synthetic 7-amino-1,2,3,9-tetramethoxydibenzo[a,c][1,3]-cycloheptadiene hydrochloride.

Racemic colchinol methyl ether was converted to the acetyl derivative in the same manner as used above for the synthetic amine. The **N-acetyl-*dl*-colchinol methyl ether** melted at 180–181° and this melting point was not depressed on mixing with 7-acetamino-1,2,3,9-tetramethoxydibenzo-[a,c][1,3]cycloheptadiene (m.p. 178–179°). It had no detectable rotation.

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[CONTRIBUTION FROM THE LANKENAU HOSPITAL RESEARCH INSTITUTE AND THE INSTITUTE FOR CANCER RESEARCH: AND THE DEPARTMENT OF CHEMISTRY, TEMPLE UNIVERSITY]

Incorporation of Glycine Carbon into Yeast Lipides[1]

BY SIDNEY WEINHOUSE, RUTH H. MILLINGTON AND MURRAY STRASSMAN

Fatty acids isolated from yeast grown in the presence of methylene-C^{14}-labeled glycine had a high content of C^{14}, distributed equally among all carbons of the chain. Fatty acids isolated from yeast grown in the presence of carboxyl-labeled glycine had negligible activity. These results suggest that fatty acids are formed from glycine through the steps: serine, pyruvate and acetate. Glycine α-carbon activity was found also in the unsaponifiable matter, and the methyl carbons of choline.

During the course of a study of glycine metabolism in yeast, it was found that when this organism was grown on glucose as the sole carbon source, together with a small quantity of glycine labeled in the methylene carbon with C^{14}, the radioactivity of the cell lipides was of such magnitude as to indicate a substantial incorporation of glycine carbon therein.[2] With carboxyl labeled glycine, however, the incorporation was so low that it could safely be concluded that the two glycine carbons follow different pathways in the conversion to lipides. The pertinent data are summarized in Table I.

TABLE I

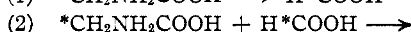
C^{14} ACTIVITIES OF LIPIDES FROM YEAST GROWN WITH METHYLENE- OR CARBOXYL-LABELED GLYCINE. VALUES ARE COUNTS/MINUTE/5 SQ. CM. DISH OF SUBSTANCES COUNTED AS SUCH

	Methylene-labeled			Carboxyl-labeled	
Expt. no.	1	2	3	1	2
Fatty acids	1650	1330	608[a]	12	5[2]
Unsaponifiable matter	2050	1190	1860	1	2
Phospholipide fatty acids	1980[b]		763	12	6
Trimethylamine chloroplatinate	1400		350[c]	..	..

[a] Three mM. of non-isotopic sodium acetate added in this experiment. [b] Phospholipides from 1 and 2 combined. [c] Hydrochloride.

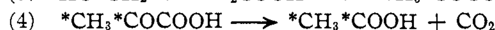
Inasmuch as acetate is already well-established as a precursor for the carbon skeleton of the fatty acids and at least part of the sterol molecule,[3] it seemed likely that the glycine methylene carbon is first incorporated into acetate. Altman[4] has found glycine methylene carbon in the bone marrow fats of rabbits and has suggested that acetate was the precursor; Sprinson[5] and Elwyn and Sprinson[6] have shown that both the glycine methylene carbon and serine β-carbon can be utilized for acetate synthesis in rats; and Barker *et al.*,[7] have demonstrated the conversion of glycine α-carbon to methyl and carboxyl of acetate in *Diplococcus glycinophylum*. On the basis of previous studies a reasonable reaction pathway for conversion of the methylene carbon of glycine to fatty acids can be formulated in the 5 equations

(1) $*CH_2NH_2COOH \longrightarrow H*COOH$



(2) $*CH_2NH_2COOH + H*COOH \longrightarrow HO*CH_2*CHNHC_2COOH$

(3) $HO*CH_2*CHNH_2COOH \longrightarrow *CH_3*COCOOH$



(4) $*CH_3*COCOOH \longrightarrow *CH_3*COOH + CO_2$

(5) $*CH_3*COOH \longrightarrow *CH_3*CH_2 \ldots\ldots *CH_2*COOH$

Reaction 1, the conversion of glycine methylene carbon to formate was noted by Sakami[8] and Siekevitz and Greenberg[9] in intact rats and in liver slices. The coupling of formate and glycine to yield serine, reaction 2, was also observed by the same authors.[9,10] The appearance of glycine carboxyl carbon in the serine carboxyl of *Torulopsis* yeast[11] can also be cited in favor in reaction 2. The conversion of serine to pyruvate has been demonstrated to occur in a variety of microorganisms and in cell-free extracts of animal liver.[12] Steps 4 and 5 require no comment since they have been well-established both in animals and yeast.[13–15]

Any fatty acids formed by this mechanism would be expected to be labeled in all positions of the chain, though not necessarily equally in all carbon atoms. Sakami[9] and Siekevitz and Greenberg[8] found that the serine formed by rats from α-labeled glycine had a preponderance of activity in

(1) Work done under contract with the U. S. Atomic Energy Commssion and aided by a grant from the American Cancer Society recommended by the Committee on Growth of the National Research Council. The labeled compounds used in this study were obtained from Tracerlab, Inc., on allocation by the U. S. Atomic Energy Commission. This work will be included as part of a thesis to be submitted by Murray Strassman to the Graduate School of Temple University in partial fulfillment of the requirements for the Ph.D. degree.

(2) The distribution of glycine carbon in other components of the yeast cell is being investigated and will be reported separately.

(3) Bloch, *Physiol. Rev.*, **27**, 574 (1947).

(4) Altman, *J. Biol. Chem.*, **177**, 985 (1949).

(5) Sprinson, *ibid.*, **178**, 529 (1949).

(6) Elwyn and Sprinson, *ibid.*, **184**, 465 (1950).

(7) Barker, Volcani and Cardon, *ibid.*, **173**, 803 (1948).

(8) Sakami, *ibid.*, **179**, 495 (1949).

(9) Siekevitz and Greenberg, *ibid.*, **180**, 845 (1949).

(10) Sakami, *ibid.*, **176**, 995 (1948).

(11) Ehrensvard, Sperber, Saluste, Reio and Stjernholm, *ibid.*, **169**, 759 (1947).

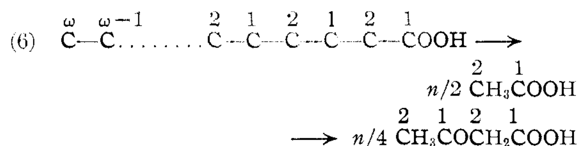
(12) Chargaff and Sprinson, *ibid.*, **151**, 273 (1943).

(13) Anker, *ibid.*, **176**, 1338 (1948).

(14) Weinhouse, Millington and Lewis, THIS JOURNAL, **70**, 3680 (1948).

(15) Pihl, Bloch and Anker, *J. Biol. Chem.*, **183**, 441 (1950).

the α-carbon, presumably because of dilution of the labeled formate arising from glycine by formate from other sources. This serine would yield acetate labeled preponderantly in the carboxyl group, and subsequently yield fatty acids labeled preponderantly in alternate carbons starting with the carboxyl. Sprinson,[5] however, found in rats that α-labeled glycine yielded acetylphenylaminobutyrate with essentially equal distribution of isotope in both acetate carbons. Such acetate would yield fatty acids labeled equally in all positions. Although no feasible method exists for determining the activity of each individual carbon atom of the fatty acid chain, a method was available for determining the activity of each of the 2 series of alternate carbon atoms. This method consists in the metabolic degradation of the fatty acids by rat liver slices to acetoacetic acid. It has previously been shown that acetoacetate arises by essentially random condensation of acetyl groups split from the fatty acid chain by successive β-oxidation.[16] Accordingly, as pictured in equation 6, the 1, 3, 5, ..., (ω-1) carbons of the fatty acid chain would appear in either the carbonyl or carboxyl positions of acetoacetate, and the 2, 4, 6, ..., ω carbons would appear in the α and γ positions.



To establish the distribution of C^{14} in the acetoacetate carbons it was degraded thermally to acetone and carbon dioxide and the former degraded further by alkaline hypoiodite to acetate and iodoform. The carbon dioxide represents the acetoacetate carboxyl (and β) carbons; whereas the iodoform represents the α- and γ-carbons.

As a test of this procedure it was carried out with fatty acids obtained from yeast grown in the presence, respectively, of carboxyl and methyl-labeled acetate. As shown in Table II, columns 3 and 4, the distribution of the labeled carbon was entirely as expected. With the fatty acids formed in the presence of carboxyl-labeled acetate more than 99% of the labeled carbon was in the carboxyl and β-carbon atoms of acetoacetate indicating essentially exclusive formation of fatty acids labeled in the 1, 3, 5, ..., (ω − 1) carbons. With fatty acids formed in the presence of methyl-labled acetate >95% of the label was in the α- and γ-acetoacetate carbons, indicating the formation of fatty acids labeled only in the 2, 4, 6, ..., ω positions. The ~5% of activity in the carboxyl and β-carbons was probably due to a randomization of the methyl carbon of acetate during its metabolic traverse through the citric acid cycle to yield some carboxyl-labeled acetate.

TABLE II

C^{14} DISTRIBUTION IN ACETOACETATE

Substance	Carbon of acetoacetate represented	Source of C^{14} activity: Acetate COOH	Acetate CH_3	Glycine $-CH_2-$
Original fatty acids		7870	5210	1980
Thermal CO_2 (as $BaCO_3$)	COOH, β	312	23	29
Iodoform (as $BaCO_3$)	α, γ	2	336	30[a]

[a] The acetic acid, arising along with iodoform in the degradation of the acetone, had an activity of 31 counts/minute (counted as $BaCO_3$), further verifying the equal distribution of C^{14} in all four carbons of the acetoacetate.

(16) Weinhouse, Medes and Floyd, *ibid.*, **155**, 143 (1944).

The reliability of the method having been established by these experiments with acetate, the fatty acids formed from methylene-labeled glycine were tested in similar fashion. As shown in column 5, Table II, the acetoacetate was labeled equally in all carbons. Because the CO_2 arising by thermal decarboxylation of acetoacetate is contaminated somewhat by extraneous carbon, it is probable that its activity is ~10% too low. Despite this slight difference, the essential equality of activity strongly suggests either that the methylene carbon of glycine is the only significant source of formate; or that conversion of the α-carbon of glycine to the β-carbon of serine does not involve its conversion to formate. There is the possibility, however, that a serine, labeled preponderantly in the α carbon, can give rise to pyruvate with an equal distribution of isotope in the α- and β-carbons if pyruvate is in equilibrium with a symmetrical 4-carbon acid.[10]

Evidence for Acetate and Formate Formation from Methylene Carbon of Glycine.—As further substantiation for the mechanism embodied in equations 1 to 5 it was possible to demonstrate the formation of labeled formate and acetate when yeast was grown in the presence of methylene-labeled glycine, together with a small quantity of normal sodium acetate to act as a "trap" for metabolic acetate. An aliquot of the yeast suspension containing the methylene-labeled glycine was removed after 24 hours of aeration and the volatile acids separately determined as described in the experimental section. The activities of both formate and acetate were high as shown in Table III. At the close of the 3-day growth period, the volatile acids were isolated by steam-distillation of the acidified medium. Again the activities in formate and acetate were high, though considerably lower than the corresponding values after one day. It is of interest in this experiment that the activity of the fatty acids falls between the values for acetate on the first and third days of growth. This is the result which could be expected if the precursor of the fatty acids was acetate which is constantly being diluted throughout the course of the growth period by normal metabolic acetate from glucose. In contrast with these results the formate and acetate isolated from the growth medium in a similar experiment with carboxyl-labeled glycine had no appreciable activity, ~5 counts per minute.

TABLE III

ACTIVITY OF ACIDS RECOVERED FROM GROWTH MEDIUM

Source of activity	Volatile acids[a] Formate[b] 1 day	Formate[b] 3 days	Acetate[c] 1 day	Acetate[c] 3 days	Fatty acids 3 days
Methylene-labeled glycine	2000	828	1860	109	608
Carboxyl-labeled glycine	..	5	..	5	12

[a] Values corrected for carrier added during isolation. [b] Counted as $BaCO_3$. [c] Counted as Na acetate.

Unsaponifiable Matter.—As expected the unsaponifiable matter isolated from yeast grown with methylene-labeled glycine had activity of the same order of magnitude as the fatty acids. In growth experiments carried out in the presence of carboxyl- or methyl-labeled acetate relatively large amounts of ergosterol were formed and no difficulty was experienced in isolating pure radioactive ergosterol by the carrier technique. It would have been of interest to isolate this sterol from yeast grown with α-labeled glycine, but strangely in none of 7 experiments in which yeast was grown in the presence either of carboxyl- or methylene-labeled glycine was there any ergosterol formed. Its absence was verified both by ultraviolet absorption analysis and by absence of radioactivity of isolated carrier ergosterol. The source of the radioactivity in the unsaponifiable matter remains for further investigation.

Phospholipides.—In addition to the activity in the phospholipide fatty acids, which was of similar magnitude to the non-phospholipide fatty acids, considerable activity was found in the methyl groups of choline, as shown by the activity of the trimethylamine obtained on degradation of the choline by the method of du Vigneaud, *et al.*[17] (Table I).[18] This observation extends and substantiates a number of recent reports that formate and glycine are precursors, in the rat, of methyl groups of methionine and choline.[19]

Experimental

Growth of Yeast.—The yeast used in these experiments was a strain of *Torulopsis utilis* No. 3015 kindly supplied by Dr. C. N. Frey of the Fleischmann Laboratories. It was subcultured on malt extract agar and the washings from 4 to 6 slants were used for inoculation in 500 ml. of the medium of Pavcek, Peterson and Elvehjem[20] containing 3 millimoles of glycine with a total radioactivity of 10 microcuries corresponding to 85,000 counts per minute per dish (as glycine). The solution was aerated by means of a sintered glass disperser for approximately 48 hours at room temperature (20–26°) at which time about 4 to 6 g. dry weight of yeast had accumulated. The yeast was harvested by centrifugation, washed repeatedly with water, and extracted successively for 6–10 hours with alcohol and ether. The total lipide thus obtained was highly variable in quantity, but ranged in most experiments between 200 and 400 mg.

Phospholipides.—The combined extracts were evaporated to dryness, taken up in about 2 ml. of water and extracted 4 times with 10-ml. portions of petroleum ether. After drying and weighing they were again dissolved in 1 or 2 ml. of petroleum ether and the phospholipides precipitated by addition of 10 volumes of acetone. These were centrifuged off, rinsed with acetone and dried. They were then hydrolyzed by refluxing approximately 18 hours with 10 ml. of 10% HCl. The fatty acids were extracted with petroleum ether and the aqueous fraction dried *in vacuo*. The dry material was taken up with 2 ml. of ethanol, insoluble matter was centrifuged off, and the solution treated with 2 ml. of a saturated solution of mercuric chloride in alcohol. After standing in the refrigerator overnight the choline–$HgCl_2$ complex was centrifuged, washed with ether, and was then degraded, without further purification, by the method of du Vigneaud, *et al.*[15] The trimethylamine was trapped in a bead tower with dilute HCl, and the hydrochloride isolated by evaporation to dryness. After counting, this was identified by conversion to the chloroplatinate; 19.2 mg. yielded 7.1 mg. Pt; calcd. for $((CH_3)_3N)_2H_2PtCl_6$, 7.1 mg.

Non-phospholipide Fractions.—The acetone-soluble lipides were saponified by 4–6 hours reflux with 2 *M* KOH. The alkaline solution was diluted by an equal quantity of water and the unsaponifiable matter extracted with petroleum ether, after which the aqueous solution was acidified and the fatty acids extracted with ether.

Biological Breakdown of Fatty Acids.—The procedures were essentially the same as described previously.[16,21] The fatty acids, neutralized with NaOH, were used in a final concentration of 0.001 *M*. Incubations were carried out in large Warburg flasks; in each experiment we used about 8 g. of liver slices in 40 ml. of medium and flasks were shaken 2 hours in oxygen at 38°. The yield of ketone bodies ranged between 0.1 and 0.2 millimole.

Acetoacetate Degradation.—After thermal decarboxylation as described previously,[16] the acetone–mercury complex was dissolved in dilute HCl and distilled into an excess of 0.1 *M* solution of iodine in 5 *M* NaOH. The iodoform was centrifuged off, washed thoroughly with water and oxidized directly to carbon dioxide by means of chromic–sulfuric acids. The carbon dioxide, caught in carbon dioxide-free alkali in a bead tower, was precipitated with $BaCl_2$ and counted as $BaCO_3$. The solution remaining after centrifugation of the iodoform was acidified and steam distilled in the presence of silver sulfate. The distillate was neutralized, evaporated to a small volume and acidified and redistilled. The distillate, consisting of acetic acid, was again neutralized, evaporated to a small volume and oxidized to carbon dioxide with persulfate.

Isolation of Formate and Acetate.—For this purpose an experiment was set up exactly as described above except that 3 mM. of normal, non-isotopic sodium acetate was added initially. After 24 hours of aeration a 50-ml. aliquot was removed and distilled with steam, yielding 0.13 mM. of volatile acid (0.02 mM. formic and 0.11 acetic as determined by Duclaux constants). This was diluted with 0.50 mM. of acetic and 0.45 mM. of formic, and the formate oxidized to carbon dioxide.[18] The residual solution, which gave Duclaux constants characteristic of pure acetic acid, was neutralized, evaporated to dryness and counted. At the close of this experiment (3 days), the same procedure was followed on the yeast centrifugate, yielding 0.73 mM. of total volatile acid consisting of 0.33 mM. formic and 0.40 mM. of acetic. In a similar experiment with carboxyl-labeled glycine the yeast centrifugate after 3 days yielded 4.2 mM. of volatile acids containing 1.08 mM. formic and 3.1 mM. acetic. Both had negligible activity.

Counting Procedures.—All samples were counted in 5 sq. cm. dishes under a thin mica window counter and radioactivities are expressed as counts per minute per dish at "infinite thickness." When "infinitely thick" samples were not available corrections were made from standard curves.

PHILADELPHIA, PENNA. RECEIVED JULY 28, 1950

(17) du Vigneaud, Cohn, Chandler, Schenck and Simmonds, *J. Biol. Chem.*, **140**, 625 (1941).

(18) The large difference in activity between the trimethylamine carbon recovered from experiments 1 and 2 and that of experiment 3 cannot be accounted for. It cannot be due to dilution by the added acetate in this experiment since trimethylamine isolated from choline formed in the presence of methyl-labeled acetate was found to have a very low activity (40 counts/minute as the hydrochloride).

(19) The utilization of formate carbon as well as the α-carbon of glycine for the synthesis of methyl groups in animals has been reported recently by Welch and Sakami, *Federation Proc.*, **9**, 245 (1950), du Vigneaud, Verly and Wilson, THIS JOURNAL, **72**, 2819 (1950), Johnson and Mosher, *ibid.*, **72**, 3316 (1950), Weissbach, Elwyn and Sprinson, *ibid.*, **72**, 3317 (1950), and Stekol and co-workers (private communication).

(20) Pavcek, Peterson and Elvehjem, *Ind. Eng. Chem.*, **29**, 536 (1937).

(21) Weinhouse and Millington, *J. Biol. Chem.*, **181**, 645 (1949).