The Oxidation of Dimethylthetin and Related Compounds to Sulphate in the Rat

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Sulphonium salts, such as the trimethylsulphonium and triethylsulphonium halides, possess certain of the pharmacological properties of quaternary ammonium compounds (Crum Brown & Fraser, 1872) and have been given some attention on this account in the past (Hunt & Renshaw, 1925, 1932; Ing & Wright, 1931). More recently, interest in sulphonium compounds has centred on the thetins and their role in transmethylation. Du Vigneaud (1942–3) first reported that dimethylthetin chloride (carboxymethyldimethylsulphonium chloride) $\{(CH_3)_2S^+CH_2CO_2H\}Cl^-$, could support the growth of rats fed on a methyl-deficient diet supplemented with homocystine, and that it could act, like choline and glycine betaine, as a methyl donor in the rat (Du Vigneaud, Moyer & Chandler, 1948). A related thetin, dimethyl-β-propiothetin, was also found to be an active methyl donor (Maw & Du Vigneaud, 1948*a*); this is of significance in view of its natural occurrence in a marine alga (Challenger & Simpson, 1947, 1948). Ethylmethylthetin is partly active in this respect, whereas diethylthetin, lacking the necessary methyl groups, is inactive (Maw & Du Vigneaud, 1948*c*). Dubnoff & Borsook (1948*a*), using rat, pig and guinea-pig liver suspensions, demonstrated the ability of dimethylthetin and dimethyl-β-propiothetin to methylate homocysteine anaerobically *in vitro* and showed that a specific transmethylase was involved (Dubnoff & Borsook, 1948*b*). Only one methyl group per molecule of thetin was transferred in these experiments. The methyl groups of these two thetins can also be rapidly oxidized to carbon dioxide by the rat (Ferger & Du Vigneaud, 1950).

A fairly clear picture has thus been established of the metabolic utilization of one of the methyl groups of dimethylthetin and related thetins, but information regarding the fate of the rest of the molecule, particularly of the sulphur, is lacking. The experiments described here are concerned with the extent to which the sulphur of such compounds is converted to sulphate by the intact rat, and with the nature of the possible intermediates in the path of sulphate formation. Dimethylthetin, in the form of its chloride, has been found to undergo rapid and extensive oxidation to sulphate. Experiments with seven other related sulphonium chlorides indicate that it is not a general catabolic reaction for this type of compound. In the limited series of compounds studied, there is evidence of a correlation between transmethylation and sulphate formation. It is considered that loss of a methyl group represents an initial and obligatory step in the catabolism to sulphate of dimethylthetin and probably other allied compounds.

MATERIALS AND METHODS

Preparation of compounds. The chlorides of dimethylthetin, ethylmethylthetin and diethylthetin were prepared by incubating a mixture of equimolar amounts of monochloroacetic acid and the appropriate sulphide at 35° for 24 hr., followed by washing of the sulphonium salt with ether and recrystallization from ethanol. Dimethyl-β-propiothetin chloride (β-carboxyethyldimethylsulphonium chloride) was most satisfactorily prepared by way of the bromide from methyl sulphide and β-bromopropionic acid (Challenger & Simpson, 1948). Sulphocholine chloride (β-hydroxyethyldimethylsulphonium chloride) and β-hydroxyethyl methyl sulphide were prepared as described by Maw & Du Vigneaud (1948*b*). Trimethylsulphonium, ethyldimethylsulphonium and triethylsulphonium chlorides were obtained from the iodides by treatment with AgCl. Sodium *S*-methylthioglycollate was made by treatment of the sodium derivative of methyl thioglycollate with methyl iodide. The resulting methylated ester was hydrolysed with dilute alkali, and after acidification, the *S*-methylthioglycollic acid was extracted with ether and converted to the Na salt. Sodium *S*-ethylthioglycollate was prepared in a similar manner from methyl thioglycollate and ethyl iodide. Sodium thioglycollate was prepared from a freshly distilled sample of the acid.

Plan of metabolism experiments. Pairs of male rats (wt. 120–160 g.) were used. The routine adopted in the animal experiments, the diet used, the urine collection and the determination of urinary sulphate were as in the preceding paper (Maw, 1953).

Administration of compounds. Although neutralized aqueous solutions of dimethylthetin chloride can be administered to rats orally or by injection in single doses of 15 mg. S/rat without any untoward effects, some of the other compounds, e.g. trimethylsulphonium and triethylsulphonium chlorides and sodium thioglycollate, are toxic at this level and caused death of the animals. All compounds were well tolerated, however, when administered by incorporation with the diet, and this mode of dosage was, therefore, adopted, since not only did it allow relatively

large doses to be employed, but the slow administration gave the maximum opportunity for absorbed compounds to be metabolized with the minimum of their renal excretion. The daily food consumption of each pair of rats was determined during an acclimatization period of 4–5 days. This amount of food was then supplied daily throughout the rest of the experiment. After a further control period of 4–5 days, the compound under study was mixed in with 1 day's allocation of food, and for a final period of 4–5 days the animals were fed on the diet alone. Where possible, the compounds were added to the diet in the dry state, but those sulphonium chlorides deliquescent were added in 4 ml. of water, the amount of compound present being calculated from the chloride content of the solution. The majority of compounds used did not cause any change in food intake when given with the diet. When, however, any food remained uneaten on the day of the administration, the uneaten portion was weighed and the actual amount of compound ingested then calculated. Urine was collected every 24 hr. from the beginning of the control period.

RESULTS

In an initial series of experiments a considerable proportion of an orally administered dose of dimethylthetin was found to be oxidized to sulphate by the rat, the resulting increase in urinary sulphate output being associated with the inorganic but not the ethereal fraction. This increase took place over a 2-day period, since the rats consumed the diet containing the compound mainly at night, towards the end of the 24 hr. period during which it was administered. It was therefore to be anticipated that catabolites of the thetin, such as sulphate, would be present in the urine on the second day after administration. The 24 hr. inorganic sulphate outputs then generally returned to pre-dosage levels, although in some experiments they were still significantly raised 4–5 days later. The excretion of inorganic sulphate during the control period was subject to as little variation as in previous experiments (Maw, 1953). Increases in sulphate output produced experimentally have therefore been assessed as the amounts excreted in excess of the average output for the control period. Table 1 gives the summarized results for four experiments and also includes results for three experiments in which neutralized solutions of dimethylthetin chloride were administered by injection. In these last experiments considerable conversion of the thetin to sulphate was again obtained, but the increase in urinary sulphate output was complete 24 hr. after the injection. The values for the percentage oxidation of injected doses were sufficiently close to those obtained when the compound was administered orally to indicate that the oxidation occurred in the tissues of the rat itself and was not to any great extent, if at all, the result of bacterial action in the intestine.

Table 1. *Oxidation of dimethylthetin chloride to sulphate by the rat*

Mode of administration	Dose (mg. S/rat)	Percentage of S of the dose excreted as inorganic sulphate: 1st day	2nd day	Total
By mouth (mixed with the diet)	16·0	57·9	0	57·9
	14·0	57·6	0	57·6
	16·1	61·1	7·6	68·7*
	8·0	47·1	9·6	56·7
By subcutaneous injection	15·3	53·2	3·9	57·1
	15·3	37·4	0	37·4
	15·3	43·7	0	43·7

* Inorganic sulphate excretion still significantly above the pre-dosage level 6 days after administration.

Table 2. *Oxidation to sulphate by the rat of compounds related structurally to dimethylthetin chloride*

(Compounds were administered by addition to the diet unless otherwise stated.)

Compound	Dose (mg. S/rat)	Percentage of S of the dose excreted as inorganic sulphate: 1st day	2nd day	Total
Diethylthetin chloride	13·2	0	0	0
	13·5	0	0	0
Ethylmethylthetin chloride	10·7	17·6	4·5	22·1
	14·6	22·1	4·3	26·4
Dimethyl-β-propiothetin chloride	13·0	24·9	42·0	66·9*
	13·3	38·9	27·4	66·3
Trimethylsulphonium chloride	6·8	0	3·8	3·8
	6·3	0	3·7	3·7
Ethyldimethylsulphonium chloride	6·0	0	0	0
	8·3	0	1	1
Triethylsulphonium chloride	10·1	0	0	0
Sulphocholine chloride	10·4	11·0	10·2	21·2
	13·6	28·2	12·4	40·6*
	13·6	21·4	0	21·4
	9·6	13·9	0	13·9
	14·1†	14·4	2·9	17·3
β-Hydroxyethyl methyl sulphide	15·4	48·6	12·6	61·2
	17·0	60·0	0	60·0

* Inorganic sulphate excretion still significantly higher than the pre-dosage level 4–5 days after administration.
† Administered by subcutaneous injection.

In view of the ease and extent of oxidation of dimethylthetin by the rat, the formation of sulphate from some related compounds was studied in order to see whether the process is specific for dimethylthetin or whether it is a general one for sulphonium compounds. The results obtained are shown in Table 2. When an increase in urinary sulphate was obtained, it took place over a 2-day period. Dimethyl-β-propiothetin was oxidized to sulphate

about as extensively as dimethylthetin itself; ethylmethylthetin was oxidized to a lesser extent, whereas diethylthetin did not form sulphate. Three simple alkylsulphonium salts, trimethylsulphonium, ethyldimethylsulphonium and triethylsulphonium chlorides were not significantly oxidized. Sulphocholine chloride, on the other hand, did form sulphate, but the percentage conversion ranged from 13·9 to 40·6. It is thus evident that oxidation to sulphate is not a general metabolic reaction for sulphonium salts.

Possible intermediates in sulphate formation. It is believed that in the animal dimethylthetin is converted to *S*-methylthioglycollic acid by transfer of a methyl group (Maw & Du Vigneaud, 1948*c*; Dubnoff & Borsook, 1948*b*), although this compound has not yet been isolated as a catabolite. It is also reasonable to suppose that the methyl donor ethylmethylthetin, on demethylation, gives rise to *S*-ethylthioglycollic acid. Sodium salts of these two alkylthioglycollic acids and thioglycollic acid itself were administered to rats; the percentages converted to sulphate are given in Table 3. Thioglycollic acid is known to be partly oxidized in this way in dogs and rabbits (Smith, 1893; Hill & Lewis, 1924). In the rat the percentages in two experiments were 35·5 and 38·8. The urine voided during the 2-day period after administration gave a negative test for SH groups but a marked positive reaction for SS groups (Brand, Harris & Biloon, 1930), suggesting partial conversion of the compound to dithiodiglycollic acid. *S*-Methylthioglycollic acid was oxidized to a considerable extent, but *S*-ethylthioglycollic acid less so. It is of interest that the values obtained for these two alkylthioglycollic acids correspond closely to those obtained for the thetins from which, it is suggested, they are derived. Table 2 includes results for the administration of β-hydroxyethylmethyl sulphide to rats. The ease with which this compound is oxidized to sulphate may implicate it as a metabolite of sulphocholine if the latter compound were to undergo enzymic demethylation.

Table 3. *Oxidation to sulphate by the rat of possible intermediates in the metabolism of thetins*

(Compounds were administered by addition to the diet.)

Compound	Dose (mg. S/rat)	Percentage of S of the dose excreted as inorganic sulphate: 1st day	2nd day	Total
Sodium thioglycollate	16·0	35·5	0	35·5
	15·1	37·3	1·5	38·8
Sodium *S*-methylthioglycollate	15·1	57·4	0	57·4
	11·8	56·4	8·0	64·4
Sodium *S*-ethylthioglycollate	15·2	25·5	4·0	29·5
	15·2	20·0	0	20·0

Many sulphonium compounds of the form $\{RS^+(CH_3)_2\}X^-$ may be degraded chemically in the presence of alkali to methyl sulphide, which is also evolved from the marine alga *Polysiphonia fastigiata* as a result of the enzymic breakdown of the naturally occurring dimethyl-β-propiothetin (Challenger & Simpson, 1947, 1948). Furthermore, the urines collected after the administration of sulphocholine to rats smelt of methyl sulphide, although whether its presence was the result of enzymic action, bacterial action, or simple chemical degradation in urine has not been studied. Methyl sulphide has therefore been examined as a likely intermediate in sulphate formation. It was administered as a solution in arachis oil either by injection or by incorporation in the diet. The results for its oral administration are undoubtedly subject to criticism on the grounds of its high volatility, and to offset losses from the food a larger amount of the sulphide was used (equivalent to 30 mg. sulphur/rat). When administered by injection it was clearly detectable in the breath of the rats within 1 hr. of dosing. In six experiments, no resultant increase in urinary sulphate was observed. Were methyl sulphide an intermediate in the catabolism of dimethylthetin to sulphate, some increase in sulphate output in these experiments might have been expected, considering the large proportion of dimethylthetin sulphur oxidized in this way.

DISCUSSION

Of the eight sulphonium chlorides administered orally to rats only dimethylthetin, dimethyl-β-propiothetin, ethylmethylthetin and sulphocholine caused an increase in urinary inorganic sulphate, indicating that the oxidation process involves a certain degree of enzymic specificity. The first three of these compounds are active as methyl donors in the rat (Maw & Du Vigneaud, 1948*a*, *c*), and the first two in liver suspensions (Dubnoff & Borsook, 1948*a*, *b*), in which one methyl group per molecule of thetin is transferred to homocysteine. The most likely intermediates in the path of oxidation of dimethylthetin and ethylmethylthetin to sulphate are their demethylated products, *S*-methylthioglycollic acid and *S*-ethylthioglycollic acid respectively. This is in accord with the finding that the two alkylthioglycollic acids undergo oxidation to sulphate in the rat. Diethylthetin is inactive in promoting rat growth on a homocystine-containing diet and is not lipotropic. It is not oxidized to sulphate, and this is most probably because, unlike ethylmethylthetin, no mechanism exists for its conversion to *S*-ethylthioglycollic acid. Trimethylsulphonium chloride is likewise not a methyl donor

(Du Vigneaud & Maw, unpublished observations) and is not oxidized to sulphate, possibly for a similar reason. It is considered that loss of a methyl group either by transmethylation or by other degradative reactions represents an initial and obligatory step in the catabolism to sulphate of dimethylthetin, ethylmethylthetin and probably dimethyl-β-propiothetin. The possibility that the two processes are quite separate metabolic pathways is not ruled out, but the case of diethylthetin argues against this.

Sulphocholine is oxidized to a certain extent and if a preliminary demethylation were involved it would give rise to β-hydroxyethyl methyl sulphide, which forms sulphate to a considerable extent. In growth studies with rats, sulphocholine supplied in a methyl-free diet has definite lipotropic activity but does not appear to act as an active methyl donor, judged by the growth criterion (Maw & Du Vigneaud, 1948*b*), although any definite conclusions are complicated by the toxic nature of the compound. However, Dubnoff (1949) has reported that sulphocholine can methylate homocysteine *in vitro* under aerobic, but not anaerobic, conditions, and he suggests that sulphocholine is initially oxidized to dimethylthetin, just as choline is oxidized to betaine before taking part in its transmethylation reactions. Under these circumstances sulphocholine would eventually be demethylated, but to *S*-methylthioglycollic acid rather than to β-hydroxyethyl methyl sulphide. The behaviour of sulphocholine *in vitro* needs further examination, but may not be irreconcilable with the rat-growth studies discussed, since *in vivo* the compound may be releasing methyl groups, either directly or after oxidation to the thetin, too slowly to be capable of acting as a sole methyl source. Dubnoff's findings would account for the oxidation of sulphocholine to sulphate, and would extend the relationship between transmethylation and sulphate formation in the sulphonium series.

The intermediates in the metabolism of *S*-methylthioglycollic acid and *S*-ethylthioglycollic acid to sulphate are not yet known. *S*-Methylthioglycollic acid is not a methyl donor either *in vivo* (Maw & Du Vigneaud, 1948*c*) or *in vitro* (Dubnoff & Borsook, 1948*b*). It might, however, give rise to thioglycollic acid or dithiodiglycollic acid by oxidative demethylation. The urines of rats fed dimethylthetin and *S*-methylthioglycollic acid invariably gave negative tests for SH and SS groups, although any thioglycollic acid formed endogenously might well have been metabolized as fast as it was produced. Other possible catabolites include methyl mercaptan, methanesulphonic acid and sulphoacetic acid. The last two compounds when administered to rats by injection are not significantly oxidized to sulphate (Maw, 1953).

Methyl sulphide also requires consideration as a possible catabolite of sulphonium compounds, since its non-enzymic formation from a number of aliphatic dimethylsulphonium salts takes place readily and since it is formed enzymically from dimethyl-β-propiothetin (Challenger & Simpson, 1947, 1948). Repeated administration of the sulphide to rats, orally or by injection, has never resulted in an increase in urinary sulphate output. No decisive conclusions as to its role as a precursor of sulphate can at present be drawn from these experiments, in which it was of exogenous and not endogenous origin. This is also true for the experiments with methanesulphonic and sulphoacetic acids. But it may be pointed out that if the oxidation of a sulphonium salt, such as dimethylthetin, to sulphate requires an initial demethylation, methyl sulphide could never be formed, except by an entirely separate catabolic process. It is hoped that *in vitro* experiments will provide further information concerning the reactions involved.

SUMMARY

1. Dimethylthetin chloride administered to pairs of rats was oxidized to inorganic sulphate to the extent of 57·6–68·7 % when given orally and 37·4–57·1 % when injected subcutaneously.

2. Of seven other sulphonium compounds only ethylmethylthetin, dimethyl-β-propiothetin and sulphocholine were oxidized in this way, indicating that the formation of sulphate is not a general mode of metabolism for this type of compound.

3. In the limited series of compounds studied, oxidation to sulphate was confined to sulphonium salts which are able to act as biological methyl donors, and it is suggested that demethylation is an initial and obligatory step in their catabolism to sulphate.

4. Possible intermediates in the oxidation are discussed.

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The Estimation of D- and L-Glutamic Acid in Proteins

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In connexion with studies on the glutamic acid content of tumour proteins it was necessary to estimate the respective amounts of the two optical isomers given on acid hydrolysis. The enzymic decarboxylation procedure of Gale (1945) was applicable to the L-isomer, but a search for a specific method for the D-isomer was not successful. Experience showed that it was not oxidized by the specific D-amino acid oxidase of kidney and that the activity of fungal oxidases (cf. Bender & Krebs, 1950; Emerson, Puziss & Knight, 1950) was very low. Moreover, the preparation from rabbit liver of an enzyme able to oxidize D-glutamic acid and D-aspartic acid (Still, Buell, Knox & Green, 1949; Still & Sperling, 1950) could not be repeated. Total glutamic acid was therefore estimated by an ion-exchange method (Consden, Gordon & Martin, 1948), which does not distinguish between the isomers, and the D-component found by difference. The present communication concerns the application of this two-step procedure to four purified proteins as well as to certain protein preparations and derivatives which have been reported by previous workers to contain a fair proportion of the D-isomer. In addition, opportunity is taken to present new evidence on the racemization of the L-isomer during acid hydrolysis.

EXPERIMENTAL

General methods

Nitrogen determinations were made by the micro-Kjeldahl procedure. Amino N was estimated by the method of Van Slyke (4 min. reaction time). L-Glutamic acid was estimated by the decarboxylase procedure of Krebs (1948), using a washed suspension of *Clostridium welchii* S.R. 12. Optical rotations were determined in a 4 dm. tube unless otherwise stated. Protein hydrolysates were prepared by boiling with 6N-HCl for 24 hr. Evaporations *in vacuo* were conducted below 40°.

Materials

The sample of L-glutamic acid hydrochloride used was recrystallized; $[\alpha]_D^{20} + 31{\cdot}4°$ (*c*, 4·0 in 2·5N-HCl). The other amino-acids were purified commercial samples. The following proteins were purified samples available in the laboratory; the N content is quoted on a moisture- and ash-free basis unless stated: Edestin, N = 18·65%; ox insulin, recrystallized according to Du Vigneaud, Miller & Bodden (1939), N = 15·58%; horse myoglobin, N = 16·6% (not corrected for ash). Preparations of casein and gelatin described below were made from commercial samples.

L-Pyrrolidonecarboxylic acid. L-Glutamic acid was heated as described by Wilson & Cannan (1937). The anhydride was extracted from aqueous solution with ethyl acetate, from which solvent it was twice recrystallized. $[\alpha]_D^{20} - 9{\cdot}92°$ (*c*, 7·5 in water).

Amino acid control mixtures. These were made up to simulate *A*, haemoglobin; *B*, gliadin; *C*, myoglobin (Table 1). *A* was rich in aspartic acid, *B* in glutamic acid and *C* had these two amino acids present in the proportions usually found in proteins.

Tobacco-mosaic-virus protein. This was prepared by heat coagulation of the nucleoprotein and was a gift from Dr J. D. Smith, Molteno Institute, Cambridge; N = 17·89% (not corrected for ash).

Dried cells of Lactobacillus casei. Strain 3253 of the National Collection of Type Cultures was grown for 50 hr. at 30° in 1 l. flasks of the medium described by Rodwell (1953). The flasks were chilled to 0° and the bacteria separated by centrifuging. They were washed thrice with