[17] A. J. ROSENBERG, *J. Biol. Chem.*, 219 (1956) 951.
[18] E. S. WEST AND R. E. RINEHART, *ibid.*, 146 (1942) 105.
[19] F. J. REITHEL AND R. P. WITHER, *J. Am. Chem. Soc.*, 71 (1949) 1879.
[20] R. MOUBASHER, *J. Biol. Chem.*, 176 (1948) 529.
[21] H. J. KAUFFMANN, *J. Am. Chem. Soc.*, 73 (1951) 4311.
[22] E. SILVERBLATT, A. L. ROBINSON AND C. G. KING, *ibid.*, 65 (1943) 137.
[23] H. G. STEINMAN AND C. R. DAWSON, *ibid.*, 64 (1942) 1212.
[24] J. H. BAXENDALE, M. G. EVANS AND G. S. PARK, *Trans. Faraday Soc.*, 42 (1946) 155.
[25] B. ROSENFELD, *J. Biol. Chem.*, 150 (1943) 281.
[26] P. HOLTZ AND G. TRIEM, *Naturwissenschaften*, 25 (1937) 251.
[27] K. H. BEYER, *J. Pharmacol. Exptl. Therap.*, 71 (1941) 394.
[28] S. UDENFRIEND, C. T. CLARK, J. AXELROD AND B. B. BRODIE, *J. Biol. Chem.*, 208 (1954) 731.
[29] M. B. MATHEWS, *ibid.*, 189 (1951) 695.
[30] A. NASON, W. D. WOSILAIT AND A. J. TERRELL, *Arch. Biochem. Biophys.*, 48 (1954) 233.

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A STUDY OF CARBON DIOXIDE FIXATION IN THE HEN OVIDUCT*

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The hen oviduct is a tissue uniquely versatile in its ability to utilize carbon dioxide. It has been shown that carbon dioxide is fixed efficiently into both carboxyl groups of glutamic acid[1] whereas in other tissues of higher organisms carbon dioxide goes almost exclusively to the α-carboxyl group. Carbon dioxide is fixed into the carboxyl groups of acetic acid and glycine under conditions where acetic acid itself is only poorly converted, if at all, to glycine[2]. The following paper describes a compound in which incorporated radioactive carbon dioxide is present in labile configuration. A preliminary report of this work has already appeared[3].

EXPERIMENTAL

The tissue system, medium, and conditions of incubation were the same as previously described[4]. After 2 hours of incubation the contents of the flask were centrifuged at 1000–2000 r.p.m. Two volumes of alcohol were added to the supernatant fluid and, after centrifugation, a few drops of 1 *N* NaOH to make the clear supernatant fluid alkaline to phenol red. This solution was concentrated nearly to dryness on the steam bath or *in vacuo* at about 50°.

Steam distillation and Duclaux procedures

The residue from the above treatment, in a volume of 1–2 ml, was placed in a Markham type steam-distillation apparatus[5]. 35 ml of distillate was collected and transferred to a Duclaux distillation apparatus of the KNETEMANN type[6], fitted with a 100 ml flask. Before distillation a 2.0 ml aliquot of the 35 ml was withdrawn for estimation of counts and acid equivalents and was replaced with 2.0 ml of distilled water. The first 30 ml of distillate were collected in three 10.0 ml volumetric flasks. The Duclaux constants represent the cumulative fraction of the material in these three successive samples, expressed as per cent of the total in the three fractions. A fast stream of N_2 was passed through all fractions for at least 2 minutes prior to neutralization in order to remove dissolved carbon dioxide.

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Counter-current distribution

8 ml of steam distillate was placed in a 25 plate counter-current apparatus which contained the mutually saturated phases, *n*-amyl alcohol and water[7]. After 25 transfers each fraction was analyzed for radioactivity, acid equivalents, or amount of carbonyl-containing compounds[8].

Synthetic procedures

β-methyl glutamic acid was synthesized in conjunction with Dr. ALTON MEISTER by the condensation of ethyl crotonate with ethyl acetoamidomalonate[9] by a process analagous to that used by SNYDER *et al.*[10].

Esters of acetoacetic acid were prepared by heating ethyl acetoacetate with an equivalent amount of the alcohol of the desired ester on the steam bath for 8–24 hours[11]. 2,4-dinitrophenylhydrazones of the esters were purified on columns of silica gel and celite developed with 10% ether in petroleum ether[12]. 2,4-dinitrophenylhydrazones were chromatographed on Whatman No. 1 filter paper which had been impregnated with olive oil[13].

Radioactivity measurements were obtained by counting the material after it had been dried on stainless steel planchets of 1.54 cm^2 area. A Geiger Müller thin-window and open flow gas counter were used. Radioactivity measurements were corrected for background and self-absorption.

RESULTS AND DISCUSSION

When 5 g of hen oviduct tissue were incubated for 2 hours with 12.5 ml of medium containing 40 μmoles per ml of $NaHCO_3$ and saturated with a 5% carbon dioxide–95% oxygen gas mixture, about 0.6–0.7% of the total carbon dioxide* was fixed into the tissues and soluble compounds of the medium (Table I). Early in the incubation (within 30 minutes) an equilibration was reached where the gas phase over the medium contained 27% of the total carbon dioxide* at essentially the same specific activity as the medium. Therefore about 1% of the carbon dioxide of the medium or at least 5 μmole had become incorporated. After 2 hours incubation the unknown material discussed in this paper accounted for from 1–8% of the fixed carbon dioxide, depending on the experiment. Approximately one half of the fixed radioactive carbon was in the amino acids, principally glutamic and aspartic acids, and about 5% in the cellular proteins. An appreciable part of the radioactivity contained in the amino acids may have passed first through the unknown material.

TABLE I

EXTENT OF CARBON DIOXIDE FIXATION

	Counts/min	*Total radioactivity %*
1. Radioactivity of carbon dioxide added at zero time	$309 \cdot 10^5$	
2. Radioactivity of carbon dioxide of medium after 2 hours incubation	$233 \cdot 10^5$	75
3. Radioactivity of carbon dioxide in gas phase above liquid.	$80 \cdot 10^5$	26
4. Radioactivity of medium after removal of carbon dioxide.	$2 \cdot 10^5$	0.65

5 mg of oviduct mince was incubated for 2 hours with a total of 500 μmoles of $NaHCO_3$ in 12.5 ml of medium which was saturated with 95% O_2 — 5% CO_2. Therefore 1% of the dissolved carbon dioxide or at least 5 μmoles of carbon dioxide was fixed.

Upon Duclaux distillation, acetic acid distributes with constants of 29, 61, 100%. The higher fatty acids distill progressively more rapidly, with octanoic acid giving constants at 91, 99, 100. The unknown steam-volatile material resulting from radioactive carbon dioxide fixation in the hen oviduct exhibited constants of 68, 94, 100**.

* By total carbon dioxide is meant the sum of CO_2, HCO_3^-, and H_2CO_3.

** Average of 4 experiments. In other experiments the first constant has been as high as 78%.

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This material was not simply a higher fatty acid because the fixed carbon dioxide was extremely labile as will be described below. Acetic acid, upon counter-current distribution in a 25-plate apparatus, with *n*-amyl alcohol as the mobile phase and water as the stationary phase, forms a peak in plate No. 12. As can be seen in Fig. 1 the steam-volatile material under study distributes around plates 18 and 20. Aliquots from the same solution gave both the material with high Duclaux constants and that which distributed in the alcohol-soluble region upon counter-current chromatography.

The counter-current fractions containing this alcohol-soluble material were dried on the steam bath at a pH just alkaline to phenol red. The dried material upon subsequent steam distillation lost all of its radioactivity. Replacing the sodium by ammonia, with a Dowex ion-exchange column resulted in complete loss of radioactivity (9500 c.p.m.) upon drying*. The radioactivity of a dried residue of the unknown material was recovered completely as carbon dioxide upon acidification (Table II).

TABLE II

RECOVERY OF RADIOACTIVITY OF THE UNKNOWN MATERIAL AS CARBON DIOXIDE

	Counts/min	*Recovery %*
Total radioactivity in the form of the unknown	4025	
Radioactivity distilled into sodium hydroxide	4310	107
Radioactivity recovered as $BaCO_3$	4430	110

Plates 18–23 inclusive representing a peak the unknown material from Fig. 2 were pooled and dried on the steam bath from an aqueous solution just alkaline to phenol red. An aliquot of a solution of the residue of this material was placed in a Warburg flask containing $HClO_4$ in the side arm and NaOH in the center well. Two hours at room temperature were allowed after tipping the acid, to collect the carbon dioxide in the center well. Aliquots from a known volume of the center well contents were counted after drying and after conversion to $BaCO_3$.

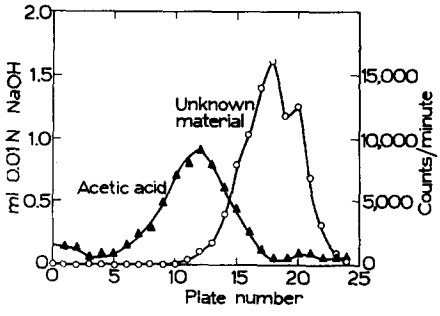

Fig. 1. After a 2 hour incubation of a hen oviduct mince with $NaH^{14}CO_3$ the supernatant fluid was deproteinized with alcohol, concentrated, and steam-distilled. This figure represents the counter-current distribution of the radioactive components from the incubation, and carrier acetic acid. Refer to text for details.

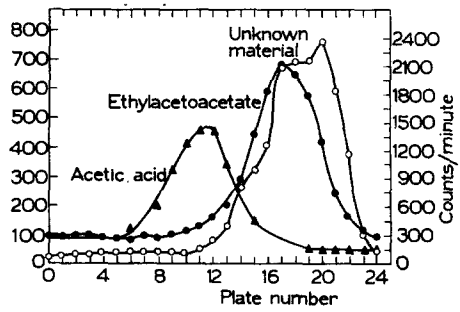

Fig. 2. This figure represents the counter-current distribution of a steam-volatile distillate obtained similarly as described in Fig. 1. Carrier acetic acid and ethyl acetoacetate were added as standards. The ordinate at left represents optical density readings for the ethyl-acetoacetate determined as described in the text and when divided by a factor of 1000, is equivalent to the number of ml of 0.01 *N* NaOH needed to titrate the acetic acid. Refer to text for details.

* $(NH_4)_2CO_3$ decomposes on heating.

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The possibility that the unknown material may have arisen as a result of a reaction of a metabolite with the ethyl alcohol added to precipitate the proteins was excluded by the finding that when acetone was used as the protein precipitant, the unknown material was similarly observed.

Since the lability of the bound carbon dioxide suggests a β-keto configuration, acetoacetic acid and ethyl acetoacetate were examined as model compounds. Acetoacetic acid was completely decomposed upon attempted steam distillation but ethyl acetoacetate gave Duclaux constants of 78, 95, 100, and formed a peak in the counter-current apparatus at plate 17. The higher esters of acetoacetic acid, up to octyl acetoacetate, formed peaks around plate 22. Carrier ethyl acetoacetate was added to a steam distillate obtained from a 2 hour incubation of hen oviduct mince with radioactive carbon dioxide, and the solution partitioned in the counter-current apparatus. In Fig. 2 it can be seen that the bulk of the material cannot be ethyl acetoacetate, although radioactive ethyl acetoacetate may also be present in the shoulder around tube 17.

In another experiment the steam-volatile material from two separate flasks were converted to 2,4-dinitrophenylhydrazones and chromatographed on paper impregnated with olive oil. The major part of the material from one flask traveled with an R_F between that for the derivatives of butyl and amyl acetoacetate. This material was uncharged at pH 7.0, as determined by electrophoresis on paper. After further purification on a silica gel-celite column, infra-red spectroscopy revealed the structure as a keto derivative of a carboxylic acid ester. Its spectrum however, was distinct from that of butyl and amyl acetoacetate derivatives. Acetone was virtually absent from the steam-volatile material. The second flask, however was found to contain almost exclusively the 2,4-dinitrophenylhydrazone of acetone with the complete absence of the keto ester which predominated in the steam-volatile fraction of the first flask. Since these flasks contained aliquots of the same tissue mince it seems possible that the keto ester of the first flask was an acetoacetyl derivative. An aliquot of solution known to contain at least 2200 counts per minute in the form of the unknown was placed with 5 μl of ethyl acetoacetate in the main compartment of a Warburg flask. 1.5 ml of 0.8% 2,4-dinitrophenylhydrazone in 2 N HCl was placed in the center well. 0.3 ml of 15 N H_2SO_4 was added to the unknown and the flask was stoppered and stored at 40° overnight. Acetone liberated by H_2SO_4 decomposition of ethyl acetoacetate was collected in the form of needle-shaped crystals of the 2,4-dinitrophenyl hydrazone, which melted exactly at 126° (reported value) and contained no radioactivity.

Preliminary experiments suggested that the ability to obtain the steam-volatile unknown material was influenced by the addition of CoA and arsenate. As can be seen in Table III, CoA decreased the number of steam-volatile counts, whereas the subsequent addition of arsenate markedly stimulated the appearance of radioactive steam-volatile material. It is interesting to note that in incubations of hen oviduct mince with radioactive carbon dioxide the ^{14}C-labeled steam-volatile material upon counter-current distribution has revealed either exclusively acetic acid or exclusively the unknown material. Mixtures of the two have not been obtained. When no free acetic acid is found, it is apparent that a derivative must be formed since radioactivity is found in carbon atoms known to originate from acetic acid (*i.e.* γ-carboxyl of glutamic acid). In experiments run on different days, the same amount of radio-

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TABLE III

EFFECT OF CoA AND ARSENATE ON STEAM-VOLATILE FRACTION FROM $^{14}CO_2$ INCUBATIONS WITH HEN OVIDUCT

		1 Control	2 +CoA	3 +CoA and Arsenate
Hen oviduct 1				
mls. of 0.01 *N* steam-volatile acid obtained		3.6	4.6	4.9
Duclaux distribution of steam-volatile titratable acid	1	27	27	26
	2	56	55	56
	3	100	100	100
Total steam-volatile radioactivity		$4.66 \cdot 10^3$	$1.12 \cdot 10^3$	$1.60 \cdot 10^4$
Radioactivity relative to control		1	0.24	3.4
Radioactivity relative to "+CoA" flask		4.65	1	14.3
Duclaux distribution of steam-volatile radioactivity	1	78	62	72
	2	85	79	88
	3	100	100	100
Hen oviduct 2				
Total steam-volatile radioactivity		$2.1 \cdot 10^3$	$1.75 \cdot 10^3$	$1.67 \cdot 10^4$
Radioactivity relative to control		1	0.83	8.0
Duclaux distribution of steam-volatile radioactivity	1	67	28	28
	2	83	60	64
	3	100	100	100

Cell flasks contained $NaH^{14}CO_3$ (0.5 mc). The concentration of CoA was 100 units/ml and potassium arsenate was at 0.05 *M*.

activity has appeared in the steam-volatile fraction, one time completely in the form of acetic acid, the other in the form of the unknown (see Table III).

One scheme, the occurrence of which is well authenticated in higher tissues, results in the incorporation of $^{14}CO_2$ into the carboxyl group of acetic acid[14].

Senecioyl CoA $\longrightarrow$ β-hydroxyl*iso*valeryl CoA $\xrightarrow{CO_2}$ β-hydroxy β-methyl glutaryl CoA (HMG) $\longrightarrow$ acetoacetate and acetyl CoA.

The Duclaux constants of senecioic* acid were determined as 51, 83, 100 and hence were too low to correspond to the properties of the unknown studied here. β-hydroxy*iso*valeric acid is more hydrophilic than *iso*valeric acid and hence would have lower constants. HMG has two carboxyl groups and would therefore not be steam-volatile. HMG is structurally similar to citric acid and if it could form α-hydroxy β-methyl glutaric acid, analogous to *iso*citric acid formation from citrate, an equilibrium concentration of the monocarboxylic acid internal ester (lactone) would be expected. This compound might be steam-volatile. β-methyl glutamic acid was synthesized in conjunction with Dr. ALTON MEISTER[9]. This compound when tested with the hydroxamic acid reagents for esters[15] was completely negative. However, after treatment with nitrous acid under conditions for replacement of the amino group by a hydroxyl group, about 20% of the resulting material reacted

* The senecoic acid was kindly contributed by Dr. M. J. COON.

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positively. None of the hydroxamic acid-reactive material was steam-volatile, indicating that the lactone of α-hydroxy β-methyl glutaric acid is not steam-volatile.

Formation of acetoacetic acid from acetic acid would result in doubly labeled acetoacetic acid and might be expected to leave the acetic acid pool appreciably radioactive in the presence of the unknown. Neither of these conditions have been observed and so it would appear that formation of acetoacetic acid by this route is unlikely. Thus, if the unknown is an acetoacetyl derivative, it would precede acetic acid in any major metabolic pathway occurring in oviduct tissue.

The properties of the unknown material exclude it from being considered in the present scheme of BACHHAWAT, ROBINSON AND COON[14]. It is possible that the compound may mirror an aspect of the above scheme not yet elucidated, or may indicate another pathway for the incorporation of carbon dioxide into the carboxyl carbon of acetic acid.

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SUMMARY

1. An unknown radioactive material arises during the course of radioactive carbon dioxide fixation in the hen oviduct.

2. This unknown material is very steam-volatile, becomes distributed in the alcohol region of a *n*-amyl alcohol-water counter-current distribution, and contains its radioactivity in a very labile form so that $^{14}CO_2$ is liberated on heating.

3. The material appears to be interconvertible with carboxyl-labeled acetic acid.

4. Present evidence indicates that the material may be one or more acyl derivatives of a β-keto acid, possibly acetoacetic acid.

REFERENCES

1 R. W. HENDLER AND C. B. ANFINSEN, *J. Biol. Chem.*, 209 (1954) 55.
2 R. W. HENDLER, *Nature*, 178 (1956) 651.
3 R. W. HENDLER, *3ème Congres intern. biochimie, Bruxelles, 1955, Res. communs.*, p. 5–32.
4 R. W. HENDLER, *J. Biol. Chem.*, 223 (1956) 831.
5 R. MARKHAM, *Biochem. J.*, 36 (1942) 790.
6 A. KNETEMANN, *Rec. trav. chim.*, 47 (1928) 950.
7 L. C. CRAIG, *J. Biol. Chem.*, 155 (1944) 519.
8 T. E. FRIEDMANN AND G. E. HAUGEN, *J. Biol. Chem.*, 147 (1943) 415.
9 A. MEISTER, L. LEVINTOW, R. E. GREENFIELD AND P. A. ABENDSCHEIN, *J. Biol. Chem.*, 215 (1955) 441.
10 H. R. SNYDER, J. F. SHEKLETON AND C. D. LEWIS, *J. Am. Chem. Soc.*, 67 (1945) 310.
11 A. R. BADER, L. O. CUMMINGS AND H. A. VOGEL, *J. Am. Chem. Soc.*, 73 (1951) 4195.
12 B. E. GORDON, F. WOPAT, H. D. BURNHAM AND L. C. JONES, *Anal. Chem.*, 23 (1951) 1754.
13 R. B. SELIGMAN AND M. D. EDMONDS, *Chemistry and Industry*, 1406 (1955).
14 B. K. BACHHAWAT, W. G. ROBINSON AND M. J. COON, *J. Am. Chem. Soc.*, 76 (1954) 3098.
15 F. LIPMANN AND C. L. TUTTLE, *J. Biol. Chem.*, 159 (1945) 21.

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