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Sulphate Formation from Dimethylthetin in Rat Liver

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Dimethylthetin chloride, i.e.

has been established as a biological methyl donor on the basis of its ability to enhance the growth rates of rats fed on a methyl-free diet supplemented with homocysteine (du Vigneaud, Moyer & Chandler, 1948; Maw & du Vigneaud, 1948) and its ability to promote methionine synthesis from homocysteine in the presence of liver preparations (Dubnoff & Borsook, 1948). Transfer of one methyl group from the thetin to homocysteine or homocystine takes place and the compound is believed to be converted into methylthioacetic acid. The methyl group of methylthioacetic acid, however, does not appear to be labile, since the acid does not have the growth-promoting properties of a methyl donor, and since the administration of trideuteriomethylthioacetic acid to rats does not result in the appearance of deuteriomethyl-labelled choline or creatine in the tissues (Maw & du Vigneaud, 1948). Furthermore, the acid is unable to methylate homocysteine *in vitro* (Dubnoff & Borsook, 1948).

In the intact rat the sulphur of dimethylthetin chloride, administered orally or by injection, has been shown to be converted to a considerable extent into urinary inorganic sulphate (Maw, 1953). This oxidation is considered to be connected with the methyl group lability, since ethylmethylthetin chloride and dimethyl- β -propiothetin chloride, i.e.

which are also methyl donors, are likewise oxidized to sulphate, whereas diethylthetin chloride and trimethylsulphonium chloride, i.e. $\{(\text{CH}_3)_3\text{S}^+\} \text{Cl}^-$, which are not methyl donors, are not so oxidized.

The present communication describes a series of *in vitro* experiments in which the formation of inorganic sulphate by rat-tissue preparations incubated with dimethylthetin chloride and some related

compounds has been followed. It has been found that in contrast to its behaviour in the whole animal, dimethylthetin chloride alone is not oxidized to sulphate by liver or kidney slices, but that a mixture of the thetin and homocysteine gives rise to a higher output of sulphate in liver-slice preparations than does homocysteine alone. This implies that the oxidation of the thetin is dependent on its preliminary demethylation, as suggested by earlier animal experiments (Maw, 1953). Methylthioacetic acid, the most likely demethylation product, is readily oxidized by liver slices and to a lesser extent by kidney slices. Conversion of this compound into sulphate in the presence of fortified liver suspensions and acetone-dried liver powders has not so far been obtained. Its oxidation by liver slices has been compared with that of a number of related compounds, and also with that of cysteine, *S*-methylcysteine and methionine.

MATERIALS AND METHODS

Preparation of compounds

Dimethylthetin chloride and the sodium salts of mercaptoacetic (thioglycollic), methylthioacetic (*S*-methylthioglycollic) and ethylthioacetic (*S*-ethylthioglycollic) acids were prepared as described in a previous paper (Maw, 1953). Dithiodiglycollic acid, i.e. $(-\text{S} \cdot \text{CH}_2 \cdot \text{CO}_2\text{H})_2$, was prepared by the method of Westerman & Rose (1927) and converted into the neutral sodium salt. Sodium *S*-phenylthioacetate (*S*-phenylthioglycollate) was made from thiophenol and ethyl chloroacetate by a method similar to that used for the two alkylthioacetates. *S*-Methyl-L-cysteine was prepared by the reduction and subsequent methylation of L-cysteine in liquid ammonia (du Vigneaud, Loring & Craft, 1934). L-Cysteine hydrochloride, DL-homocysteine, L-methionine and adenosine 5'-phosphate (AMP) were obtained from Roche Products Ltd., diphosphopyridine nucleotide (DPN) and cytochrome *c* from L. Light and Co. Ltd.

Procedure in metabolism experiments

Male black and white rats (wt. 120–150 g.) were killed by a blow on the head, the livers removed and slices cut in the usual way. Slices of approximately 200 mg. wet wt. were placed in 50 ml. Pyrex beakers containing 8 ml. of liquid medium, the composition of which is given below. The beakers were placed in an apparatus similar to that designed by Dubnoff (1948). This consisted of a rectangular wire tray fitted with rows of spring clips for holding the beakers, the tray being suspended in a thermostatically controlled water bath. The level in the bath was so adjusted that the beakers were immersed to a depth of 3.5 cm. (the depth of liquid within the beakers was 0.8 cm.). The beakers were totally enclosed by an airtight Perspex hood resting on the bottom of the tray and fitted with an inlet tube connected to a cylinder of the appropriate gas mixture, and an outlet tube. Beakers of volume 50 ml. were employed as reaction vessels because they allowed of a relatively large ratio of surface area to volume of the contents and thus of good contact with the gas phase within the hood. The tray was shaken mechanically in the bath at the rate of 100 oscillations/min. with an amplitude of 2.3 cm. The temperature of the bath was $37.0 \pm 0.1^\circ$.

The medium in each beaker consisted of 5 ml. of either 'phosphate saline' or 'bicarbonate saline' (Krebs & Henseleit, 1932) in which MgSO_4 was replaced by MgCl_2 (Pirie, 1934*a*), together with 3 ml. of water containing the various compounds under investigation and any added reagents, all previously adjusted to pH 7.4. Metabolism experiments were of 2 hr. duration unless otherwise stated. During the first 30 min. the appropriate gas mixture (100% O_2 or N_2 in the case of the phosphate saline medium, and 95% O_2 –5% CO_2 with the bicarbonate medium) after being saturated with water in a bubbler, was passed rapidly through the hood, the flow being reduced during the remaining 90 min. At the end of the period, the tissue slices were removed with forceps, washed with water, dried at 104° for 3 hr. and weighed. The contents of each beaker were deproteinized by the addition of 2 ml. of 20% trichloroacetic acid (TCA), and after standing for 30 min. filtered through a Whatman no. 1 paper.

Suspensions of fresh liver were prepared with the apparatus of Potter & Elvehjem (1936). The liver (2 g.) was ground up with a little ice-cold saline, made up to 50 ml. with more cold saline and filtered through two layers of muslin, 5 ml. of this suspension being used in each beaker in metabolism experiments, together with 3 ml. of water containing the added compounds. Acetone-dried liver powders were prepared in the usual way. Suspensions were prepared from 400 mg. of the powder made up to 50 ml. with saline and 5 ml. used in each beaker.

Tissue from one liver only was employed in each experiment. Beakers containing the tissue controls and the compounds under test were always prepared in duplicate, and the mean of each pair of resulting sulphate values taken.

Estimation of inorganic sulphate

The method used was that of Cuthbertson & Tompsett (1931) with certain minor modifications. A 0.5% solution of benzidine in acetone (5 ml.) was pipetted into finely tapered 15 ml. centrifuge tubes followed by 2 ml. of the TCA filtrates, the contents of each tube were mixed with a fine glass rod, and the tubes then placed in crushed ice in a

stoppered thermos jar. A period of 1 hr. in ice has been suggested by Cope (1931) and by Medes & Floyd (1942) as being adequate for quantitative precipitation of benzidine sulphate under these conditions. Pirie (1934*a*) suggested a minimum period of 2 hr. but in the present experiments at least 4 hr. was considered necessary, and it was found more convenient to leave the tubes in ice overnight (18–19 hr.). The sides of each tube were then washed down with 0.5 ml. of ice-cold acetone and the tubes centrifuged for 10 min. at 1250 g at $2-4^\circ$. Centrifuging at room temperature led to variable analytical results. Dodgson & Spencer (1953) in their experiments experienced difficulty in removing the supernatant liquid without disturbing the precipitate. This was not encountered with filtrates obtained from the metabolism experiments described here, although occasionally it did happen when the method was being tested on pure solutions of sodium or potassium sulphate. The tubes were drained by inverting them on a hard filter paper and the precipitates resuspended in 2 ml. of ice-cold acetone and recentrifuged under the same conditions for 5 min. The tubes were again drained of supernatant liquid and the washing of the precipitates in acetone and the centrifuging repeated. To each tube, drained finally of acetone, was added 1 ml. of N-HCl ; the tubes were placed in a boiling-water bath for 2 min. and the contents then washed out into 25 ml. graduated flasks with about 15 ml. of water.

The benzidine sulphate was estimated colorimetrically by the addition to each flask of 1 ml. of 0.1% NaNO_2 followed 1 min. later by 2 ml. of 1% thymol in 20% KOH . The volumes were made up to 25 ml. after 10 min. and the colour intensities compared after a further 10 min. against a water blank in a Hilger Spekker absorptiometer using Ilford 603 filters (max. transmission, 470–520 $\text{m}\mu$). Sulphate-S values were read from a calibration curve prepared from readings obtained with solutions of potassium sulphate containing the same amount of TCA as did the samples from metabolism experiments.

The above procedure gave very consistent results and the original calibration curve was checked frequently over a period of a year without showing any change. The reagents employed in the estimation gave rise to a small blank reading, constant for each set of reagents and filter papers used, and ranging from 4 to 10 μg . throughout the experimental series. The appropriate blank has been subtracted from all sulphate-S values recorded. It was determined by making up 5 ml. of the saline together with 2 ml. of 20% TCA to 10 ml., filtering the solution through a Whatman no. 1 paper and using 2 ml. of the filtrate in the above estimation. This procedure was carried out whenever a new batch of saline or other reagents was prepared, or whenever a new box of filter papers was opened.

All glassware was cleaned with nitric acid–chromic acid mixture and all solutions prepared with de-ionized water.

RESULTS

Inorganic sulphate in incubated liver slices

Relatively little data is available in the literature on the oxidative conversion of sulphur-containing compounds into sulphate by tissue slices and extracts. Pirie (1934*a, b*) observed the oxidation to sulphate of cysteine, cystine, glutathione, methionine and a number of other compounds by slices of

rat tissues, of which liver and kidney were the most active. Medes (1939) and Medes & Floyd (1942) confirmed these findings and extended their experiments to liver breis and extracts. Sulphate was also produced by tissue slices and extracts without the addition of sulphur-containing compounds to the medium. Medes (1939) gave average values for these tissue sulphate 'blanks' corresponding to 28.7 μ g. sulphate-S/100 mg. dry wt. of tissue for liver slices and 54.1 μ g. for kidney slices, both incubated for 2 hr. at 38° in a bicarbonate-containing medium.

In the present series of experiments sulphate-S determinations, carried out on liver slices incubated alone for 2 hr. at 37° in either phosphate saline or bicarbonate saline, gave values ranging from 14.0 to 35.4 μ g. sulphate-S/100 mg. dry wt. of tissue (average of thirty-nine values, 24.8 μ g.). Duplicate estimations in any one experiment generally agreed to within 5 μ g. There was no noticeable difference between blank values obtained with either saline medium, and addition of 10 mg. of glucose to the medium had no effect on the amount of sulphate accumulating. Similar values were obtained with kidney slices (average 33.2 μ g. of

sulphate-S/100 mg. dry wt. of tissue/2 hr.). Sulphate estimations carried out on liver slices which were allowed to stand in either medium for 10 min. at room temperature gave values approximately half those for incubated slices. The sulphate appearing at room temperature may have arisen by diffusion from the slices during their short period in the medium, and it is probable, therefore, that the sulphate obtained after incubation was the result of further diffusion from the slices rather than of any oxidation occurring during the experiment.

Experiments with dimethylthetin chloride

When dimethylthetin chloride was incubated with liver slices in either phosphate saline or bicarbonate saline, no increase in sulphate output was obtained (Table 1). This was an unexpected finding, in view of the extensive oxidation of the thetin which takes place in the whole animal. However, when DL-homocysteine was added to the medium, a larger amount of sulphate was formed than was obtained with homocysteine alone (Table 2). Homocysteine and homocystine are recognized methyl acceptors, both *in vivo* and *in vitro* and the data in Table 2 have been interpreted as indicating that dimethylthetin requires to be demethylated before being metabolized to sulphate. Glycocyamine, dimethylethanolamine and nicotinamide, known to be methyl acceptors specific for methionine, did not produce this sulphate increase in the presence of the thetin. The increase obtained with homocysteine although consistent was not large. This is probably accounted for by the fact that transmethylation reactions *in vitro* are relatively slow and, furthermore, those involving homocysteine are markedly inhibited by the presence of oxygen (Borsook & Dubnoff, 1947; Dubnoff & Borsook, 1948), an essential condition of the present experiments. In addition, the homocysteine was being lost from the system by oxidation to homocystine and sulphate and also by conversion into methionine by methyl donors

Table 1. *Sulphate formation by rat-liver and -kidney slices in the presence of dimethylthetin chloride*

Contents of each beaker: 5 ml. saline, 3 ml. water containing 10 mg. dimethylthetin chloride neutralized to pH 7. Wet wt. of slices, approx. 200 mg. Incubation period, 2 hr. at 37°. Sulphate expressed as μ g. S/100 mg. dry wt. of tissue.

Tissue	Type of saline used	Sulphate formed in the presence of	
		Control slice	Dimethylthetin chloride
Liver	Bicarbonate	26.1	28.2
Liver	Bicarbonate	32.5	29.4
Liver	Bicarbonate*	21.3	23.3
Liver	Phosphate	26.3	24.1
Kidney	Bicarbonate	34.9	39.5

* Containing 10 mg. added glucose.

Table 2. *Sulphate formation by rat-liver slices in the presence of dimethylthetin chloride and DL-homocysteine*

Contents of each beaker: 5 ml. saline, 10 mg. dimethylthetin chloride in 2 ml. water, neutralized to pH 7, and/or DL-homocysteine in 1 ml. water, neutralized to pH 7, and water to 8 ml. Wet wt. of slices, approx. 200 mg. Incubation period, 2 hr. at 37°. Sulphate expressed as μ g. S/100 mg. dry wt. of tissue.

Type of saline used	Amount of homocysteine present (mg.)	Sulphate formed in the presence of			
		Control slice	Dimethylthetin chloride	Homocysteine	Dimethylthetin chloride + homocysteine
Bicarbonate	5	27.1	26.5	108.7	139.6
Bicarbonate	5	31.2	—	73.8	123.7
Bicarbonate	10	22.9	23.8	113.7	174.6
Bicarbonate	10	—	—	141.3	174.9
Phosphate	10	23.8	—	86.5	124.6
Phosphate	10	27.0	—	84.2	110.4

Table 3. *Sulphate formation by rat-liver slices in the presence of sodium methylthioacetate and some related compounds*

Contents of each beaker: 5 ml. saline, 3 ml. water containing the compound under study (amount of each compound present equivalent to 64 μ moles, unless otherwise stated). Wet wt. of slices, approx. 200 mg. Period of incubation, 2 hr. at 37°. Sulphate expressed as μ g. S/100 mg. dry wt. of tissue.

Compound under study	Amount in each beaker (mg.)	Type of saline used	Sulphate formed in the presence of	
			Control slice	Added compound
Sodium methylthioacetate	8.2	Bicarbonate	20.7	135.6
	8.2	Bicarbonate	35.3	131.6
	8.0	Phosphate	14.9	82.8
	8.2	Phosphate	26.3	190.7
	6.0*	Phosphate†	19.5	16.2
Sodium mercaptoacetate	7.3	Bicarbonate	22.5	50.2
	7.3	Phosphate	18.1	54.5
Sodium ethylthioacetate	9.1	Bicarbonate	35.3	71.4
Sodium phenylthioacetate	12.2	Bicarbonate	35.3	31.2
Disodium dithiodiglycollate	7.2	Bicarbonate	22.5	68.8
	7.2	Phosphate	18.1	43.2
L-Cysteine hydrochloride	10.1	Bicarbonate	28.8	261.0
S-Methyl-L-cysteine	8.7	Bicarbonate	28.8	88.0
L-Methionine	9.5	Bicarbonate	33.7	58.8

* Equivalent to 47 μ moles.

† Under anaerobic conditions (100% N₂).

Fig. 1. Relationship between the amount of sulphate formed by rat-liver slices and the amount of sodium methylthioacetate present. The results shown were obtained from three separate experiments.

initially present in the tissue, as observed by Borsook & Dubnoff (1947). The sulphate increase reported could have arisen not only from the oxidation of the demethylation product of the thetin, but also from the oxidation of the methionine formed in equivalent amounts from homocysteine. Methionine is oxidized at a slower rate than is methylthioacetic acid, however (see Table 3), and would contribute only partially to the increase. In any case, whether the sulphate increase originated

from methylthioacetic acid, from methionine, or from both compounds, it would still implicate methyl transfer as the initial metabolic step.

Oxidation of methylthioacetic acid and related compounds by liver slices

Methylthioacetic acid was oxidized aerobically, but not anaerobically, to sulphate by liver slices, and also to a slight extent aerobically by kidney slices. In Table 3 are shown figures for its oxidation by liver slices together with data for some related compounds including L-cysteine, S-methyl-L-cysteine and L-methionine, all compounds being studied at comparable concentrations. The acid was oxidized to about the same extent as DL-homocysteine (Table 2), less readily than L-cysteine and more so than S-methyl-L-cysteine or L-methionine. It also gave rise to sulphate to a greater extent than mercaptoacetic, dithiodiglycollic and ethylthioacetic acids, a similar situation to that observed in the whole animal (Maw, 1953). S-Phenylthioacetic acid was not oxidized to sulphate, and this has also been found to be the case in the whole rat (Maw, unpublished observations).

Fig. 1 shows the relationship between concentration of methylthioacetic acid (as the sodium salt) in the medium and the amount of sulphate formed, the latter being expressed as μ g. S/2 hr., from which the tissue sulphate 'blanks' have been subtracted. The wet weights of the tissue slices used were approximately 200 mg. The largest amount of sulphate was obtained at a level of methylthioacetic acid (sodium

salt) of 4–6 mg., corresponding to about 4% oxidation. At lower levels the percentage oxidation was higher, 6% at a level of 2 mg. and 10% at a level of 1 mg. This is of the same order as that obtained with equivalent amounts of cysteine, both in the present experiments and in those of Pirie (1934*a*) and Medes (1939).

Experiments with liver suspensions and acetone-dried powders

Attempts made to study the conversion of methylthioacetic acid into sulphate in more detail with suspensions in saline of fresh liver and acetone-dried liver powders, ranging in concentration from 2.5% to 10%, have so far been unsuccessful. Liver so treated completely lost its ability to oxidize the compound. Furthermore, addition of the following reagents either singly, in groups or altogether, did not restore the activity: glucose (7×10^{-3} M), MgCl_2 (10^{-2} M), MnCl_2 (10^{-3} M), phosphate buffer, pH 7.4 (1.2×10^{-3} M, used with bicarbonate saline only), DPN (3.8×10^{-4} M), cytochrome *c* (4.8×10^{-6} M), AMP (7.2×10^{-4} M) and sodium fumarate (5.4×10^{-3} M), the figures in brackets referring to the final concentrations of the reagents in each beaker. The last two reagents were used in place of ATP as in the experiments of Borsook & Dubnoff (1947).

The ability of suspensions of fresh liver or acetone-dried liver powders to oxidize mercaptoacetic acid was very much diminished, and in some experiments lost. An unfortified 2.5% suspension containing the same amount of tissue as used in the liver-slice experiments, was still able to oxidize L-cysteine, although only at about one-third to one-half the rate obtained with slices. DL-Homocysteine also formed sulphate under these conditions, but at a third of the rate of slices, whereas L-methionine was not oxidized, even in the presence of added phosphate, MgCl_2 , AMP, sodium fumarate and a methyl acceptor, such as nicotinamide or glycocyamine. S-Methyl-L-cysteine also was not oxidized.

DISCUSSION

Dubnoff & Borsook (1948) have shown that dimethylthetin chloride is demethylated by liver preparations under anaerobic conditions in the presence of homocysteine, one methyl group being lost. Dubnoff (1949) subsequently reported that the product was methylthioacetic acid, although no supporting experimental evidence was provided. Since dimethylthetin chloride is oxidized to sulphate in the whole rat, the present finding that no oxidation took place in liver slices, but that together with homocysteine it resulted in a greater formation of sulphate than from homocysteine

alone, suggests that demethylation is the initial step in the oxidation. This finds a parallel in the whole rat, in which out of a number of sulphonium compounds administered orally, only those which behaved as methyl donors to homocysteine were converted into sulphate (Maw, 1953). The apparently specific role of homocysteine in sulphate formation is in accord with the fact that it is the specific acceptor in methyl transfer reactions from glycine betaine and the thetins.

Liver suspensions were unable to oxidize methylthioacetic acid, in contrast to liver slices. It seems likely that the loss of activity in disintegrated tissue is due to dislocation of enzyme systems involved in the earlier stages of the breakdown, e.g. those concerned with the removal of the S-methyl group or with fission of the sulphur from the rest of the molecule. In the terminal stages of the oxidation the forms in which the sulphur is most likely to be released from organic combination are hydrogen sulphide or sulphur dioxide, and these are both oxidized by liver and other tissue preparations (Smythe, 1943; Heimberg, Fridovich & Handler, 1953). It is of interest that liver suspensions were also unable to oxidize methionine and S-methylcysteine, possibly for a similar reason.

A survey of the literature shows that oxidation to sulphate in the animal is restricted to a relatively small number of organic sulphur compounds. Thus, in addition to the sulphur-containing amino acids and some of their derivatives, only certain aliphatic thiols and related disulphides, certain aliphatic thioethers, generally containing the CH_3S — group, and a few miscellaneous sulphur compounds are oxidized in this way (Hill & Lewis, 1924; Westerman & Rose, 1928; Shen & Lewis, 1946; Fromageot, 1947). The compounds methylthioacetic acid and ethylthioacetic acid studied here, together with β -hydroxyethyl methyl sulphide (Maw, 1953) provide an addition to the group of thioethers which can give rise to sulphate, while the behaviour of S-phenylthioacetic acid in the whole rat and in rat-liver slices strengthens the original view of Hill & Lewis (1924) that aromatically bound sulphur is poorly oxidized, if at all. Sulphonium compounds which possess labile methyl groups must be considered as a further group of compounds capable of giving rise to sulphate. In the light of the present work it is suggested that there is no direct pathway for the oxidation of the sulphur of these compounds, but that oxidation only takes place after their conversion into thioethers by transmethylation. Their position in the general scheme of sulphur metabolism may therefore be compared with that of the sulphate-forming disulphides referred to above, which also require to undergo preliminary conversion to directly oxidizable compounds, in this case thiols.

SUMMARY

1. Dimethylthetin chloride incubated with rat-liver or -kidney slices in either bicarbonate or phosphate salines was not oxidized to sulphate, but the compound incubated with homocysteine gave rise to a larger amount of sulphate than was obtained with homocysteine alone.

2. Methylthioacetic acid was oxidized to sulphate by liver slices and to a lesser extent by kidney slices. Ethylthioacetic, mercaptoacetic and dithiodiglycollic acids were also oxidized by liver slices, whereas *S*-phenylthioacetic acid was not.

3. Suspensions of fresh liver or acetone-dried liver powders were unable to oxidize methylthioacetic acid. Addition of the following reagents singly, in groups, or altogether did not restore the oxidative ability of the tissue: $MgCl_2$, $MnCl_2$, glucose, diphosphopyridine nucleotide, cytochrome *c* and adenosine 5'-phosphate with sodium fumarate.

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Studies on the Volatile Fatty Acids of Sheep Blood with Special Reference to Formic Acid

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The production of volatile fatty acids (VFA's) in the rumen, and their subsequent absorption is well established. The recognition of the nutritional significance of these acids to the ruminant (cf. Phillipson, 1948) has led to many investigations of the VFA's in the blood of these animals. Reid (1950) made a careful study of the uptake of VFA from the peripheral circulation by the tissues of the sheep, and VFA levels in the blood of cattle were reported by McClymont (1951*a*). In earlier work values were reported for the concentration of circulating VFA in men and dogs (McClendon, 1944), and in the sheep, pig, pony and rabbit (Barcroft, McAnally & Phillipson, 1944). Dog blood was subsequently investigated by Phillipson (1947).

* This work was started while the author was seconded to the Rowett Research Institute, Bucksburn, Aberdeenshire.

Investigation of the nature of the blood VFA's, however, had to await the development of reliable methods of analysis, based on partition chromatography (Elsden, 1946; Moyle, Baldwin & Scarisbrick, 1948). Reid (1950), using chromatographic methods, found that about 90% of the VFA of sheep blood was acetic acid, and similar findings with respect to cattle blood were reported by McClymont (1951*a*). The VFA's of goat blood have recently been examined chromatographically by Craine & Hansen (1952).

In each of these chromatographic investigations of blood VFA's the acids were first distilled from mercuric oxide by the method of Friedemann (1938) to remove acidic substances which might interfere with the subsequent analysis. Formic acid is destroyed by this procedure. Furthermore, the