- Ouchterlony, O. (1953). *Acta path. microbiol. scand.* **32**, 231.
 Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
 Partridge, S. M. & Morgan, W. T. J. (1940). *Brit. J. exp. Path.* **21**, 180.
 Pirotsky, I. (1938). *C.R. Soc. Biol., Paris*, **127**, 98.
 Pollitzer, R. (1954). *Plague*. Geneva: World Health Organization.
 Rowland, S. (1914). *J. Hyg., Camb.*, **14** (Plague suppl. no. 3), 418.
 Schar, M. & Thal, E. (1955). *Proc. Soc. exp. Biol., N.Y.*, **88**, 39.
 Schütze, H. (1932). *Brit. J. exp. Path.* **13**, 284, 289.
 Schütze, H. (1939). *Brit. J. exp. Path.* **20**, 235.
 Seal, S. C. (1951). *Proc. Soc. exp. Biol., N.Y.*, **77**, 675.
 Silverman, S. J. (1954). *J. Lab. clin. Med.* **44**, 185.
 Slein, M. W. & Schnell, G. W. (1953). *Proc. Soc. exp. Biol., N.Y.*, **82**, 734.
 Somogyi, M. (1937). *J. biol. Chem.* **117**, 771.
 van Heyningen, W. E. (1950). *Bacterial Toxins*. Oxford: Blackwell.
 Weidel, W., Koch, G. & Bobosch, K. (1954). *Z. Naturf.* **9b**, 573.
 Westphal, O., Lüderitz, O. & Bister, F. (1952a). *Z. Naturf.* **7b**, 148.
 Westphal, O., Lüderitz, O., Eichenberger, E. & Keiderling, W. (1952b). *Z. Naturf.* **7b**, 536.

Thetin-Homocysteine Transmethylase

A PRELIMINARY MANOMETRIC STUDY OF THE ENZYME FROM RAT LIVER

By G. A. MAW

Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E. 1

(Received 21 October 1955)

Transmethylation reactions involving the formation of methionine from homocysteine have been shown to take place in the presence of slices and suspensions of liver and kidney from the rat, pig and guinea pig (Borsook & Dubnoff, 1947; Dubnoff & Borsook, 1948). These tissues contain two distinct enzyme systems capable of catalysing the transfer of methyl groups to homocysteine, one requiring dimethylthetin or dimethyl- β -propiothetin, and the other requiring glycine betaine, as the specific methyl donor. Dubnoff & Borsook (1948) referred to these enzymes as dimethylthetin

formed. More recently, Ericson, Williams & Elvehjem (1955) have made a further study of the betaine transmethylase.

If the overall chemical changes involved in the reactions catalysed by these two enzymes are examined, it is evident that, in both cases, the methylation of the sulphhydryl group of homocysteine under physiological conditions must result in the liberation of a proton, since according to the data of Rykkan & Schmidt (1944) the magnitude of the pK attributable to the sulphhydryl group is such that the ionization of the group is small:

transmethylase and betaine transmethylase respectively, and showed that they could be distinguished from one another by their different stabilities in acid solution. They were both found to possess a pH optimum in the region of 7.8 and to function in anaerobic conditions. The thetin transmethylase was able to bring about transfer of one methyl group only, per molecule of dimethylthetin. The action of both enzymes was followed at pH 7.4 by colorimetric estimation of the methionine

In the reaction involving dimethylthetin (reaction 1) there are no other changes in the number of acid or basic groups initially present. The net result is the production of acid, and if the reaction be carried out in a bicarbonate medium it should be possible to follow it manometrically by the amount of CO_2 evolved. Smythe (1936) has studied the non-enzymic alkylation by iodoacetate of cysteine and other thiols in a bicarbonate buffer by such a procedure.

With betaine as the methyl donor (reaction 2), however, proton formation is accompanied by the production of dimethylglycine, i.e. of an extra basic group. In this case, therefore, one would expect no production of acid and no evolution of CO_2 in a bicarbonate medium.

Experiments with rat-liver preparations as sources of the two transmethylases have shown that the above predictions are in fact correct. The action of the thetin transmethylase can be followed manometrically in a bicarbonate buffer, whereas that of the betaine transmethylase does not involve any gas exchange. The former enzyme has been partially purified by ammonium sulphate fractionation and some of its characteristics have been examined with the aid of this simplified assay procedure. Ericson *et al.* (1955) have renamed the betaine enzyme betaine-homocysteine transmethylase. It is suggested that the thetin enzyme be termed similarly thetin-homocysteine transmethylase, not only to emphasize the nature of the methyl acceptor, but also because the enzyme is capable of utilizing other thetins, in addition to dimethylthetin, as sources of methyl groups (G. A. Maw, unpublished observations). A preliminary account of some of the results in this paper has already been published (Maw, 1955).

MATERIALS AND METHODS

Materials

Substrates. Dimethylthetin chloride was prepared by heating an equimolar mixture of dimethyl sulphide and monochloroacetic acid under gentle reflux for 5 hr. The resulting solid was washed with ether and twice recrystallized from methanol. DL-Homocysteine was prepared from DL-homocystine by reduction with sodium in liquid ammonia (Riegel & du Vigneaud, 1935-36).

Preparation of tissue suspensions. Liver (6 g.) from a freshly killed male rat (wt. 150-200 g.) was homogenized with 24 g. of ice-cold 0.0128M phosphate buffer, pH 7.4, containing NaCl, KCl and MgSO_4 (Cohen & Hayano, 1946), and filtered through two layers of muslin. Such a preparation contained approx. 55 mg. dry wt. of tissue/ml. In the majority of experiments it was then centrifuged at 1250 g in a cold room at 4° for 15-20 min. to remove cell debris and other inactive material. The supernatant fraction contained approx. 35 mg. dry wt. of tissue/ml.

Analytical method for following transmethylation

The liver suspension obtained as described above was pipetted into 50 ml. beakers, 1 ml. in each, together with 2 ml. of a neutralized solution of the thetin chloride and 2 ml. of DL-homocysteine, both made up in the phosphate buffer. Beakers were also prepared containing suspension alone, suspension + homocysteine, and suspension + thetin chloride, together with sufficient buffer to give a final volume of 5 ml. in each beaker. The beakers were placed in a thermostat previously described (Maw, 1954) and shaken at 36.8° for 2 hr. while being gassed with 100% N_2 . In

a number of experiments the liver suspensions were made up in a 0.02M sodium bicarbonate buffer containing the same concentrations of NaCl, KCl and MgSO_4 as the phosphate buffer, and the beakers were then gassed with a mixture of $\text{N}_2 + \text{CO}_2$ (95:5, v/v).

At the end of the 2 hr. period, the beakers were removed from the bath, the contents were deproteinized by the addition of 1 ml. of 20% (w/v) trichloroacetic acid (TCA) and centrifuged at 1250 g for 10 min., after being allowed to stand for 30 min. The supernatant was used for methionine estimations.

Estimation of methionine

Certain difficulties were encountered in the estimation of methionine under the particular experimental conditions employed, and these are discussed in some detail.

In their transmethylation studies, Dubnoff & Borsook (1948) used a modification of the colorimetric method of McCarthy & Sullivan (1941) for the estimation of methionine. They had found previously (Borsook & Dubnoff, 1947) that the presence of homocysteine can inhibit the colour obtained with methionine. The products of demethylation of the thetins studied, and also dimethyl- β -propiothetin itself, give colours with the reagents similar to that formed by methionine. Dubnoff & Borsook stated that the intensity of colour in each case is a constant fraction of that produced by equimolar amounts of methionine, and they therefore deducted the contributions of these chromogenic compounds from the total colour given by solutions in their enzyme experiments.

Effect of homocysteine on colour formation. It has been found that homocysteine can inhibit the colour formed with methionine by combining at a comparable rate with the sodium nitroprusside reagent. A number of other compounds, including various amino acids, have the same effect as shown by Horn, Jones & Blum (1946). When the concentration of homocysteine present is below about 0.01M the depression of colour formation may be completely overcome by using sufficient sodium nitroprusside to react with both amino acids. This amounts to a final concentration of 0.2% in the solution for analysis. When the homocysteine concentration is much above 0.01M it is not possible to compensate completely for inhibition of colour development, and furthermore, increasing the amount of sodium nitroprusside used itself causes a diminution of the methionine colour.

Effect of methylthioacetate and dimethylthetin chloride. The methylation of homocysteine by dimethylthetin chloride involves the formation of methylthioacetic acid, which gives a positive McCarthy-Sullivan reaction. The ratio of colour intensities obtained from equimolar amounts of methylthioacetic acid and methionine is not strictly constant, but varies from 0.55 to 0.58 over the concentration range 0.003M. The need for correcting the observed methionine value for the colour due to methylthioacetic acid is obviated by removing the latter compound from the solution before colour development. This may be achieved by ether extraction, since the solution for analysis is sufficiently acid at this stage, owing to the presence of the excess of TCA. Three extractions with equal volumes of ether served to remove methylthioacetic acid completely from aqueous solution under these conditions. The ether extraction also removed the excess of TCA, so making it unnecessary to neutralize the solution before addition of the

colour reagents. Neither the process of ether extraction nor the presence of ether had any effect on the colour obtained with methionine. Ether-extracted solutions occasionally became green on addition of sodium nitroprusside and NaOH, but this colour was discharged by acidification. Dimethylthetin chloride at the concentrations used in these experiments had no effect on colour formation and was not itself chromogenic.

Effect of temperature. After the addition of sodium nitroprusside and NaOH, Dubnoff & Borsook kept the solution for analysis at 38° for 5–10 min. In the present experiments, temperature was not found to be a critical factor in colour development, maximum colour formation being obtained over the range 15–40°. On final acidification the solution became noticeably warm, but this was without effect on the colour intensity obtained. All estimations have therefore been carried out at room temperature without regard to temperature control at any stage.

Procedure for the estimation of methionine. The following modification of the McCarthy–Sullivan method was employed. The deproteinized solution containing up to 2 mg. of methionine/5 ml. was shaken three times in a 30 ml. stoppered glass tube with equal volumes of anaesthetic ether, the two layers being allowed to separate and the ether layer drawn off each time by means of a capillary tube. To 3 ml. of the clear aqueous layer were added 2 ml. of water, 0.5 ml. of 2% (w/v) freshly prepared sodium nitroprusside and 0.5 ml. of 5*N*-NaOH; the whole was well mixed and allowed to stand for 10 min. Two ml. of 89% (w/w) H₃PO₄ (A.R.), sp.gr. 1.75 (Horn *et al.* 1946), was then added slowly with vigorous swirling, the mixture was allowed to stand for a further 10 min. and the colour which developed was compared with that obtained from a control tube containing water in place of the ether-extracted sample, with an EEL photoelectric colorimeter (Evans Electroselenium Ltd.) and a Chance OG1 filter (max. transmission 530 mμ.). Methionine concentrations were read off a calibration curve constructed from a series of aqueous L(+)-methionine solutions of known strength. This curve was coincident with that obtained from a series of methionine solutions containing added homocysteine,

dimethylthetin, sodium methylthioacetate and TCA, and subjected to ether extraction.

The iodometric method of estimating methionine of Bakay & Toennies (1951) is undoubtedly more sensitive than the method described above. However, as samples for analysis contain unreacted homocysteine, which also reacts with iodine, this particular method becomes impracticable in the experiments described here, unless the homocysteine is first removed by some procedure such as that of Lavine & Floyd (1954).

Manometric procedure for following transmethylation

This was carried out in the conventional Warburg apparatus. Each flask contained the neutralized substrates in 2 ml. of the 0.02*M* bicarbonate buffer described previously, with 0.5 ml. of liver suspension or enzyme preparation in the side arm. When high concentrations of the thetin chloride were used, the compound was first dissolved in water, neutralized with NaOH and then an equal volume of bicarbonate buffer of twice the required final strength was added. The flasks were gassed with N₂ + CO₂ (95:5, v/v) and, 5 min. after mixing the enzyme with the substrates, CO₂ evolution was measured for a period of 2 hr. in the case of liver suspensions and for 1 hr. when more active preparations were used. The initial reaction velocity has been expressed as the volume of CO₂ liberated during the 10 min. period commencing 5 min. after 'zero' time.

Homocysteine has frequently been used at a concentration of 0.03*M*, and at this strength is responsible for a considerable CO₂ retention amounting to 17% of the gas liberated. This retention has been taken into account in assessing CO₂ output, and the assumption has been made that the retention due to the methionine formed in the reaction is equal to that of the homocysteine being utilized. At lower concentrations of homocysteine (e.g. 0.003*M*) there was no measurable retention and none was attributable to the other compounds used or to the tissue preparations, whatever the concentrations employed.

Table 1. *Methionine formation from DL-homocysteine in the presence of rat-liver suspensions, with dimethylthetin chloride and betaine hydrochloride as methyl donors*

Liver suspensions 4%; substrates 0.003*M*; saline media, pH 7.4; flasks gassed with either 100% N₂ or N₂ + CO₂ (95:5, v/v) according to the nature of the buffer present; length of experiments 2 hr. at 36.8°. The figures represent μg. of methionine formed.

(a) Methyl donor dimethylthetin chloride

Medium	Liver alone	Liver + thetin	Liver + homocysteine	Liver + thetin and homocysteine
Phosphate-saline	30	70	135	745
	—	—	185	730
Bicarbonate-saline	—	—	115	820
	—	—	150	840
		Suspension frozen at -21°		870
		Suspension heated to 100°		130

(b) Methyl donor betaine hydrochloride

Medium	Liver alone	Liver + betaine	Liver + homocysteine	Liver + betaine and homocysteine
Bicarbonate-saline	0	0	60	365
	0	20	130	435

RESULTS

Experiments with rat-liver suspensions

Methionine formation. The methylation of homocysteine by dimethylthetin and by betaine in the presence of rat-liver suspensions has been studied by direct estimation of the methionine formed, the results obtained being given in Table 1. They are in close agreement with those obtained originally by Dubnoff & Borsook (1948) under somewhat similar experimental conditions. There was negligible methionine formation by the tissue, either in the absence of the substrates or in the presence of the methyl donors alone, but some synthesis occurred when the tissue was incubated with homocysteine alone. This amount was markedly increased by the further addition of either of the methyl donors. The addition of dimethylthetin gave rise to approximately twice as much extra methionine as did an equivalent amount of betaine. The synthesis occurring in the presence of the thetin corresponded to a methylation of 27 % of the total DL-homocysteine present.

Fig. 1. CO_2 formation from DL-homocysteine and dimethylthetin chloride in the presence of a rat-liver suspension. Saline medium containing 0.02M- NaHCO_3 ; substrates 0.003M; liver suspension 4%; flasks gassed with $\text{N}_2 + \text{CO}_2$ (95:5, v/v); temp. 36.8°. ▲, Substrates incubated alone without liver suspension; ○, suspension with homocysteine; ●, suspension with homocysteine and dimethylthetin chloride; ◐, homocysteine and dimethylthetin chloride with the same suspension kept for 14 days at 4°.

Heating the suspension to 100° for 15 min. destroyed its enzymic activity, whereas freezing it to -21° had no effect. The extent of methionine synthesis appeared to be the same whether the medium contained phosphate or bicarbonate buffer. Furthermore, the suspension lost none of its activity after centrifuging for 30 min. at 1250g, which removed a considerable amount of cell debris. The substrate mixtures in the absence of tissue are responsible for a small methionine value, but whether this is a true non-enzymic synthesis or merely a 'reagent blank' has not been determined.

Manometric experiments. On incubation of centrifuged rat-liver suspensions with homocysteine and dimethylthetin in Warburg flasks, a small but steady evolution of CO_2 was observed. Fig. 1 shows the results of a 2 hr. manometric experiment, in which the substrates were present at concentrations corresponding to those used in the previous

Fig. 2. CO_2 formation from DL-homocysteine in the presence of a rat-liver suspension, with dimethylthetin chloride and betaine hydrochloride as methyl donors. DL-Homocysteine 0.03M; methyl donors 0.1M; other details as for Fig. 1. ▲, Substrates incubated alone without liver suspension, coincident with the curve for liver suspension incubated without substrates; ○, suspension with homocysteine, coincident with the curve for suspension with homocysteine and betaine hydrochloride; ●, suspension with homocysteine and dimethylthetin chloride.

series of experiments (see Table 1). A negligible amount of CO_2 was formed from a mixture of the substrates alone or from the tissue alone. The tissue suspension in the presence of homocysteine gave rise to a small gas evolution, and this was greatly increased by the additional presence of the thetin. The ability of the suspension to produce CO_2 under these conditions was still marked after storage for 14 days at 4° , but was completely destroyed after the preparation had been heated to 100° for 15 min.

In Fig. 2 are shown the CO_2 -output curves obtained in a 1 hr. experiment with both dimethylthetin and betaine as methyl donors. The concentrations of thetin and DL-homocysteine used in this experiment approximated to their optimum values as determined with partially purified preparations of the thetin-homocysteine transmethylase described below. The betaine was used at the same concentration as the thetin. Again, only a small evolution of CO_2 was produced by the suspension together with homocysteine, whereas a relatively copious evolution occurred on the addition of dimethylthetin. On the other hand, betaine in place of the thetin as the methyl donor produced no such increase in CO_2 output, despite the synthesis of methionine which was taking place.

Correlation between CO_2 output and methionine formation. Table 2 gives the results of a series of manometric experiments, with dimethylthetin as the methyl donor, in which the amount of methionine formed in the reaction was determined colorimetrically at the end of the 2 hr. period. The concentration of both substrates was 0.003 M. The total CO_2 output in each experiment has been corrected for the small amount of gas produced by the mixture of substrates alone, and also for the amount formed by the tissue suspension in the presence of homocysteine alone. The methionine values have been corrected in the same manner for the 'substrates blank' and the 'tissue + homocysteine blank'. Good agreement between the

corresponding amounts of the two reaction products formed was obtained in all experiments, which justifies the adoption of the manometric procedure as a measure of methionine synthesis under the particular experimental conditions. The correlation has not been tested at higher substrate concentrations, however, owing to the limitations in the method of estimation of methionine.

Partial purification of the thetin transmethylase

With the manometric procedure of assaying enzyme activity, some degree of purification and concentration of the transmethylase has been achieved by ammonium sulphate fractionation. A liver suspension was prepared by homogenizing 18 g. of rat liver in 72 g. of 0.0128 M phosphate buffer, pH 7.4, filtering it through muslin and centrifuging it at 1250 g for 20 min. The volume of the supernatant fluid was made up to 100 ml. with more phosphate buffer before the addition of ammonium sulphate. The most active fraction was precipitated at 0.35–0.45 saturation, although the 0.45–0.55 fraction was also quite active. The precipitate was spun down at 1600 g for 10 min., and dissolved in 10 ml. of phosphate buffer and dialysed against running tap water for 15 hr. at room temperature. It was then centrifuged at 1250 g to remove a bulky precipitate, the major part of the activity remaining in the supernatant portion. This was finally made 0.02 M with respect to NaHCO_3 . The preparation (vol. 15 ml.) contained approx. 8 mg. dry weight of material/ml., and 0.5 ml. of this was able to liberate 250–300 $\mu\text{l.}$ of CO_2 /hr. from 2 ml. of a mixture of dimethylthetin chloride (0.1 M) and DL-homocysteine (0.03 M). When stored at 4° , it remained stable for several weeks without appreciable loss of activity.

Dubnoff & Borsook (1948) obtained highly active preparations of the transmethylase from extracts of Viobin liver (a commercially prepared desiccated-liver preparation) by ethanol fractionation at 0° . This has also been attempted with rat-liver suspensions. The rat-liver enzyme appears to be more ethanol-soluble than that of the Viobin preparation since most of the activity was found in the fraction precipitated over the range 20–40% ethanol, rather than in the 11–18% fraction as found by the American workers.

Experiments with enzyme preparations

The active thetin-transmethylase preparations obtained by ammonium sulphate fractionation of rat-liver suspensions have been shown to produce CO_2 at low concentrations of the substrates in amounts equivalent to the methionine synthesized, as in the suspensions themselves. These preparations have been used in experiments concerned with the effect of substrate concentration and

Table 2. *Correlation between CO_2 evolved and methionine formed from DL-homocysteine and dimethylthetin chloride in the presence of rat-liver suspensions*

Liver suspensions 4%; substrates 0.003 M; bicarbonate-saline medium, pH 7.4; flasks gassed with $\text{N}_2 + \text{CO}_2$ (95:5, v/v); length of experiments 2 hr. at 36.8° .

Expt.	Methionine formed		CO_2 liberated	
	$\mu\text{g.}$ (corr.)	μmoles	$\mu\text{l.}$ (corr.)	μmoles
1	600	4.02	86	3.84
2	480	3.22	71	3.17
3	590	3.96	84	3.75
4	520	3.48	76	3.39
5	545	3.66	86	3.84

enzyme concentration on enzyme activity, this being assessed as the initial reaction velocity given by the particular preparation.

Effect of concentration of substrates. By varying the concentration of one substrate while maintaining that of the other constant, attempts were made to determine the optimum concentration values for dimethylthetin and homocysteine. When the thetin concentration was varied with the homocysteine concentration kept at 0.03M (see below), optimum activity was never completely reached, even at a concentration as high as 0.3M (see Fig. 3). Nevertheless, it was clearly being approached, since raising the concentration from 0.1 to 0.3M increased the initial rate by only 11%. In most subsequent experiments, the thetin chloride has been used at a strength of 0.1M. The relation between initial reaction rate (v) and thetin concentration (s) was a hyperbolic one and a plot of $1/v$ against $1/s$, according to the method of Lineweaver & Burk (1934), gave a straight line. The value of the Michaelis constant for this substrate calculated from the data was 10.2×10^{-3} M.

By keeping the strength of thetin chloride constant at 0.1M, the concentration of DL-homocysteine required for optimum activity was found to be a fairly well-defined value in the region of 0.03M (see Fig. 4), allowance being made for the CO_2 retention by this substrate at the higher concentrations. However, the shape of the activity/concentration curve deviated from a hyperbola at

Fig. 3. Relationship between the initial reaction velocity of a thetin-homocysteine transmethylase preparation and the concentration of dimethylthetin chloride. Initial reaction velocity expressed as volume of CO_2 in $\mu\text{l.}$ produced by the preparation over the period 5–15 min. after mixing the enzyme with the substrates. Medium 0.02M- NaHCO_3 gassed with $\text{N}_2 + \text{CO}_2$ (95:5, v/v); DL-homocysteine 0.03M; temp. 36.8° .

low homocysteine concentrations (Fig. 5), and a plot of $1/v$ against $1/s$ in this case was not linear. This type of deviation might be expected if a constant amount of the homocysteine present was not available to the enzyme, either because of its decomposition or because of reaction with substances other than the enzyme in the preparations. If a hyperbola be fitted to as many of the experimental points as possible, as shown in Fig. 5, it cuts the abscissa at a value of approximately 0.002M homocysteine. If the assumption be made that this amount of the substrate is not available

Fig. 4. Relationship between the initial reaction velocity of a thetin-homocysteine transmethylase preparation and the concentration of DL-homocysteine. Dimethylthetin chloride 0.1M. Other details as for Fig. 3.

Fig. 5. Variation of initial reaction velocity of a thetin-homocysteine transmethylase preparation over the DL-homocysteine concentration range 0–0.016M. Other details as for Fig. 4.

and $1/v$ is plotted against $1/(s-0.002)$, a good straight-line relationship is obtained. With this correction, the Michaelis constant calculated for this substrate was found to be $5.3 \times 10^{-3} \text{ M}$, or approximately half the value obtained for the thetin; it implies a greater affinity of the enzyme for the methyl acceptor.

As experiments have not yet been carried out with the separate isomers of homocysteine, it is not known whether they are both methylated by the transmethyrase. It is likely that only the L-isomer is the true substrate, in which case the Michaelis constant would be lower than the value quoted above.

Effect of enzyme concentration. Contrary to expectations, the relationship obtained between the initial reaction rate, as measured by CO_2 output, and the amount of enzyme preparation present was not a linear one (see Fig. 6). This result was found not only for the partially purified preparations, but also for unfractionated liver suspensions both at low and high substrate concentrations. In view of the fact that a linear activity/concentration relationship is a general characteristic of enzymes, particularly under conditions favourable for optimum activity, it seemed necessary to determine whether or not environmental factors were producing this effect. A number of possible explanations have been examined.

(i) Carbon dioxide evolution may not be a true measure of the enzymic reaction. However, at low substrate concentrations, CO_2 formation has been shown to correspond to methionine synthesis. Furthermore, in several experiments the same activity/concentration relationship was obtained

when activity was measured chemically in terms of the amount of methionine formed.

(ii) Inhibition of the enzyme by substances present in the preparations or by products of the reaction might be responsible. Addition of a heat-inactivated enzyme preparation to the system had no significant effect on the reaction rate, although undoubtedly such an inhibitor might be heat-labile. Against its existence is the finding that two enzyme preparations precipitated from the same liver suspension by two different concentrations of ammonium sulphate gave identical activity/concentration curves, and it seems unlikely that the enzyme and any such inhibitor would be present in the same proportions in both preparations.

The reaction products L-methionine and sodium methylthioacetate have also been tested for their influence on the reaction rate. Neither compound produced any significant effect.

(iii) Interference by side reactions might be responsible. Simultaneous reactions resulting in the removal of substrates, the formation of inhibitory products, or the production of changes in the number of acid or basic groups present, with a consequent effect on CO_2 evolution from the main reaction, have been considered. Since the experimental conditions were anaerobic, the only side reaction likely to cause interference appeared to be the action of homocysteine desulphurase, which would give rise to H_2S and ammonia. However, the preparations employed produced negligible quantities of these products both at low and high concentrations of DL-homocysteine, and neither compound was found to exert any significant effect on the activity of transmethyrase preparations.

It is also possible that as a result of homogenizing the tissue or preparing the active fractions some factor has been lost or has been considerably diluted. Addition of various metal ions to the preparations, including Ca^{2+} , Mg^{2+} , Mn^{2+} , Co^{2+} and Zn^{2+} , was without effect on enzyme activity, but the influence of added substances needs further study.

DISCUSSION

The manometric method for the study of thetin-homocysteine transmethyrase has numerous advantages over procedures involving the estimation of the methionine formed, even though its use is limited to the pH range covered by the H_2CO_3 - NaHCO_3 buffer system. According to Dubnoff & Borsook (1948) the activity/pH curve for the enzyme from Viobin liver preparations has a very flat peak over the pH range 7.0-8.0, so that enzyme action may be studied manometrically under conditions of optimum activity. The method enables the kinetics of the enzyme to be followed in the

Fig. 6. Activity/concentration relationship for a thetin-homocysteine transmethyrase preparation. Dimethyl-thetin chloride 0.1M; DL-homocysteine 0.03M. Other details as for Fig. 3.

presence of high concentrations of homocysteine as well as other thiols, in the presence of methionine and its derivatives and of other compounds giving a positive McCarthy-Sullivan reaction, e.g. dimethyl- β -propiothetin.

No explanation can be offered for the non-linear relationship between enzyme activity and concentration for the liver preparations used in the present series of experiments, and the tentative assumption has been made that the particular experimental conditions employed are responsible. Whether or not it is a true property of the enzyme may be

follow this particular transmethylation reaction manometrically in a bicarbonate medium, as in the thetin-homocysteine reaction already described, thereby avoiding the somewhat troublesome estimation of the creatine formed. Cantoni & Vignos (1954), in a footnote, also refer to the possibility of studying creatine synthesis in this manner. It also appears from reaction 4 that choline formation might be followed manometrically, but this does not appear likely in the methylation of nicotinamide (reaction 3), since the latter reaction is not accompanied by proton formation.

established only when purer preparations are available. It means however that no clear assessment can here be made of the degree of concentration of the enzyme achieved by the ammonium sulphate fractionation.

It is of interest to compare the transmethylation reaction involving dimethylthetin with reactions undergone by 'active methionine' or *S*-adenosyl-methionine. This compound, into which methionine is believed to be converted before its participation in transmethylation reactions (Cantoni, 1951), exhibits the properties of a sulphonium compound (Cantoni, 1953) and its structure has been confirmed by synthesis (Baddiley & Jamieson, 1954). The compound is akin to dimethylthetin, being a γ -*n*-butyrothetin derivative. It is able to act as a direct methyl donor to nicotinamide (Cantoni, 1952), glycocyamine (Cantoni & Vignos, 1954) and probably dimethylethanolamine. It is believed that reactions 3, 4 and 5 take place (Ad represents the adenosyl group).

It will be seen that the last reaction (reaction 5) closely resembles the methylation of homocysteine (reaction 1), in that a proton is liberated as a result of the methylation of the glycocyamine. In the region of pH 7, creatine and glycocyamine exist in their zwitterion forms, and both compounds would be expected to have little tendency to combine with protons. It should therefore be possible to

SUMMARY

1. The methylation of homocysteine by dimethylthetin in the presence of rat-liver suspensions has been studied by estimation of the methionine formed. The estimation of methionine under the particular experimental conditions employed is discussed.

2. A manometric method for following the course of the transmethylation, based on the formation of acid in the reaction, is described.

3. The enzyme involved, thetin-homocysteine transmethylase, has been partially purified from rat liver by ammonium sulphate fractionation and some of its properties have been investigated.

The author wishes to thank Dr M. B. Thorn for many helpful discussions during the course of this work. Grateful acknowledgement is also made to the Endowment Fund of St Thomas's Hospital for aid in the purchase of materials and equipment.

REFERENCES

- Baddiley, J. & Jamieson, G. A. (1954). *Chem. & Ind.* p. 375.
- Bakay, B. & Toennies, G. (1951). *J. biol. Chem.* **188**, 1.
- Borsook, H. & Dubnoff, J. W. (1947). *J. biol. Chem.* **169**, 247.
- Cantoni, G. L. (1951). *J. biol. Chem.* **189**, 745.
- Cantoni, G. L. (1952). *J. Amer. chem. Soc.* **74**, 2942.
- Cantoni, G. L. (1953). *J. biol. Chem.* **204**, 403.

- Cantoni, G. L. & Vignos, P. J. (1954). *J. biol. Chem.* **209**, 647.
 Cohen, P. P. & Hayano, M. (1946). *J. biol. Chem.* **166**, 239.
 Dubnoff, J. W. & Borsook, H. (1948). *J. biol. Chem.* **176**, 789.
 Ericson, L.-E., Williams, J. N. & Elvehjem, C. A. (1955). *J. biol. Chem.* **212**, 537.
 Horn, M. J., Jones, D. B. & Blum, A. E. (1946). *J. biol. Chem.* **166**, 313.
 Lavine, T. F. & Floyd, N. F. (1954). *J. biol. Chem.* **207**, 97.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
 McCarthy, T. E. & Sullivan, M. X. (1941). *J. biol. Chem.* **141**, 871.
 Maw, G. A. (1954). *Biochem. J.* **58**, 665.
 Maw, G. A. (1955). *Biochem. J.* **60**, xxxii.
 Riegel, B. & du Vigneaud, V. (1935-36). *J. biol. Chem.* **112**, 149.
 Rykman, L. R. & Schmidt, C. L. A. (1944). *Arch. Biochem.* **5**, 89.
 Smythe, C. V. (1936). *J. biol. Chem.* **114**, 601.

Structure of a Naturally Occurring Antagonist of Dihydrostreptomycin

BY J. W. CORNFORTH AND A. T. JAMES

National Institute for Medical Research, Mill Hill, London, N.W. 7

(Received 12 August 1955)

The isolation from culture filtrates of *Pseudomonas pyocyanea* of a crystalline product, which antagonizes under certain conditions the antibacterial action of dihydrostreptomycin, has been reported elsewhere (Lightbown, 1950, 1954). This paper records a chemical examination of the product, which has proved to be a mixture, and the synthesis of its principal constituents. The natural product is referred to hereafter as the 'antagonist'.

RESULTS AND EXPERIMENTAL

Use of partition chromatography for isolation of the antagonist. The yields of antagonist from the initial butanol extract of culture filtrates could be increased by evaporating most of the butanol in a rotary evaporator at approximately 30°, adding benzene until the mixture contained about 30% (v/v) benzene, and then running the solution on a kieselguhr column with a stationary phase of 0.1M phosphate buffer (pH 11.9) and butanol-benzene (7:3, v/v) as mobile phase. The column (3 ft. × 1½ in.) contained 200 g. of kieselguhr (Hyflo Super Cel) for a charge containing about 200 mg. of antagonist. The antagonist ran rapidly through the column and emerged free from most of the coloured impurities as well as from some contaminants absorbing in the ultraviolet. The product was further purified by crystallization from benzene-light petroleum.

Properties of the antagonist. The antagonist separated from ethanol or ethyl acetate in ill-defined plates. The melting point, which varied in different specimens, was in the range 136-148° and was little affected by repeated recrystallization. The antagonist was insoluble in water and dilute mineral acids, soluble in aqueous NaOH or Na₂CO₃,

to a yellow solution, and sparingly soluble in ether. It gave an orange-red colour with FeCl₃ in ethanol; it reacted instantly with Br₂-CHCl₃ with liberation of HBr; an aqueous alkaline solution reduced KMnO₄ rapidly in the cold. It did not reduce Fehling's solution; it liberated no iodine from NaI in isopropanol-acetic acid; it gave no azo-dye with diazotized sulphanilic acid. The ultraviolet absorption spectrum in aqueous solution at pH 9 showed a broad maximum at about 350 mμ. (*E*_{1%}^{1cm.} 250) and a sharper peak at 250 mμ. (*E*_{1%}^{1cm.} 700). Analysis indicated an approximate formula C₁₇H₂₃O₂N. (Found: C, 75.0, 74.8; H, 8.45, 8.2; N, 5.0; OMe, 0%.)

Isolation of the antagonist from an organism which has already been shown (Hays *et al.* 1945; Wells, Elliott, Thayer & Doisy, 1952; Wells, 1952) to produce 2-alkyl-4-hydroxyquinolines (I) suggested a chemical relationship, a suggestion further

supported by the general similarity of the ultraviolet absorption spectra. The only essential difference suggested by elementary analysis was the presence of an additional oxygen atom. One