Betaine-Homocysteine-Methyl-Transferases

II.   Isolation and Properties of the Transferase of Pig Liver

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Procedures for the purification of the betaine-homocysteine-methyl-transferase of pig liver are described and discussed. Using these procedures, an enzyme preparation was obtained which was essentially homogeneous, as judged by sedimentation, chromatography and electrophoresis experiments. The molecular weight of the transferase monomer was found to be about 270 000. The transferase did not appear to be a metalloenzyme and no bound co-factors related to vitamin B_{12}, vitamin B_6, folic or folinic acid could be detected in the purified enzyme. The transferase showed a single light absorption maximum near 276 mμ. The apparent pH-optimum of the reaction catalyzed by the enzyme was approximately 7.5. In this reaction one molecule of betaine and homocysteine gave one molecule of dimethylglycine and methionine. Reversibility could not be demonstrated.

Enzymes that catalyze the transfer of an intact methyl group from betaine or some thetins to homocysteine thereby making methionine seem to occur in the livers of most vertebrates.[1] Enzymes with this property have been purified from rat liver by Ericson et al.[2], Maw [3], and Fromm and Nordlie [4], and from horse liver by Durell et al.[5]

In this laboratory we have for some years been engaged in a study of procedures for the purification of betaine-homocysteine-methyl-transferases from a variety of sources. Most work has been devoted to the methyl-transferase of pig liver and we have in a previous publication reported a method for the purification of this enzyme [6]. Since then some modifications have been made in the fractionation procedure and it is the purpose of this paper to describe in detail the method now in use in our laboratory, as well as some of the properties of the purified enzyme.

EXPERIMENTAL

The pig livers were purchased from a slaughter-house. They were removed and packed in ice immediately after the animals had been killed and bled. In the laboratory the livers were kept frozen before use. No decrease in the betaine-homocysteine-methyl-transferase activity of frozen livers could be detected over a period of several months. The methyl-transferase activity of different fresh livers varied considerably, in extreme cases by a factor of four or five. A similar variation in activity has been observed for instance with human livers [1] as well as with the livers of rats given various diets [7-9].

The activity of the enzyme solutions obtained during the fractionation was estimated by determining the amount of methionine formed aerobically per hour at 37°C during the initial phase of the reaction. The volumes of the different enzyme solutions used in the tests were chosen so that approximatively the same amount of methionine (and of dimethylglycine) was formed per hour from each enzyme fraction. All incubation mixtures contained 0.11 M betaine hydrochloride (California Corporation for Biochemical Research, Cal., USA, or L. Light & Co., Ltd., England) and 0.025 M DL-homocysteine (Nutritional Biochemicals Corporation, Ohio, USA). Methionine was determined chemically [9,10] or microbiologically [11]. The protein content of the different enzyme preparations was estimated by the biuret reaction [12] or by the optical method suggested by Kalckar [13]. In some cases the results were checked with the Kjeldahl procedure of Perrin [14].

The fractionation procedure for the preparation of purified betaine-homocysteine-methyl-transferase comprised the following steps:

Step 1

Alternative 1. 200 g of pig liver were cut into pieces and homogenized in 600 ml ice-cold water in a Waring blender. The pH of the homogenate was 6.4—6.8. The homogenate was centrifuged in a Spinco Model L ultracentrifuge at 44 000 × g for 25 min. and the supernatant fluid was adjusted to approximatively pH 7.7. The precipitate was washed with 250 ml ice-cold water and again spun down at 44 000 × g for 25 min. The supernatant fluid was combined with the fluid obtained after the first centrifuging. The total volume of the enzyme solution was approximately 810 ml.

Alternative 2. 200 g of frozen pig liver were cut into pieces and homogenized in 600 ml ice-cold water in a Waring blender. The homogenate was kept at 0°C while the pH was adjusted to approximately 5.1 with 1 N H_2SO_4. The slurry obtained was centrifuged in a Spinco ultracentrifuge at 44 000 × g for 15 min. The pH of the supernatant fluid was immediately adjusted to 7.5—8.0 with 1 N NaOH. The precipitate was suspended in 250 ml of ice-cold water and homogenized in a Waring blender. The suspension was centrifuged at 44 000 × g for 15 min. and the supernatant fluid was added to the fluid obtained after centrifuging the homogenate. The total volume was generally of the order of 890 ml. As the enzyme is very labile at pH 5.1 it is essential to keep the temperature near 0°C and to work quickly.

Foaming can be controlled with a few drops of *iso*octyl alcohol.

Step 2

The material from step 1 was adjusted to pH 7.75 and poured into test tubes with a diameter of 17 mm. The tubes were put in a water bath having a temperature of 80°C and stirred for 80—90 sec. The manual stirring of all tubes simultanously was accomplished by means of a rake-like contrivance having two rows of prongs fitting into the two rows of test tubes. After the heating, the tubes were immediately transferred to an ice-bath where the stirring was continued for 2—3 min. The fluid was thereafter centrifuged at 44 000 × g for 15 min. The supernatant fluid contained the enzyme. The precipitate was washed with 100 ml of water in a Waring blender and again centrifuged at 44 000 × g for 15 min. The combined supernatant fluids had a volume of approximately 800 ml when alternative 1, step 1, was used, and over 870 ml when alternative 2, step 1, was employed.

At this stage of the purification the enzyme can be stored for many months in the frozen state without loss of activity.

Step 3

In this step the enzyme was first adsorbed on and then eluted from calcium phosphate gel.

The calcium phosphate gel was prepared according to Keilin and Hartree [15] and was stored at room temperature. The day before the gel was to be used it was washed three times with 0.01 M phosphate buffer pH 8.0 and finally centrifuged at 4 500 × *g* in an MSE "Major Refrigerator" centrifuge. The dry weight of the washed gel was determined after it had been heated at 105°C for 2 h.

Alternative 1. If in step 1 the enzyme had been purified according to alternative 1, the amount of gel added to the solution obtained in step 2 corresponded to 10 mg dry gel per ml. With the facilities at hand it was found convenient to treat only 200 ml enzyme solution at a time. The gel was suspended in the solution by means of an electric stirrer for 30 min at 0°C and was thereafter recovered by centrifuging at 4 500 × *g* for 2 min. (Table 1). The supernatant fluid which contained only a few per cent of the activity but more than 50 % of the protein of the step 2 material was discarded. The gel was washed three times at 0°C with a total of 100 ml 0.05 M sodium phosphate buffer pH 8.0. Between each washing, the suspension was centrifuged at 4 000—4 500 × *g* for a few minutes (Table 1). The supernatant fluids from the washings were also discarded. They generally contained 5—10 % of the activity and about 25 % of the protein.

The elution of the enzyme from the gel was accomplished by means of five successive washings at 0°C, the first two with 0.15 M sodium phosphate buffer pH 8.5, the following three with a solution containing 0.15 M sodium phosphate and 0.1 M sodium citrate at pH 8.5 (Table 1). Vigorous stirring was used in order to get an even suspension of the gel in the eluting agent. The first two elutions lasted 40 min, the third and the fourth 30 min and the last 10 min. The volumes of the buffer solutions used were 25, 25, 20, 20, and 20 ml, respectively. After each of the first two elutions the suspensions were centrifuged at 4 500 × *g*, after the third and the fourth at 26 000 × *g* and after the fifth at 105 000 × *g*. The volume of the eluate was 110—115 ml.

Alternative 2. When the alternative 2 of step 1 was used, the amount of gel added to the enzyme solution from step 2 corresponded to only 6.5 mg dry gel per ml. All other operations followed the procedure outlined in alternative 1 of step 3.

The conditions used for the adsorption and desorption of the transferase from the calcium-phosphate gel are summarized in Table 1. If necessary a few drops of *iso*octyl alcohol were added to control the foaming.

Table 1. The conditions used for the purification of the transferase by treatment with calciumphosphate gel. All procedures were carried out at 0°C.

Process	No.	Washing or eluting agents	Volume ml	pH	Time min	Gel recovered by centrifuging at
Adsorption			200	8.0	30	4 500 × *g* 2 min
Washing	1st	0.05 M phosphate	50	8.0	15	4 000 × *g* 2 min
	2nd	0 05 M »	30	8 0	10	4 000 × *g* 2 min
	3rd	0.05 M »	20	8.0	3	4 500 × *g* 10 min
Elution	1st	0.15 M phosphate	25	8.5	40	4 500 × *g* 2 min
	2nd	0.15 M »	25	8.5	40	4 500 × *g* 2 min
	3rd	0.15 M phosphate + 0.10 M citrate	20	8.5	30	26 000 × *g* 10 min
	4th	—»—	20	8.5	30	26 000 × *g* 10 min
	5th	—»—	20	8.5	10	105 000 × *g* 10 min

Step 4

Alternative 1. The pH of the step 3 eluate was adjusted to 7.80 and the material poured into a row of test tubes. These tubes were submerged into a water bath having a temperature of 80°C. Stirring was performed as in step 2. After 80 sec. the tubes were put in ice-water and the stirring was continued for 2—3 min. The precipitate was removed by centrifuging at 44 000 × g. It was washed with 10 ml of water and again spun down at 44 000 × g.

This fractionation step was used only with enzyme preparations purified according to the alternative 1 of the first and the third steps. No purification was obtained when it was applied to enzyme solutions made according to alternative 2.

The preparation was dialyzed over night at 2°C against 2 l of distilled water.

Alternative 2. The pH of the eluate from step 3, alternative 2, was adjusted to 7.2 and ice-cold acetone to a final concentration of 55 % was slowly added to the enzyme solution. The mixture was kept at 0°C for 1 h and then centrifuged at 4 500 × g for 15 min. The precipitate that contained the enzyme was transferred to a dialysis bag by washing with approximately 5 ml of water. The dialysis was carried out over night at 2°C against 2 l of a 0.0025 M sodium phosphate buffer having a pH of 8.2.

Step 5

Alternative 1. Any precipitate formed during the dialysis was removed by centrifuging at 20 000 × g for a few minutes. The pH of the supernatant was then adjusted to 7.2 and ice-cold acetone was added to a final concentration of 60 %. The mixture was kept at 0°C for 2—3 h and then centrifuged at 22 000 × g for 25 min. The precipitate was dissolved in 10—15 ml of 0.010 M sodium phosphate buffer at pH 8.0.

Alternative 2. The precipitate formed during the dialysis was removed by centrifuging at 20 000 × g for 10 min. The supernatant was adjusted to pH 8.0 before step 6 was carried out.

Step 6

In this step the enzyme was poured through a column of triethylaminoethyl cellulose (TEAE-cellulose) [16] under conditions which retained on the column the remaining impurities in the step 5 preparation but let most of the enzyme pass through. The cellulose ion exchanger was purchased from the Serva Laboratories, Heidelberg, Germany. It was suspended in 0.010 M sodium phosphate at pH 8.0 and after about 45 min. the smallest particles were removed by decanting. Approximately 1 g of TEAE-cellulose was used per 70 mg of protein. The suspension was deaerated before being used for making the column, which had an inner diameter of 17 mm. The material was poured into the column as a slurry and was allowed to settle on the glass filter disc at the bottom. When most of the material had come to rest, a flow of buffer was passed through the column until the pH of the effluent was 8.0. A filter paper disc was then put on the top of the column.

The enzyme solution from step 5 was let through the column at a rate of approximately 0.5 ml per min and at a temperature of about 2°C. The effluent, which was virtually colourless, was collected in fractions of 2 or 5 ml. Fractions containing a substantial amount of activity were freeze-dried. It was possible to store the enzyme in this state for many months at temperatures below 0°C without loss of activity. However, the enzyme was not stable at room temperature.

Tables 2 and 3 summarize the steps used and the yield and purification obtained in the fractionation of betaine-homocysteine-methyl-transferase from pig liver according to alternative 1 and 2 respectively.

Other fractionation experiments

It may be of interest to mention briefly some of the other fractionation methods that have been tried, although they have given less satisfactory results. Precipitation with ammonium sulfate or ammonium phosphate [17] has in our hands consistently given low yields. Fromm and Nordlie [4] had the same experience in attempts to purify thetin-homocysteine transmethylase from rat liver with ammonium sulfate. Durell *et al.*[5], on the

other hand, used ammonium sulfate fractionation successfully for the purification of the thetin homocysteine methylpherase from horse liver.

Organic solvents, e.g. methanol, ethanol, n-propanol, isopropanol, acetone, methylethylketone and dioxane were also tried for the precipitation of the methyl-transferase at different stages of the purification procedure. Although good results were occasionally obtained, particularly with methanol, ethanol and acetone, we had difficulties in obtaining reproduceable results. Only when an excess of the precipitating agent was used (which led to little or no increase in specific activity) as in step 5, alternative 1, could a good yield consistantly be obtained.

Chromatography on the cation exchange resin Amberlite XE-64, treated as recommended by Hirs [18], was also unsuccessful as the degree of separation was poor. Chromatography on a column of the calcium phosphate (hydroxylapatite) prepared by the method of Tiselius, Hjertén and Levin [19] failed, due to irreversible adsorption of the transferase. Also attempts to use this particular calcium phosphate preparation for batch-wise adsorption and desorption (as in step 3) were unsuccessful, as it was very difficult to elute the enzyme from the "gel". To improve the yield when eluting the transferase from this "gel" or the gel prepared according to Keilin and Hartree, we added betaine, homocysteine, sodium chloride or sodium pyrophosphate to the eluting agent without success. Increasing the temperature during the elution step lowered the yield.

Zone electrophoresis on starch [20] or on specially treated cellulose [21] was tried, but low yields were obtained in both cases, probably due to irreversible adsorption of the transferase to the column material. Paper electrophoresis was also unsuccessful for the same reason. Electrophoresis in a density gradient according to Svenson et al.[22] was possible and separated the partially purified (step 5) transferase from coloured impurities [6]. However, we consider this method more elaborate and much more time consuming than the TEAE-cellulose treatment of step 6. The latter procedure can moreover be adapted to larger quantities and it does not require that a person works in a cold-room for long periods of time.

It should be pointed out that our failure to use the above mentioned methods for the purification of betaine-homocysteine-methyl-transferase does not mean that they cannot be successfully applied for this purpose under different conditions.

RESULTS AND DISCUSSION

Comments on the fractionation procedure. It can be seen from Table 3 that the purification of the betaine-homocysteine-methyl-transferase of pig liver according to alternative 2 gave satisfactory results in a comparatively large number of cases. The experiments on which the data are based were carried out during 1956, 1957 and the first part of 1958. When the investigations on the transferase were resumed in the summer of 1959, we were surprised to find, on the one hand that the transferase activity of the pig livers then obtained was on the average about three times higher than previously, on the other hand that the purification method that we had successfully applied in earlier experiments failed. This was particularly so in step 1 but also steps 2 and 3 gave unsatisfactory results. As it was impossible to account for all the activity in the different fractions from these steps, it appeared that the methyl-transferase of the high-activity livers was more labile than that of the livers with low transferase activity. Thetin homocysteine methylpherase has been shown by Durell et al.[5],[23] to consist of a mixture of polymers and this seems to be the case also with the enzyme described here. It is conceivable that in a liver with a high concentration of the betaine-homocysteine-methyl-transferase less depolymerization of the enzyme to the monomer form takes place. The more polymerized form may be more labile than the monomer. This speculation finds some support from the observation that oxidizing agents (iodine and air) were found to make the

Table 2. Data relevant to the purification of betaine-homocysteine-methyl-transferase according to alternative 1.

Step	Total protein (approx.)	Total volume (approx.)	Step to step recovery (approx.)	Over all recovery (approx.)	Over all purifica- tion (approx.)
	mg	ml	%	%	
Homogenate	40 000	760	100	100	1
1 Centrifuging at pH 6.4 — 6.8	20 000	810	85	85	1.7
2 Heating at 80°C	6 900	800	95	80	4.7
3 Treatment with calcium phosphate gel	400[1]	115[1]	70	57	14
4 Heating at 80°C and dialysis	220	115	90	50	23
5 Precipitation with acetone	97	15	75	38	39
6 TEAE-cellulose column	38	20	75	28	75

[1] Only 200 ml of the material from step 2 was treated at a time.

enzyme more heat labile than reducing agents (cysteine and homocysteine). This observation can, however, not be considered to prove the hypothesis as it is open to several objections, and the explanation for the above-mentioned phenomenon will have to await further and more eleborate experiments in which polymer and monomer forms are quantitatively prepared. It should also be mentioned that we have not yet been able to trace the cause of the increase in transmethylase activity of the pig livers between 1958 and 1959. It is possible that dietary factors play a part.*

The purification of betaine-homocysteine-methyl-transferase from pig liver according to alternative 1 (Table 2) has not been repeated as many times as alternative 2 (Table 3). However, when the procedure had been developed, a skilled technician was able to carry out the fractionation with satisfactory results (Table 2) in five consecutive experiments. The purification took four days. By using a fractionation procedure that is different from alternative 1 or 2, we have occassionally obtained reasonably pure transferase preparations in one day, but it has so far not been possible to get reproduceable results with this procedure.

Purity. The purity of transferase preparations obtained as described under Experimental (alternative 1) have been studied using sedimentation, chromatography and electrophoresis. Fig. 1 shows two photographs from a

* We have since learned from Dr. H. Fredholm of the Swedish Federation of Butchers, Stockholm, that the pigs slaughtered in 1959 and 1960 were on the average younger than in previous years.

Table 3. Data compiled from a number of attempts to purify betaine-homocysteine-methyl-transferase according to alternative 2.

Step	Total protein (approx)	Total volume (approx)	Step to step recovery Average ±standard error	Highest and lowest value	Over all recovery (approx)	Over all purification (approx)
Homogenate	mg 40 000	ml 760	% 100	% 100	% 100	1
1 Centrifuging at pH 5.1—5.2	15 900	890	83.5[2] ± 0.9	77.4—88.0	83.5	2.1
2 Heating at 80°C	3 700	870	98.5[3] ± 1.5	86 —100	82	8.9
3 Treatment with calcium phosphate gel	125[1]	115[1]	77.4[4] ± 1.3	61.5—89.0	64	51
4—5 Precipitation with acetone and dialysis	78	25	77.7[5] ± 3.8	51.5—100	49	64
6 TEAE-cellulose column	25	30	74[6]	53 —80	36	150

[1] Only 200 ml of the material from step 2 was treated at a time.
[2] Average for 12 runs over a period of 18 months.
[3] " " 20 " " " " " 24 "
[4] " " 26 " " " " " 18 "
[5] " " 13 " " " " " 18 "
[6] Only four runs performed.

sedimentation experiment in a Spinco Model A ultracentrifuge, taken after 32 and 64 min at 59 780 r.p.m. The enzyme was dissolved in a 0.01 M phosphate buffer having a pH of 8.0. The sedimentation constant s_{20} was approximatively 8.5 Svedberg units. Only if DL-homocysteine was added to the transferase solution was a sedimentation pattern with one peak obtained. In the absence of reducing agents, three peaks could be observed. This indicates that betaine-homocysteine-methyl-transferase can exist in monomer and polymer forms and that the ratio of monomer to polymer is influenced by reducing agents, *e.g.* SH-groups. This situation is analogues to the one discovered for thetin homocysteine methylpherase by Durell *et al.*[5,23], and suggests a close similarity between the two enzymes.

Fig. 2 shows the result of a *chromatography* experiment on a preparation of betaine-homocysteine-methyl-transferase using a TEAE column. In this experiment the enzyme was eluted with increasing concentrations of phosphate buffer at pH 8 and a careful analysis was made of the distribution of protein and enzyme in the effluent. It can be seen that the curves for protein content and enzyme activity are symetrical about the same fraction number. The enzyme activity was determined using either betaine or dimethyl-β-pro-

Fig. 1. Sedimentation pattern of purified betaine-homocysteine-methyl-transferase. The pictures were taken after 32 and 64 min at 59 780 r.p.m. in a Spinco Analytical Ultracentrifuge Model E.
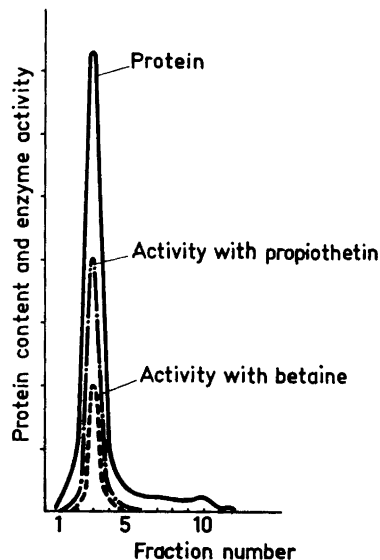
Fig. 2. Chromatography of purified betaine-homocysteine-methyl-transferase on a column of TEAE-cellulose. The units of protein concentration and of enzyme activity (measured either with !betaine or dimethyl -β-propiothetin as methyl donor) are arbitrarily chosen to keep the three curves separate from each other.

piothetin as the methyl donor and it is noteworthy that there is no separation of the two activity curves.

In Fig. 3 the *electrophoretic* behaviour of the purified betaine-homocysteine-methyl-transferase is shown. The electrophoresis was carried out in the micro-cells of a Spinco Model H apparatus. A sodium borate buffer (pH = 9.36, $\mu = 0.1$) containing 1 mg DL-homocysteine per ml was used. Prior to the electrophoresis, the enzyme solution was dialysed against the same buffer, again in the presence of homocysteine.

Figs. 1—3 demonstrate, that although ultracentrifuging, chromatography and electrophoresis revealed minor impurities in the betaine-homocysteine-methyl-transferase purified from pig liver, the transferase protein seemed to dominate according to these criteria of purity.

Molecular weight. An attempt was made to estimate the molecular weight of the transferase monomer using the principle of centrifuging towards sedimentation equilibrium [24]. Calculations at the meniscus and at the cell bottom from a series of concentration gradient patterns, obtained in a run performed in 0.01 M sodium phosphate and at 8 000 r.p.m. in a Spinco Model A ultracentrifuge, gave a mean value of approximatively 270 000.

Absorption spectrum. The light absorption spectrum of a solution of purified betaine-homocysteine-methyl-transferase in a phosphate buffer pH 7.8

Fig. 3. Electrophoresis of purified betaine-
-homocysteine-methyl-transferase in a so-
dium borate buffer at pH 9.36, $\mu = 0.1$. The
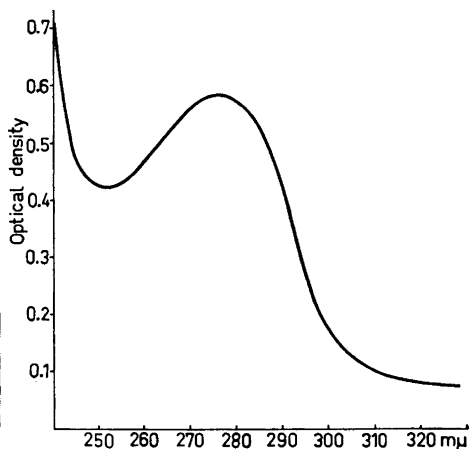picture was taken after 261 min.

Fig. 4. Light absorption spectrum between
240 and 330 mμ of purified betaine-homo-
cysteine-methyl-transferase.

was determined in a Beckmann Model DU spectrophotometer. The curve
shown in Fig. 4 was obtained. This curve, that shows a single absorption
peak at 276 mμ, does not suggest the presence of any bound co-factors of,
for instance, a vitamin nature. In some preparations a small peak was noticed
at 415 mμ. However, we believe that this was caused by an impurity as we
have not been able to correlate it with the enzyme activity.

    Content of vitamin B_{12}, folic- and folinic acid, and vitamin B_6. Several inves-
tigators have noticed that the betaine-homocysteine-methyl-transferase ("be-
taine-homocysteine-transmethylase") activity is lower in the livers from rats
given a diet deficient in vitamin B_{12} than in the livers from normal rats.
However, no determination of the vitamin B_{12} content of a purified methyl-
transferase has ever been made. A solution of the betaine-homocysteine-
methyl-transferase isolated from pig liver was therefore boiled for 10 min.
at pH 6.0 in the presence of potassium cyanide in order to release bound vita-
min B_{12} from the enzyme. The vitamin B_{12} content of the solution was there-
after determined using the mutant 113—3 of *E. coli* [25] in the medium suggested
by Diding [26]. The result of this determination is given in Table 4. It should
be mentioned that not only vitamin B_{12} (cyanocobalamin) itself but a large
number of vitamin B_{12}-like compounds can support the growth of *E. coli*
113—3. The theoretical content of vitamin B_{12} (and of the other vitamins and
growth factors studied, see Table 4) was calculated on the assumption of a
one to one relationship between the "co-factor" and the monomer form of
the protein moiety and on the basis of a molecular weight of 270 000 for this
form of the transferase. It appears unlikely in view of the result of the micro-
biological assay (Table 4) that vitamin B_{12} or a vitamin B_{12} derivative is pre-
sent in the betaine-homocysteine-methyl-transferase described here. The fact

Table 4. Calculated and experimentally determined concentrations of microbial growth factors related to vitamin B_{12}, folic- and folinic acid, and vitamin B_6 in solutions of purified betaine-homocysteine-methyl-transferase.

Vitamin or growth factor	Calculated concentration	Experimentally determined concentration
	mμg/ml	mμg/ml
Vitamin B_{12}	30	< 0.004
Folic acid	6	0*
» »	6	< 0.1 **
Folinic acid	6	< 0.3 *
» »	6	< 0.1 **
Vitamin B_6	30	< 1.0

* Direct determination.
** Determination after incubation with chicken pancreas conjugase.

that the enzyme is virtually colourless and that its absorption spectrum shows no maximum near 360 mμ also supports this conclusion. The effect of vitamin B_{12} deficiency on the transferase activity must consequently be indirect and is possibly mediated by certain metabolites, which have been shown to affect the enzyme activity *in vitro* [6].

Numerous studies indicate that an intimate relationship exists between the biological function of vitamin B_{12} and of "folic- and folinic acid". An example of this relationship is the series of reactions leading to the *synthesis* of methyl groups, in which both vitamin B_{12} and pteridine co-factors play a rôle. The function of various pteridine derivatives in the transfer of 1-C fragments at the oxidation level of formate or formaldehyde is well established. It was consequently considered of interest to assay the purified betaine-homocysteine-methyl-transferase for microbial growth factors related to folic- and folinic acid, especially in view of the recent findings by Sauberlich, which show a decrease in mouse liver transmethylase activity due to folic acid deficiency[27]. In order to release folic- and folinic acid factors from the enzyme and to obtain microbially active conjugates of these compounds, aliquots of the enzyme solutions were incubated for 20 h at pH 6.8 and 37°C with a chicken pancreas preparation prior to assay. *Streptococcus faecalis* ATCC 8043 and *Pediococcus cereviseae (Leuconostoc citrovorum)* ATCC 8081 were used as test organisms in the turbidimetric tube assay media described by Dr. A. Bolinder of this laboratory [28]. Pteroylglutamic acid and N^5-formyl-tetrahydro-pteroylglutamic acid (Leucovorin, Lederle Laboratories Division, Pearl River, N.J., USA) were used as reference substances. As can be seen from Table 4, the transferase could not replace these two substances as a growth factor for *S. faecalis* or *P. cereviseae* either before or after treatment with chicken pancreas. No evidence was thus obtained for the existence of a pteridine-like co-factor in the betaine-homocysteine-methyl-transferase of pig liver.

Fromm and Nordlie have suggested that pyridoxine is a tightly bound co-factor in the thetin homocysteine transmethylase of rat liver [4]. The sugges-

tion was based on the fact that their transmethylase preparations showed flourescence and absorption at 415 mμ. An attempt was therefore made to determine the vitamin B_6 content of the betaine-homocysteine-methyl-transferase of pig liver. A tube assay with *E. coli* 154—59L [29] was used[30]. This organism responds well to pyridoxine, pyridoxal and to a lesser degree also to pyridoxamine. The vitamin B_6 was released from the enzyme by boiling a solution of the transferase at pH 1.0 for 30 min or by autoclaving at pH 1.7 for 60 min. It was not possible to detect any compound in the enzyme solutions treated in this way that could support the growth of *E. coli* 154—59L (Table 4). In a separate experiment it was also shown that the acid treated transferase failed to stimulate the growth of the B_6-requiring *Saccharomyces carlsbergensis* [31]. As we have only occassionally been able to find an absorption maximum at 415 mμ in our transferase preparations (see above) our data do not provide any evidence for the participation of vitamin B_6 in this type of methyl group transfer. Ericson and Harper have previously shown that a vitamin B_6 deficiency does not decrease the transferase activity of rat liver[8].

Dependance on metals. Two solutions of purified betaine-homocysteine-methyl-transferase were dialysed at 2°C for 50 h, one solution against 0.025 M phosphate buffer pH 8.0, the other against 0.025 M phosphate buffer pH 8.0 containing 2×10^{-3} M EDTA (ethylenediamine tetraacetic acid). There was only a slight decrease but no difference in the transferase activity of these two enzyme solutions after the dialysis, which indicates that the activity is not dependent on bound metals. In other experiments it was found that the addition of potassium cyanide, sodium citrate, sodium pyrophosphate or 8-hydroxy-quinoline to the incubation mixtures had no effect on the transferase activity. This is in accordance with the results from similar studies with the methyl transferases purified from rat [2,32] and horse liver [5]. Microbial transferases, on the other hand, appear to be metalloenzymes [33].

Activators and inhibitors. Although no evidence was found for the presence of bound co-factors in the betaine-homocysteine-methyl-transferase described in this communication, it can be shown that the activity of the enzyme is greatly influenced by a number compounds, *e.g.* organic acids, amino acids and peptides [6]. This phenomenon will not be further elaborated upon here, but the author hopes to return to the problem of activation and inhibition in connection with a study of some kinetic properties of the enzyme.

Apparent pH-optimum. Incubation mixtures containing betaine-homocysteine-methyl-transferase, betaine and DL-homocysteine in phosphate buffer were adjusted at 0°C to pH-values ranging from approximatively 5.5 to 9.5. They were thereafter kept at 37°C for 3 h whereupon their methionine content was determined. When the activity of the enzyme was plotted against the pH a curve of the type shown in Fig. 5 was obtained. This indicates that the apparent pH-optimum of the pig liver transferase is near 7.5. It should be mentioned that the apparent pH-optimum — as would be expected — is influenced by a number of factors, *e.g.* the type of buffer, the concentration of the components of the incubation mixture, *etc.*

Stoichiometry of the reaction. To study the stoichiometry of the reaction, betaine containing ^{14}C in one of the N-methyl groups was used. This compound was synthesized from dimethylglycine and ^{14}C-methyl iodide (Radio-
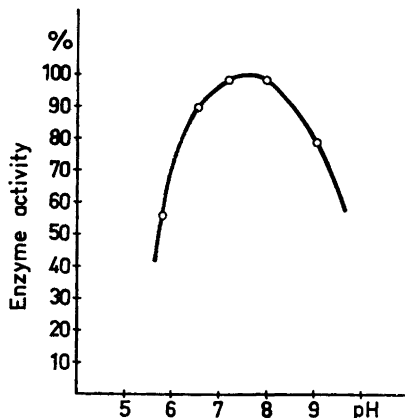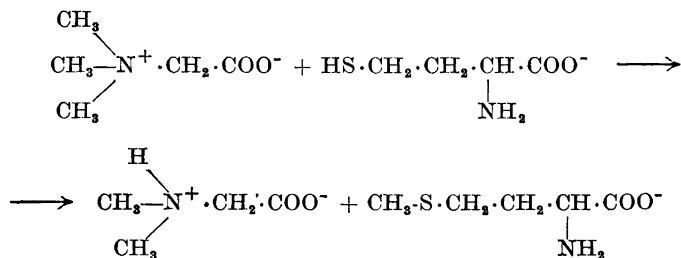
Fig. 5. The apparent pH-optimum of puri-
fied   betaine - homocysteine - methyl - trans-
ferase.

chemical Center, Amersham, England). Carrier betaine was added to make
possible crystallization of the product.

The ^{14}C-methyl betaine was incubated overnight together with the enzyme
and an excess of DL-homocysteine at 37°C and pH 7.8. The reaction was
terminated by boiling and the mixture clarified by centrifuging. The super-
natant fluid was chromatographed in a solvent system containing methyl
ethyl ketone: acetone: NH$_3$ (ca. 35 %) at a ratio of 60:27:13 for 30 h at 22°C.
After drying, the radioactivity along the chromatogram was measured using
a Tracerlab TGC2 Geiger-Müller tube and a LKB Scaler Type Nr 117. At the
areas where betaine, dimethylglycine and methionine were located on the
chromatogram the following net counts per minute were found in two separate
experiments:

	betaine	dimethylglycine	methionine
Expt. 1	204	90	44
Expt. 2	360	180	89

These results show that the radioactivity in counts per min. was twice as
high for dimethylglycine (that contains two methyl groups) as for methionine
(that contains one methyl group). This suggests that for each molecule of
dimethylglycine, one molecule of methionine is formed and that the reaction
takes place according to the over all scheme:

$$\underset{\underset{CH_3}{\diagup}}{\overset{\overset{CH_3}{\diagdown}}{CH_3-N^+}} \cdot CH_2 \cdot COO^- + HS \cdot CH_2 \cdot CH_2 \cdot \underset{\underset{NH_2}{|}}{CH} \cdot COO^- \longrightarrow$$

$$\longrightarrow \underset{\underset{CH_3}{\diagup}}{\overset{\overset{H}{\diagdown}}{CH_3-N^+}} \cdot CH_2 \cdot COO^- + CH_3 \cdot S \cdot CH_2 \cdot CH_2 \cdot \underset{\underset{NH_2}{|}}{CH} \cdot COO^-$$

At the apparent pH-optimum of the reaction, dimethylglycine exists mainly in the "zwitterion" form shown above, as indicated by the pK_a and pK_b values determined by Bjerrum[34]. Only insignificant amounts of H_3O^+-ions therefore result from this reaction in contrast to the reaction in which a thetin serves as the donor of the methyl group[3,5].

An attempt was also made to determine whether the transferase reaction is reversible or not. L-Methionine containing ^{14}C in the methyl group (Radiochemical Center, Amersham, England), dimethylglycine and the transferase were incubated for several hours at 37°C and the reaction mixture was thereafter boiled, centrifuged and chromatographed. It was not possible to detect any radioactivity at the site of betaine on the chromatogram.

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