate, is now measurable. The asymmetrical peaks throughout the chromatogram in Fig. 3 indicate additional labelled solutes in the extracts.

The similar specific activities (see legend Figs. 2 and 3) of the acids, when the homogenates are incubated aerobically with pyruvic acid as well as with 3-phosphoglyceric acid, would seem to indicate that the organic acids in each case are in equilibrium or that there is steady state synthesis of the acids. Then the difference in the

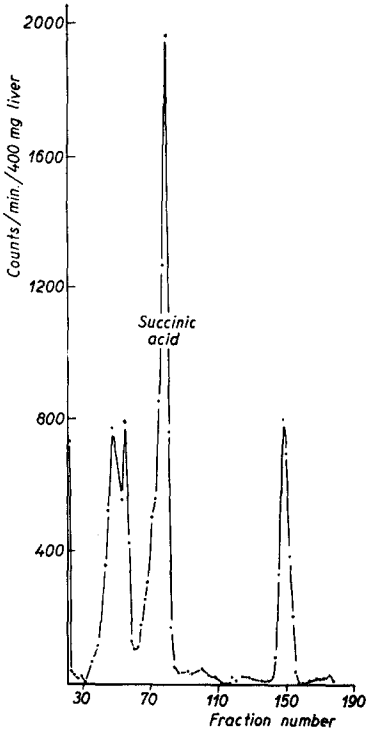

Fig. 2. Chromatogram showing the incubation of 3-phosphoglyceric acid with liver homogenates in oxygen atmosphere for conditions otherwise like those for Fig. 1. Specific activity of the fumarate (peak at fraction 47), succinate and malate peak at 145) was $1.3 \cdot 10^2$, $1.3 \cdot 10^2$ and $1.0 \cdot 10^2$ cts/min/micromol respectively for the dose of 0.01 mc $NaH^{14}CO_3$ g liver. Fifty-four per cent. of the total activity of the extract was due to succinate. The fumarate, degraded by acid permanganate, showed all the activity on carboxyl groups.

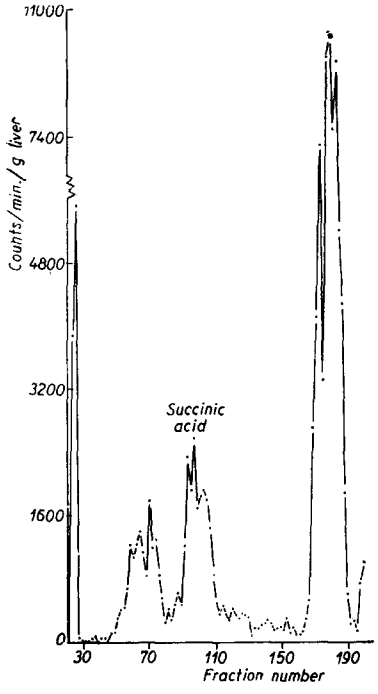

Fig. 3. Chromatogram showing the incubation of pyruvic acid with liver homogenates in oxygen atmosphere for conditions otherwise like those for Fig. 1. Specific activity of the fumarate, succinate and malate was $2.5 \cdot 10^3$, $5 \cdot 10^3$ and $4.5 \cdot 10^3$ cts/min/micromol for the dose of 0.01 mc $NaH^{14}CO_3$. Radioactivity from fraction 110 to 115 accounts for labeled α-ketoglutarate and aconitate. The percentage of the total activity of the extract due to succinate is 13 per cent.

percent of total activity (54% with 3-phosphoglyceric acid and 13% with pyruvate) indicates that the two substrates function by different mechanisms in the fixation of carbon dioxide into succinate. When the compounds listed in Table IV were incubated with the homogenates the percentage of the total radioactivity of the extract due to succinate was less than 50% under aerobic conditions. These data can only now suggest that the capacity of these substrates to provide radioactive succinate aerobically in the presence of $NaH^{14}CO_3$ is either less than or, at least, the mechanism by which they

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function in this capacity is different than that of 3-phosphoglyceric acid in these liver homogenates.

TABLE IV

PERCENTAGE OF TOTAL ACTIVITY RESIDING IN CERTAIN ORGANIC ACIDS FOLLOWING THE INCUBATION OF LIVER HOMOGENATES WITH ADDED SUBSTRATE AND $Na^{14}CHO_3$

	*Per cent. of total activity of extract****				*Per cent. of total activity of extract****		
Added	*Fumarate*	*Succinate*	*Malate*	*Added*	*Fumarate*	*Succinate*	*Malate*
Malate	35.1	13.3	27.7	Ketoglutarate	11.5	22.5	39.6
Malate	29.6	18.7	36.5	Propionate	7.9	12.9	63.5
Malate	16.2	16.5	46[7]	Propionate	***	24.1	37.1
Malate	17.5	20.3	48	Propionate	23.6	10.5	13.1
Cis-Aconitate	9.9	11.6	40.1	Sarcosine	***	10.2	42.8
Cis-Aconitate	2.4	17.3	69.8	Sarcosine	***	11.7	36.6
Oxalacetate	16.7	26.8	38.9	Acetaldehyde	21.2	3.6	39
Oxalacetate	30.8	30.6	29.6	Acetaldehyde	33.4	13.3	33.9
Fumarate	10.3	16.6	32.5	Glycine	16.5	14.4	44.0
Fumarate	30.0	16.6	32.5	Control	26.42	20.48	30.7
Pyruvate	11.25	7.6	58.7	Control	18.6	18.8	26.3
Pyruvate	11.00	13.4	59.9	Control	37.19	12.05	27.7

* All incubations were 10 minutes long.

** Percent of activity in total extract $= \frac{\text{Activity of organic acid}}{\text{Activity of total effluent}}$

*** The chromatographic peaks were insufficiently discreet to justify calculation for percent.

SUMMARY

When succinate or 3-phosphoglyceric acid was incubated anaerobically with liver homogenates and ^{14}C labelled bicarbonate, the percentage of the total radioactivity in the chromatographically separated organic acids was greater in succinate than in any other acid. For the incubation with 3-phosphoglyceric acid, the specific activity of succinate, was greater than that of fumarate or malate. Aerobically the behaviour of 3-phosphoglyceric acid, with respect to carbon dioxide fixation into succinate, differed from that of pyruvate.

RÉSUMÉ

Quand du succinate ou de l'acide 3-phosphoglycérique sont incubés en anaérobiose avec des broyats hépatiques et du bicarbonate marqué par ^{14}C, c'est dans le succinate que le pourcentage de la radioactivité totale dans les acides organiques isolés par chromatographie est le plus grand. Après incubation avec l'acide 3-phosphoglycérique, l'activité spécifique du succinate est plus élevée que celle du fumarate ou du malate. En aérobiose, le comportement de l'acide 3-phosphoglycérique vis à vis de l'incorporation du CO_2 dans le succinate diffère de celui du pyruvate.

ZUSAMMENFASSUNG

Nach der anaerobischen Bebrütung von bernsteinsaurem Salz oder 3-Phosphoglycerinsäure mit Leberhomogenaten und mit ^{14}C-markiertem Bikarbonat war der Prozentsatz der Gesamtradioaktivität in den chromatographisch abgetrennten organischen Säuren in den bernsteinsauren Salzen höher als bei irgend einer anderen Säure. Bei der Bebrütung mit 3-Phosphoglycerinsäure war die spezifische Aktivität des bernsteinsauren Salzes grösser als die des fumarsauren oder des apfelsauren Salzes. Das aerobische Verhalten von 3-Phosphoglycerinsäure bezüglich des CO_2-Einbaus in bernsteinsaures Salz unterschied sich von dem des brenztraubensauren Salzes.

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