On the Role Played by Formate in Serine Biosynthesis

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Evidence for the conversion of glycine to the α- and carboxyl carbons of serine in rat-liver homogenates has been obtained by Winnick, Moring-Claesson & Greenberg (1948) and evidence for the existence of the same reaction in live rats has been given by Sakami. Sakami also found that formate, which had been administered to the rats along with glycine, served as a source for the β-carbon of serine; the possibility, that this incorporation was brought about via carbon dioxide, was excluded.

Sakami suggested that the process was the reverse of that postulated by Shemin (1946) for the conversion of serine to glycine

$$(HCOOH + CH_2NH_2COOH \rightarrow CH_2OHCHNH_2COOH).$$

The simultaneous incorporation of the two substances does not necessarily imply, however, that the incorporations are due to a condensation between glycine and formate or even between some derivatives of these. The carbons from the two substances might be incorporated by entirely independent processes. Sakami (1949*a*,*b*) reported the conversion, *in vivo*, of labile methyl groups and of the α-carbon of glycine to the β-carbon of serine. This latter conversion was also observed *in vitro* by Siekevitz & Greenberg (1949); they also obtained results with rat-liver slices which made them suggest that the transformation of the α-carbon of glycine into the β-carbon of serine proceeded via formate. These findings, together with the demonstration of an incorporation of formate-carbon into the 2- and 8-positions of uric acid (Sonne, Buchanan & Delluva, 1948), raise the question of whether formate is normally produced in intermediary metabolism. In particular, it might be asked whether the α-carbon of glycine and the carbon of labile methyl groups enter the β-position of serine via formate and whether formate should be considered the only immediate precursor of the β-carbon of serine. Further, it might be considered a possibility that the oxidation of formate in the animal body was brought about through incorporation into serine.

As an approach to these problems, the *in vitro* incorporation of formate carbon into serine has been studied. Slices and cell-free homogenates of rat liver, known to form serine from glycine, were used. The incorporation of formate carbon into the free serine of these preparations was measured following incubation with ^{14}C-labelled formate. In parallel experiments formation of serine from glycine was checked by incubation with carboxyl-^{14}C-labelled glycine. It was found that glycine and formate are both incorporated into serine by liver slices, but in homogenates glycine but not formate is incorporated; it appears, therefore, that formate is not the only or is not the immediate precursor of the β-carbon of serine. Related results were very recently reported by Siekevitz & Greenberg (1950). Ethionine was found to inhibit the formation *in vitro*, presumably in rat-liver slices, of formate from the α-carbon of glycine, while leaving the incorporation of glycine into serine unaffected.

Since it was further found that formate is oxidized at an appreciable rate in rat-liver homogenates, the conclusion can also be drawn that an incorporation of formate into serine is not a necessary step in its oxidation in rat liver. The oxidation of formate in preparations of rat-liver homogenates has been further studied in an attempt to characterize the enzyme systems responsible for this process.

EXPERIMENTAL

Isotope preparations

Sodium formate containing ^{14}C (giving 70,000 counts/min./μmol. under the prevailing counting conditions) was synthesized by the procedure of Melville, Rachele & Keller (1947). The formic acid content as determined by the microcolorimetric method of Grant (1948) accounted for 97% of the steam-distillable acids.

Carboxyl-^{14}C-labelled glycine (58,000 counts/min./μmol.) was prepared from acetate (Olsen, Hemingway & Nier, 1943). After four recrystallizations the product had a constant melting point (254–255° compared with an authentic sample which had m.p. 255–256°); a paper chromatogram contained practically all the activity within the glycine spot.

Tissue preparation and incubation

Homogenates of rat liver were prepared in the cold from 1 part of liver and 2·5 parts of medium and freed from unbroken cells by low-speed centrifugation (about 200 *g* for 5 min.). The media employed were: (1) the standard medium (*SM*) of Winnick *et al.* (1948) and (2) a bicarbonate-free medium (*BFM*) of the following percentage composition: KCl, 0·65; NaCl, 0·25; $MgSO_4.7H_2O$, 0·06; K_2HPO_4, 0·18; glucose, 0·10; sodium citrate, 0·03. The pH was adjusted to 7·3 and 100 ml. mixed with 2 ml. 1% $CaCl_2$ just before use. Rat-liver slices were cut free-hand in the cold.

Incubations were carried out by shaking in flasks at 38° in an atmosphere of 5% CO_2–95% O_2, when *SM* was used, and in 100% O_2, when *BFM* was employed. Usually 2–3 ml. homogenate were used, and there was no dilution other than by a small volume of the solution of radioactive compound. In the case of slices an amount representing 65–85 mg. dry wt. was suspended in 5 ml. of *SM*. In both cases nearly equimolar amounts of labelled formate or glycine, namely 2·3 μmol. $H^{14}COONa$ (~ 160,000 counts/min.) or 2·93 μmol. $CH_2NH_2{}^{14}COONa$ (~ 171,000 counts/min.), each contained in 0·2 ml. solution, were added to the flasks just before the incubation; 2·93 μmol. non-radioactive glycine were added together with the $H^{14}COONa$ and 2·3 μmol. non-radioactive formate together with the $CH_2NH_2{}^{14}COONa$. In some of the later experiments formate and glycine, both labelled with ^{14}C, were added to the same flask, as earlier experience had shown the activity in serine to be located exclusively in the β-carbon after incubation with $H^{14}COONa$ and in the carboxyl group after incubation with $CH_2NH_2{}^{14}COONa$. In most cases 1 mg. DL-serine was added to each flask before the start of the incubation to trap the serine synthesized.

Isolation of free serine

At the end of the incubation DL-serine (250 mg.) was added as a carrier to the homogenates and to suspensions of liver slices. The proteins were removed from the whole homogenates and from the tissue slice medium (plus wash water of slices) by heat coagulation at pH 5 and filtration. The filtrates were treated with norite and subsequently evaporated to about 2·5 ml. Ethanol (2 vol) was added to produce crystallization in the cold. After six or seven similar recrystallizations and repeated removal by centrifugation of substances insoluble in hot water, a product was obtained which had m.p. 209–211° almost identical with that of authentic DL-serine (210–211°), and contained practically all the ^{14}C activity in the serine-glycine spot of a paper chromatogram.

Separate experiments, in which 3 μmol. $CH_2NH_2{}^{14}COONa$ and 2 mmol. DL-serine were dissolved in water or in deproteinized solution from homogenates, showed that about 2% of the glycine activity was not removed from serine by the crystallization procedure mentioned above. However, this incomplete separation does not invalidate the results since less than 0·05% of the activity appeared in the CO_2 (representing the carboxyl group of serine) when this serine was degraded by periodate oxidation.

Degradation of serine and counting

The periodate method devised by Sakami (1948) was employed by which each of the three carbons of serine are obtained separately as CO_2. The CO_2 was precipitated as $BaCO_3$ (10–20 mg.) and plated on filter-paper disks. All counts were corrected to zero layer thickness. At least 2000 counts were recorded. Counts/min. lower than 2 above background were considered insignificant; in such cases the incorporation is stated as zero.

Determination of formate oxidation in homogenates

To determine the extent of the formate conversion to CO_2 in homogenates, CO_2 formed in some of the above experiments with formate was absorbed in KOH in the centre cup of the Warburg vessels used for incubation. However, for the closer study of this process in homogenates and preparations of these, another technique was adopted, mainly to allow constant cyanide concentrations to be maintained during incubation. Filtering flasks (50 ml. capacity) were used. The side tube was provided with a rubber cap and the mouth with a two-hole rubber stopper with inlet and outlet glass tubes which were connected by rubber tubes to a compressed gas source and a CO_2 absorber respectively.

The homogenate preparation was incubated in the flasks which were continuously shaken in a Warburg bath. When the effect of cyanide was tested the rubber tubes were kept clamped during incubation; otherwise a steady stream of the appropriate gas was directed through the flasks and absorbers during incubation. The rubber caps served for injections of $H^{14}COONa$ at the beginning of the incubation period and of H_2SO_4 at the end. To measure the total ^{14}C activity of the CO_2 absorbed, the KOH solution of the CO_2 absorber was subsequently diluted with a suitable Na_2CO_3 solution and portions of the mixture used for $BaCO_3$ plating. Bicarbonate-free medium was used in these experiments.

RESULTS

The percentages of the total activity, added as $CH_2NH_2{}^{14}COOH$ or $H^{14}COOH$, which could be recovered in the free serine at the end of the incubation periods are shown in Tables 1 and 2. All results entered under a particular experimental number were obtained with the same liver tissue.

As can be seen from Table 1 a considerable conversion of glycine as well as formate to serine takes place in liver slices, even though formate is incorporated to a lesser extent than glycine.

In the case of homogenates the situation is different (Table 2). There the incorporation of glycine/mg. tissue used in the preparation is of the same order of magnitude as in slices, but no incorporation at all could be demonstrated for formate, although formate of high activity was used.

In some of the above experiments with $H^{14}COONa$ in homogenates a considerable fraction of the activity was recovered in the respiratory carbon dioxide (cf. Table 2, Exps. 7 and 8).

Some of the results of separate experiments in which the formate-carbon dioxide conversion was studied under varying conditions appear in Tables 3, 4 and 5. They indicate that: (1) The enzyme system involved could not be sedimented by 2 × 30 min. centrifugation at about 20,000 *g* (Table 5); it was further found that a suspension of washed mitochondria showed little activity. (2) The enzyme activity is abolished by anaerobic conditions (Table 3). (3) The enzymic activity could be reduced by ageing and dialysis, and restored to its previous level by addition of coenzyme I (Co I) (Table 5). (4) The enzymic activity was inhibited by cyanide (Table 4); it was further found that this inhibition could not be overcome by methylene blue in molar concentrations up to seven times that of cyanide.

Table 1. *Incorporation of* $CH_2NH_2{}^{14}COOH$ *and* $H^{14}COOH$ *into serine in rat-liver slices*

(In all cases the slices were suspended in 5 ml. *SM*.)

Exp. no.	Incubation time (min.)	Labelled substances: Glycine* (μmol.)	Labelled substances: Formate* (μmol.)	DL-Serine added before incubation (μmol.)	Percentage of added activity found in serine: Carboxyl carbon	Percentage of added activity found in serine: β-Carbon
2	75	2·93	—	0	6·3	—
	75	—	2·3	0	—	1·16
3	90	2·93	—	10	9·6	—
	90	—	2·3	10	—	1·19
4* *a*	90	2·93	2·3	10	11·0	1·57
b	90	2·93	2·3	10	10·3	1·73

* In Exp. 4*b* 200 μg. aminopterin was added before the incubation, while no such addition was made in 4*a*. As seen from the figures this does not influence the glycine or formate incorporation.

Table 2. *Incorporation of* $CH_2NH_2{}^{14}COOH$ *and* $H^{14}COOH$ *into serine in cell-free homogenates of rat liver*

Exp. no.	Medium used	Homogenate used (ml.)	Incubation time (min.)	Labelled substances: Glycine (μmol.)	Labelled substances: Formate (μmol.)	DL-Serine added before incubation (μmol.)	Percentage of added activity found in serine: Carboxyl carbon	Percentage of added activity found in serine: β-Carbon	Percentage of added activity found in respiratory CO_2
1	*SM*	1·8	75	2·93	—	0	3·33	—	—
		1·8	75	—	2·3	0	—	0	—
6	*SM*	3·0	90	2·93	—	10	7·0	—	—
		3·0	90	—	2·3	10	—	0·1	—
7	*BFM*	2·5	120	2·93	—	10	7·8	—	—
		2·5	120	—	2·3	10	—	0	87
8	*BFM*	2·5	120	—	4·6	0	—	0	88
		2·5	120	—	4·6	150	—	0	85
		2·5	120	2·93	—	150	7·6	—	—

SM = Standard medium. *BFM* = Bicarbonate-free medium.

Table 3. *Influence of aerobic and anaerobic conditions on the conversion of formate to* CO_2 *in rat-liver homogenate*

(3 ml. samples of a final supernatant, obtained by three successive centrifugations (10 min. each at about 5000 *g*) of a homogenate (1 part of liver + 2 parts of medium), were incubated at 38° for 60 min. with 0·2 ml. solution containing 7 μmol. $H^{14}COONa$ with 16,000 counts/min.)

	Incubation atmosphere	Percentage of added activity recovered as $^{14}CO_2$
1	Oxygen	26
2	Nitrogen	Less than 0·2

Table 4. *Inhibition by cyanide of the conversion of formate to* CO_2 *in rat-liver homogenate*

(To 3 ml. samples of a supernatant, obtained by 15 min. centrifugation at 200 *g* of a homogenate (1 part of liver + 2 parts of medium) KCN was added to the final concentrations indicated. Incubated at 38° for 60 min. with 0·4 ml. solution containing 5 μmol. $H^{14}COONa$ with 25,600 counts/min. Incubation atmosphere: atmospheric air.)

Final KCN concentration (M)	0	3×10^{-3}	10^{-3}	3×10^{-4}	10^{-4}	3×10^{-5}
Percentage of added activity recovered as $^{14}CO_2$	58·5	2·9	17	48	56·5	58·5
Inhibition (percentage) of conversion	—	95	71	18	3	0

Table 5. *Influence of ageing, dialysis and* Co I *on the conversion of formate to* CO_2 *in a preparation of rat-liver homogenate*

(A supernatant was prepared from homogenate (1 part of liver + 3 parts of medium) by preliminary centrifugation for 15 min. at 5000 *g*, followed by two successive centrifugations, each for 30 min. at 20,000 *g*. The resulting final supernatant appeared practically particle-free on microscopy.

Part of this supernatant (*A*) was immediately used for an experiment, while another part (*B*) was kept for 6 hr. at 4° and a third part (*C*) dialysed against medium for 6 hr. at 4°. In all experiments 0·2 ml. of a solution containing 5 μmol. $H^{14}COONa$ with 25,600 counts/min. was added to each flask. Incubation: 60 min. at 38° in atmospheric air.)

	1	2	3	4	5
Supernatant and medium (*M*) used (ml.)	1·5 *A* + 1·5 *M*	1·5 *B* + 1·5 *M*	1·5 *C* + 1·5 *M*	1·5 *C* + 1·5 boiled *B*	1·5 *C* + 1·5 *M*
Further additions	0·2 ml. water	0·2 ml. water	0·2 ml. water	0·2 ml. water	2 mg. Co in 0·2 ml. water
Percentage of added activity recovered as $^{14}CO_2$	24·2	7·1	2·0	7·7	25·3

DISCUSSION

The fact that none of the activity added to a homogenate as labelled formate is incorporated into serine, while, as indicated by the simultaneous glycine incorporation, new formation of serine takes place at an appreciable rate, seems inconsistent with the idea of formate as the only immediate precursor of the β-carbon of serine. If formate really held this position such results might be obtained: (*a*) if the turnover of free formate was so extremely fast that its specific activity approached zero very soon after the beginning of the incubation, or (*b*) if the specific activity of the residual formate had approached zero towards the end of the incubation period and if, in addition, the turnover in the β-carbon of the free serine was very fast so that its activity would only mirror the activity in formate at the end of the incubation.

The following experimental observations, however, exclude these possibilities. Formate incorporation was lacking in cases where considerable formate activity was left in the homogenate at the end of the incubation, and its specific activity not demonstrably decreased (samples of a vacuum distillate, prepared according to Grant (1948), were examined for total formate activity by mercuric chloride degradation after addition of carrier formate and for total formate content, by Grant's colorimetric method). In Exp. 6 of Table 2, for example, 15% of the activity added could be recovered as formate. If (*b*) took place, the use of an increased serine pool during the incubation should lead to greater trapping of formate activity in serine. This, however, did not occur, as is shown by the result of Exp. 8, Table 2, in which the addition of 15 mg. DL-serine before incubation caused no formate incorporation although the addition of serine (as indicated by the glycine incorporation) caused no decrease in the new formation of serine. Thus it appears that formate as such cannot be the only precursor of the β-carbon of serine.

It should be pointed out that several explanations could account for the fact that formate is not incorporated into serine, while glycine is. Thus if glycine and formate were incorporated by entirely independent processes, the one responsible for formate incorporation could be blocked in the homogenates. On the other hand, serine formation might actually be brought about by a coupling of glycine and a formate derivative. In this case such conversions of glycine which possibly were necessary before its coupling with a formate derivative would be produced in the homogenate, but the transformation of formate into its appropriate derivative would not be effected. Other carbons, possibly including the β-carbons from serine undergoing decomposition, would then serve as sources for the β-carbon of newly formed serine in homogenates. It is possible that formate is first reduced to formaldehyde and that this in turn combines with the glycine; and the enzyme system for conversion of formate to formaldehyde might be destroyed in homogenates.

In the case of the non-homogeneous system of slices suspended in medium there are of course several possible explanations why formate is incorporated to a lesser extent than glycine. This finding could however be satisfactorily explained by assuming, as suggested by the above discussion, that formate has to be converted into a derivative, also formed from other sources, before it can be incorporated into serine.

The occurrence in homogenates of serine synthesis without formate incorporation suggests the use of homogenates in testing whether other substances which are known to act as β-carbon sources *in vivo*, do so through the formation of formate or not. A few experiments were carried out with methyl-labelled choline instead of formate, but since in homogenates

only a questionably significant activity was found in the β-carbon of serine (while glycine was incorporated to a great extent), these experiments do not allow us to decide whether labile methyl groups may be transformed into the β-carbon by another route than via formate.

The oxidation of formate in homogenates and the simultaneous absence of formate incorporation into serine indicate that such an incorporation cannot be a necessary step in the oxidation of formate in the rat liver.

From the results obtained in the closer study of formate conversion to carbon dioxide in homogenate preparations (Tables 3–5) it seems likely that formate decomposition is caused by a non-particle-bound dehydrogenase, requiring Co I as a coenzyme and further dependent on the presence of a cyanide-sensitive electron mediator. Formate oxidation was also demonstrated in homogenates of rabbit liver and kidney cortex.

SUMMARY

1. In rat-liver slices formate carbon and the carbon of the labile methyl groups of choline are incorporated in the β-carbon of free serine.

2. Such incorporation could not be demonstrated in rat-liver homogenates even though the simultaneous incorporation of glycine indicated that new formation of serine took place.

3. Formate is oxidized to carbon dioxide in rat-liver homogenate; the enzyme system responsible for this process has been studied.

4. Based on these findings the conclusions are drawn that (*a*) formate is not the only (or is not the) immediate precursor of the β-carbon of serine, (*b*) the oxidation of formate in rat liver does not involve an incorporation into serine.

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The Vitamin B_1 Sparing Action of Fat and Protein

4. THE EFFECT OF CARBOHYDRATE IN DIETS DEFICIENT IN VITAMIN B_1 UPON THE SURVIVAL AND VITAMIN B_1 CONTENT OF THE RAT

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Previous papers in this series (Banerji, 1940, 1941) have shown that rats, given no vitamin B_1, can thrive provided that the diet is free from sucrose. Subsequently it was demonstrated that on diets devoid of the vitamin, irrespective of the presence or absence of sucrose and so irrespective of the development or otherwise of signs of deficiency, kidney slices of rats show a specific metabolic defect, defective oxidation of pyruvate *in vitro*, which is restored when the vitamin is added to the respiring slices (Banerji & Yudkin, 1942). From these results, the conclusion was drawn that signs of deficiency are not due to the inability *per se* to carry out an essential metabolic function—the proper metabolism of carbohydrate—but to an attempt to metabolize dietary carbohydrate in the absence of vitamin B_1. In other words, the animal can grow and thrive in the absence of vitamin B_1 provided it does not have to metabolize dietary carbohydrate. The signs of deficiency appearing when carbohydrate is fed would, on this hypothesis, be due to toxic products of incomplete or perverted carbohydrate metabolism.

Some of the consequences of this hypothesis have been examined in the work to be described. First, it was of interest to see whether and to what extent variation in the amount of dietary carbohydrate affected the rate of development of signs of deficiency. Especially it was interesting to see whether animals