Table II
Formation of methionine from methyl tetrahydrofolate and L-adenosylhomocysteine

The complete incubation mixture contained methyl tetrahydrofolate, 3.0 μM; Mg, 5.0 μM; TPNH, 1.0 μM; ATP, 5.0 μM; L-α-acetylaminoacidomethionine, 5.0 μM; Tris buffer, pH 7.5, 100 μM. Total volume: 2 ml containing 35 mg of protein. L-Adenosylhomocysteine, 5.0 μmoles, was added as indicated. Incubation time, 2 hours.

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Methionine formed μmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete (boiled enzyme)</td>
<td>0.02</td>
</tr>
<tr>
<td>Complete</td>
<td>0.63</td>
</tr>
<tr>
<td>Acetylhomocysteine replaced by adenosylhomocysteine</td>
<td>0.24</td>
</tr>
<tr>
<td>Acetylhomocysteine replaced by adenosylhomocysteine. ATP omitted</td>
<td>0.04</td>
</tr>
<tr>
<td>ATP and acetylhomocysteine omitted</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Table III
Formation of methionine from purified methyl tetrahydrofolate

The complete incubation mixture contained ATP, 5.0 μM; Mg, 5.0 μM; L-α-acetylaminoacidomethionine, 5.0 μM; purified methyl tetrahydrofolate, 0.43 μM; TPNH, 1.0 μM; Tris buffer, pH 7.5, 100 μM. Total volume: 2 ml containing 35 mg of protein. Incubation time, 2 hours.

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Methionine formed μmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete (boiled enzyme)</td>
<td>0.02</td>
</tr>
<tr>
<td>Complete</td>
<td>0.46</td>
</tr>
<tr>
<td>TPNH omitted</td>
<td>0.35</td>
</tr>
<tr>
<td>Methyl tetrahydrofolate omitted</td>
<td>0.07</td>
</tr>
</tbody>
</table>

300 and 340 ml and comprised 30% of the nonvolatile radioactive material. The solution was completely colorless. Less than 3% of the C¹⁴ of the fraction was converted to formaldehyde C¹⁴ with 0.4 m HCl under the conditions described above.

As shown in Table III purified methyl tetrahydrofolate was efficiently converted to methionine in the pig liver enzyme system. When the gradient elution was carried out with water instead of the 0.13 m Tris buffer, two radioactive fractions were obtained before the active component was collected. These were eluted largely between 80 and 250 ml, and comprised 30% of the nonvolatile radioactivity of the fraction. The active fraction was obtained between 610 and 650 ml under these conditions. The first and second fractions accounted for ~10% and ~50% of the nonvolatile material. Neither of these yielded methionine when incubated with the methionine-synthesizing system without TPNH.

The chemical identity of the biologically active methyl tetrahydrofolate has not yet been unequivocally established. It is not 10-methyl tetrahydrofolate. The latter compound has been synthesized from 10-methyl folic acid essentially by the procedure for the reduction of folic acid described by Hafez et al. (5) and did not yield methionine under the conditions of our experiment. It is considered that the active methyl tetrahydrofolate fraction consists of a mixture of the isomers of 5-methyl-L-tetrahydrofolate. If 5-methyl tetrahydrofolate is an intermediate of methionine methyl formation, the ATP required by this process could be involved in the conversion of the nitrogen atom in position 5 to a quaternary amine by the addition of an adenosyl group. This would labilize the 5-methyl in a manner similar to the activation of methionine for transmethylation.

Evidence supporting the biological formation of a methyl tetrahydrofolate has been obtained in this laboratory. A radio-active compound has been isolated from a system containing the pig liver enzyme, folic acid-C¹⁴, TPNH, Mg, and tetrahydrofolate by chromatography on Amberlite CG-45. The yield was decreased by the exclusion of acetylhomocysteine in the incubation mixture and by the omission of TPNH or tetrahydrofolate. This substance does not yield formaldehyde on acid treatment and, therefore, is not a formaldehyde-tetrahydrofolate addition compound. Wilmanns et al. have mentioned the accumulation of a similar compound in an enzyme system from pig liver (4). Also, the isolation of a methyl compound from incubation mixtures containing an enzyme system from Escherichia coli has been briefly reported by Larrabee and Buchanan (6). The properties of the bacterial compound suggest that in the experiments of Larrabee and Buchanan 5-methyltetrahydrofolate accumulated during incubation and was oxidized in the process of isolation. This would account for the observation that DPNH was required both for the formation of the intermediate from formaldehyde and tetrahydrofolate, and also in the transfer of the methyl group of the compound to homocysteine after isolation.

Our results differ from those of Wilmanns et al. (4) who report that neither ATP nor TPNH is required for methionine biosynthesis from formaldehyde or their methylated derivative of folic acid and adenosylhomocysteine. This apparent disagreement is being investigated.

REFERENCES

The Disulfide of L-Cysteine and L-Homocysteine in Urine of Patients with Cystinuria*

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Cystinuria is associated with increased excretion of cystine (1), lysine, arginine (2), and ornithine, the last identified by

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† Senior Research Fellow, New York Heart Association.
by the following procedures. Aliquots of urine (500 ml) from Stein (3). With the use of ion exchange resin columns, he also moved urea and much pigment. The amino acids were eluted cystine, and unlike the blue of isoleucine.

H+ and washed through with 5 liters of water. This re-
tus continually records the reaction of the effluent with ninhydrin
at both 570 and 440 nm, it was possible to distinguish a peak
in these determinations.

amounts of the compound sufficient for analysis were obtained
by the following procedures. Aliquots of urine (500 ml) from
the patients were passed into a 4 x 30 cm column of Dowex 50-
H+ and washed through with 5 liters of water. This re-
sults in the ion exchange chromatogram.

Urine from ten patients with cystinuria was analyzed with
the automatic recording apparatus of Spackman, Stein, and Moore
with 30-50° operation as recommended for physiological fluids
(4). A peak invariably appeared between the locations of leucine
and isoleucine. Separation from isoleucine is perhaps due to the
newer method, which employs 150-cm columns, whereas
Stein previously used 100-cm columns (3). Because the appar-
atus continually records the reaction of the effluent with ninhydrin
at both 570 and 440 nm, it was possible to distinguish a peak
that gave a reddish color with ninhydrin, similar to that of
cystine, and unlike the blue of isoleucine.

Amounts of the compound sufficient for analysis were obtained
by the following procedures. Aliquots of urine (500 ml) from
the patients were passed into a 4 x 30 cm column of Dowex 50-
H+ and washed through with 5 liters of water. This removed
urea and much pigment. The amino acids were eluted with 6 n HCl at 4° and the HCl was removed on a rotary evaporator.

The resulting mass was dissolved and passed into a 1.8 x 165 cm column of Amberlite IR-120 equilibrated with 0.2 N sodium citrate buffer, pH 3.25 (4). Elution was begun with 0.2 N sodium citrate buffer, pH 4.25 (4) at 120 ml per hour, and 5-ml fractions were collected. Fractions containing the unknown compound were pooled and further purified on a narrow-walled column, 0.9 x 150 cm. Fractions containing the compound were pooled and evaporated. The material was dissolved in water, adjusted to pH 5, and permitted to stand at 4°, whereupon a white precipitate formed. This was filtered off, dissolved in 0.5 N acetic acid, and passed through Dowex 1-acetate to remove residual HCl and resins dissolved from the preparatory work (5). The acetic acid was removed by lyophilization and the powder was recrystallized from water.1

1 An abstract in which the occurrence of this compound was noted as a peak on the ion exchange chromatogram was submitted

At this point it was called to my attention by Professor Vincent du Vigneaud that the mixed disulfide of cystine and homocysteine resulting from the hydrolysis of an oxytocin analogue containing the mixed disulfide of L-cysteine and L-homocysteine, with the carboxyl group of the half homocysteine residue attached to tyrosine, gave a peak in the isoleucine area of the ion exchange chromatogram. Subsequent studies in his laboratory with a synthetic mixed disulfide of L-cysteine and L-homocysteine showed that the synthetic compound and that resulting from hydrolysis of the analogue were identical.2

The mixed disulfide was synthesized by aerating L-cysteine and L-homocysteine at pH 7.5. Ion exchange chromatography revealed three peaks: cystine, homocystine, and one identical to that of the unknown compound. The synthetic mixed disulfide was isolated as described above. When the natural and synthetic compounds were combined and chromatographed on the automatic apparatus, there resulted a single, symmetrical peak identical to that obtained from the untreated urine. Paper chromatography of the combined materials revealed a single ninhydrin-positive spot in five solvent systems, with RF value between those of cystine and homocystine. Performic acid oxidation (7) of both the natural and synthetic substances yielded equimolar amounts of cysteic acid and homocysteic acid (8) on paper chromatography and no other ninhydrin-positive spots. The infrared absorption spectrum for the compound isolated from urine was identical to that of the synthetic compound (Fig. 1). Insufficient amounts of the natural substance were obtained for elemental analysis. However, the synthetic compound was analyzed to confirm its composition.

C2H14N4O8S2 (254.3)

Calculated: C 33.06, H 5.55, N 11.02, O 25.16, S 25.02

Found: C 33.07, H 5.42, N 11.00, O 25.39, S 25.30

Optical rotation in n HCl at 25° for the synthetic compound was −60.8 ± 0.6° whereas that for the natural compound was −52.2 ± 0.6°. The slight discrepancy may be accounted for by racemization of the natural compound during evaporation of the 6 n HCl in the preparative work. However, the optical rotation demonstrates that the natural substance is of the L-L configuration.

Ion exchange chromatography of a weighed amount of the pure compound allowed an estimate of the amount of mixed disulfide excreted in six patients from whom 24-hour urine collections were obtained. As shown in Table I, there was no apparent relationship to the amount of cystine excreted. This compound was not found in urine from normal subjects or from patients with other aminocacidurias associated with high cystine excretion. Traces of the mixed disulfide were found in cystine calculi.

It is believed that this is the first description of the natural occurrence of the mixed disulfide of L-cysteine and L-homocysteine. Although this amino acid may represent an oxidation of cysteine and homocysteine after excretion, the absence of homocysteine makes this unlikely. Furthermore, the absence of homocysteine in the urine precludes the possibility of the mixed disulfide being formed by disulfide interchange from cysteine and homocysteine. The origin of this compound and its significance in cystinuria will be the subject of continued investigations in this laboratory.

1 Jarvis, M. Bodanszky, and V. du Vigneaud, manuscript in preparation.

Fig. 1. Infrared spectra of the natural and synthetic mixed disulfide compounds in KBr pellets. These are compared to the clearly different spectrum obtained for cystine. A Perkin-Elmer model 221 infrared spectrophotometer with NaCl optics was used in these determinations.
A New Enzyme in the Conversion of Propionyl Coenzyme A to Succinyl Coenzyme A*

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From the Department of Biochemistry, New York University School of Medicine, New York 16, New York

(Received for publication, June 1, 1961)

The conversion of propionyl coenzyme A to succinyl coenzyme A in animal tissues has been shown to proceed via the reaction sequence

\[ \text{Propionyl-CoA} \rightarrow \text{ATP, CO}_2 \rightarrow \text{methylmalonyl-CoA} \rightarrow \text{sucinyl-CoA} \]

involving propionyl carboxylase and methylmalonyl isomerase.

* Aided by grants from the National Institute of Arthritis and Metabolic Diseases (Grant A-1845) of the United States Public Health Service, and the Rockefeller Foundation.

† Fellow of the Rockefeller Foundation. Permanent address, Laboratory of Biochemistry, College of Science, St. Paul’s University, Ikebukuro, Tokyo, Japan.

It also seems that methylmalonyl-CoA (a) is easily racemized upon brief heating at 100° and neutral pH.

When methylmalonyl-CoA (a), which is not isomerized by Fraction 1 alone, is heated, it is now converted to succinyl-CoA by this fraction to the extent of approximately 50% (Table II, Column 2). When Fraction 2 is added to the reaction mixture, the heated methylmalonyl-CoA (a) is converted to succinyl-CoA to an extent approaching 100% (Table II, Column 3). Similar observations were made when the decarbosylation of methylmalonyl-CoA (a) by reversal of the propionyl carboxylase reaction was studied. These results are illustrated in Fig. 1. It may be seen (Curve 1) that methylmalonyl-CoA (a) is decarbosylated almost quantitatively, but as shown in Curve 2, it is decarbosylated to an extent of approximately 50% after heating. However, the decarbosylation goes to completion when Fraction 2 is also added. It has been noticed that upon longer incubation, the decarbosylation of heated methylmalonyl-CoA (a) in the absence of Fraction 2 proceeds beyond 50% but at a much slower rate (Fig. 1, broken line). This may be attributed to nontypical racemization because it is unlikely that the highly purified Fraction 2 enzyme, and in part to spontaneous racemization (which may not be insignificant at 30°).

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