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QUANTITATIVE ESTIMATION OF SULFATES IN LIPID EXTRACTS

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SUMMARY

The existing methods for quantitative sulfatide determination are based on a determination of their characteristic group, the sulfate group. The necessity for hydrolysing or purifying the sulfatides before the sulfate determination, tends, however, to make these methods complicated and may also introduce errors.

A method for the determination of sulfate in lipid extracts has been worked out. The method is based on a reductive sulfate analysis. No hydrolysis step is necessary, and the method can be applied directly to a lipid extract.

INTRODUCTION

The presence of sulfur-containing lipids, sulfatides, in mammalian tissues has been known since the nineteenth century[1]. In 1933 BLIX[2] described the isolation of one such lipid, which he identified to be cerebron sulfuric acid ester. The more detailed structure of these lipids has later been elucidated by TANNHAUSER *et al.*[3], and by JATZKEWITZ[4]. The only known sulfur-containing lipids in mammalian tissues are sulfuric acid esters of cerebrosides. The existence of lipids containing both sulfate and phosphate has never been conclusively proved, and there are reasons to believe that the phosphorus that has been demonstrated in different sulfatide preparations comes from contaminating phospholipids[4,5].

Recently interest has been focused on this group of lipids. This has partly been due to the detection that the so-called metachromatic form of diffuse cerebral sclerosis is, in fact, a metabolic disorder, a sulfatidosis, which is characterized by the accumulation of sulfatides in different organs and the excretion of large amounts in the urine[6–8]. During the last years several investigations have been published in which the biosynthesis and metabolism of the sulfatides have been studied. This intensified study of the sulfatides has aroused the need for methods for their quantitative determination in biological materials. Such a method ought to be simple enough to allow the rapid analysis of large series of samples.

EXPERIMENTAL

Principle

The sulfate ester groups are split off and reduced to sulfide in one single step in a mixture of hydroiodic, hypophosphorus and hydrochloric acid. The reduction mixture is that described by ARCHER[9]. The hydrogen sulfide is led by a stream of nitrogen into

a buffered solution of bismuth nitrate, glycerol and gum arabic[10]. A bismuth sulfide sol is then formed, the absorption of which is measured in a spectrophotometer.

Apparatus

The apparatus was constructed chiefly according to ARCHER[9]: A ground test tube, a, 1.7 × 10.5 cm is connected to a small reflux condenser, b, with a gas inlet tube reaching almost to the bottom of the test tube. The upper end of the condenser is drawn out to a tube with two right-angle bends, d. Via a short piece of polyvinylchloride tubing this tube is connected to a capillary tube, e, which can be stuck down into another test tube, f, 1.5 × 16 cm. The tube a is placed in a water bath, g, which can be heated to boiling, and the tube f is placed in another termostated water bath, h, with a temperature of 27°. The gas inlet, c, is connected to a nitrogen bomb with a reducing valve. Sulfur-containing impurities of the nitrogen gas are removed by passing it through a flask with 30 % sodium hydroxide. For all tubing connections polyvinylchloride tubing was used, as rubber or latex tubes gave high blank values. A certain absorption of hydrogen sulfide seemed to take place in the tube between d and e when a fresh piece of tubing was used. After a few runs of a standard of 100 μg SO_4^{2-} no more of such absorption was observed. (It is, however, recommended to keep this tube as narrow and short as possible). For the regulation of the gas stream a pinchcock was mounted on the gas tubing leading to the apparatus. Ten apparatuses were run in parallel (Fig. 1).

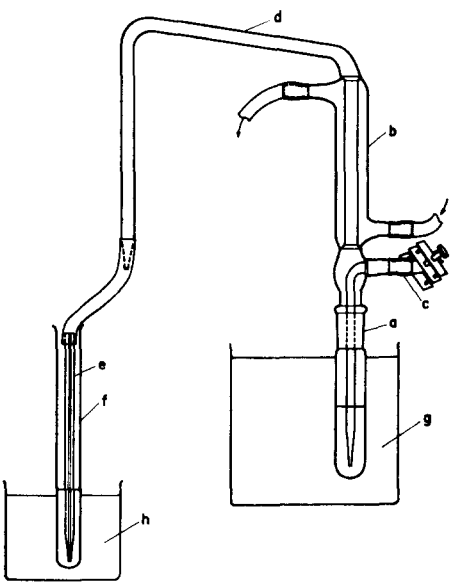

Fig. 1. The apparatus used for lipid sulfate determination.

Reagents

Reducing reagent: 250 ml hydroiodic acid, d = 1.7 (Merck p.a.), 62.5 ml 50 % hypophosphorus acid (Riedel de Haen), 250 ml hydrochloric acid (Merck p.a.), 125 ml hydroiodic acid, d = 1.96 (Merck p.a.) are mixed and boiled under reflux for 3 h with

a stream of nitrogen bubbling through the mixture. Any sulfate-containing impurities in the mixture will then be reduced. The reagent is stored in a dark bottle with a tight fitting glass stopper. It will keep its reducing properties for several months.

Bismuth nitrate reagent: 2.2 g $Bi(No_3)_3 \cdot 5H_2O$ (Merck DAB 6) is dissolved in 250 ml of 3.2 % mannitol (Merck). 80 ml redistilled glycerol (Merck p.a.), 360 ml of 2.5 % gum arabic (Riedel de Haen, purified) and 310 ml of 0.2 N acetate buffer with a pH of 4.8 (sodium acetate–acetic acid (65:35)) are added. The mixture is finally made up to 1000 ml with distilled water. The final pH shall then be 4.6. If necessary it has to be corrected to this value with glacial acetic acid or a saturated solution of sodium acetate. The reagent is stored in a dark bottle and is ready for use after two days of ageing. It will keep stable for two to three weeks.

Octanol-1

Absolute ethanol

Procedure

Lipid extracts containing 15–150 μg SO_4^{2-} are pipetted down into the ground test tubes a. The extracts are evaporated to dryness on a water bath under a stream of air or nitrogen. The lipids are then dissolved in 0.5 ml absolute ethanol under warming. To each tube is added 5 ml of the reducing reagent and 0.5 ml octanol-1 to prevent foaming. Into the test tubes f 4 ml of the bismuth nitrate reagent is pipetted, and to prevent foaming one drop of octanol-1 is added. The tubes are then connected to the apparatus as shown in Fig. 1 and nitrogen is bubbled through. The stream of nitrogen gas shall be so adapted that it precisely can keep up a continuous stream of bubbles in the test tubes (0.1–0.2 l/min for ten parallel apparatuses). The water bath g is now heated to boiling. After 30 min of boiling the process is stopped and the absorbancy of the bismuth nitrate reagent is read in a Beckman B spectrophotometer against water at 400 mμ (1 cm light path).

The sulfate values are read from a standard curve, which is prepared by running different amounts of a standard ammonium sulfate solution. With each series of samples a standard and a blank are run as controls.

Comments on the procedure

The reduction

Reducing reagent: Several methods exist in which the reducing properties of hydroiodic acid have been used for the quantitative determination of sulfate[9,11–15]. In the method here employed the reducing mixture has been reported to reduce sulfate quantitatively[9], and it has also a comparatively low concentration of the expensive hydroiodic acid.

Temperature: The reduction is performed at 100°, but the reduction goes quickly even around 90°. At room temperature practically no reduction takes place.

Time factor: After about 20 min of boiling no more hydrogen sulfide passed over to the bismuth nitrate reagent when a sample of ammonium sulfate was analysed. In the analysis of sulfatide preparations or lipid extracts the reduction time was even somewhat shorter. As a routine the reaction was run for 30 min.

Prevention of foaming: During the analysis of lipid extracts there was usually very heavy foaming. To prevent this octanol-1 was added. To obtain the desired effect in all cases as much as 0.5 ml was needed. This did not affect the results.

The hydrogen sulfide determination

Absorption curve: The absorption curve shows no maximum (Fig. 2). 400 mμ was chosen as a suitable wavelength for reading. At shorter wavelengths the extinction of the blank rises steeply.

Stability of the colour: The colour was stable for at least 2 h. After 24 h there was an increase in the absorption of about 10 %.

pH of the reagent: As a suitable pH of the bismuth nitrate solution 4.6 was recommended by KOREN AND GIERLINGER. To obtain this pH an acetate buffer of pH 4.8 was added instead of an acetate buffer of pH 5.2, as described in the original paper by KOREN AND GIERLINGER[10]. This discrepancy might depend upon different qualities of the other chemicals in the reagent. It is therefore recommended to titrate out the pH of the acetate buffer that will give the desired final pH. How the absorbancy varies with pH can be seen from Fig. 3. At higher pH the reagent was unstable.

Temperature of the reagent: Fig. 4 shows the influence of the temperature of the reagent on the absorbancy. The reaction has been run at 27°, which is about on the middle of the horizontal part of the curve.

Trap efficiency: The trap efficiency of the reagent was tested by letting the hydrogen sulfide containing gas mixture first pass through one tube with reagent and then through a second one. No bismuth sulfide could be detected in the second tube.

Prevention of foaming: Foaming of the bismuth nitrate reagent was prevented by adding one drop of octanol-1. Octanol-2 could not be used as it made the reagent turbid.

Regulation of the gas stream: It is of greatest importance that the gas stream through the bismuth nitrate reagent is carefully adjusted. If the bubbling in the tubes is too heavy, the condensor will be insufficient, and vapours and droplets from the reduction mixture will pass over into the bismuth nitrate reagent causing a turbidity.

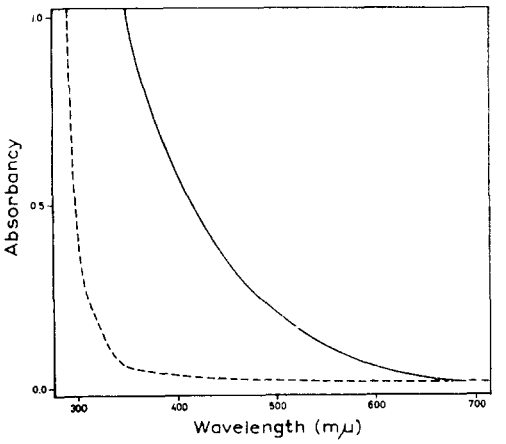

Fig. 2. Absorption curves of the bismuth sulfide sol, given by 100 μg SO_4^{2-}, ——, and of the reagent blank, – – –. These curves were read against water in a Cary No. 14 spectrophotometer.

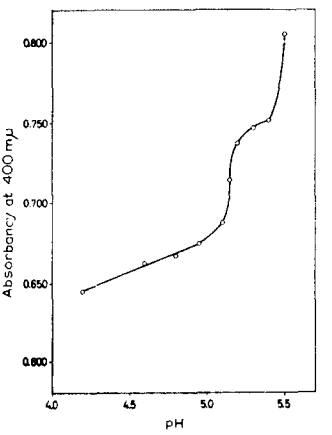

Fig. 3. Change in absorbancy with pH of the bismuth nitrate reagent. The absorbancy was read against a reagent blank at 400 mμ with 100 μg SO_4^{2-}.

If the gas stream, on the other hand, is too slow, there will be precipitation of bismuth sulfide on the inside wall of the capillary e, and consequently too low values.

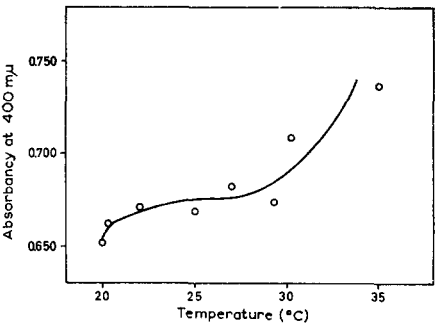

Fig. 4. Change in absorbancy with the temperature of the bismuth nitrate reagent. The absorbancy was read against a reagent blank at 400 mμ with 100 μg SO_4^{2-}.

Standard curve and accuracy of the method

The standard curve was a straight line up to an absorbancy of at least 1.500.

100 μg SO_4^{2-} gave a mean absorbancy of 0.739 against water, $\sigma = 0.009$ (23 determinations, 16 different runs). The blank value was 0.036, $\sigma = 0.005$ (16 determinations, 16 runs). These values were obtained with the same preparation of bismuth nitrate reagent. Different reagent preparations could vary in their mean absorbancy values within about 10 %. Thus it was necessary to make a new standard curve for each reagent preparation.

Application on sulfatides

With the reduction method employed, the sulfate group of the sulfatides was reduced to sulfide without any previous hydrolysis.

Samples of sulfatides from two different sulfatide preparations from human brain (the samples being a gift from Dr. L. SVENNERHOLM) were analysed for sulfate. The hexose content was determined according to SVENNERHOLM[16], and C, H and S were determined at the micro-analysis laboratory of Uppsala. The result can be seen in Table I.

No theoretical values can be given, as the exact fatty acid composition of the preparations was not known.

TABLE I

ANALYSIS VALUES OF TWO DIFFERENT SULFATIDE PREPARATIONS

	Microanalysis values %				SO_4^{2-} *determined with the authors method)* %	*Hexose (as galactose)* %	*N:SO_4^{2-}:Hexose*
	C	*H*	*N*	*S*			
Sulfatide preparation I (905/6)	64.7	10.5	1.56	3.37	10.3 (3.43% S)	19.2	1.04:1.01:1
Sulfatide preparation II* (Lindberg)	57.6	9.64	1.42	2.92	8.53 (2.84% S)	16.2	1.12:0.99:1

* This substance was isolated after chromatography on silicic acid, and contains about 12 % inorganic matter.

To estimate the recovery, known amounts of sulfatide preparations were added to lipid extracts of different origins and the mixtures analysed (see Table II).

TABLE II

RECOVERY OF SULFATIDES ADDED TO LIPID EXTRACTS

Source of lipid extract	*SO_4^{2-} in lipid extract (μg)*	*SO_4^{2-} in added sulfatides (μg)*	*Recovery of added sulfatide sulfate (μg)*	*Recovery (%)*
Human brain				
Total lipid extract	52.1	53.0	52.1	98.3
Total lipid extract	52.1	106.0	106.8	100.8
Total lipid extract	52.1	158.9	156.4	98.4
Total lipid extract	82.8	66.1	65.7	99.4
Impure mixture of neutral sphingolipids (10.6 mg)	5.5	59.1	58.9	99.7
Human kidney				
Total lipid extract	46.6	46.0	42.4	92.1

Analysis of lipid extracts

Accuracy: 4.50 mg of a lipid extract from calf brain gave a mean absorbancy of 0.122, $\sigma = 0.009$ (28 determinations, 16 different runs), and 25.70 mg of the same extract gave a mean absorbancy of 0.586, $\sigma = 0.032$ (27 determinations, 9 different runs).

Non-lipid sulfate: A certain amount of non-lipid sulfate compounds are extracted with lipids. The amounts and the nature of the substances will probably vary greatly from organ to organ. Of special interest in this regard are the kidneys, where several compounds are excreted as sulfate esters. Lipid extracts from this organ can therefore be expected to have a high concentration of non-lipid sulfate.

When determining sulfatide sulfate, non-lipid sulfate must be eliminated. This can be accomplished with the solvent partition method of FOLCH[17]. A detailed description for the general application of the FOLCH method cannot, however, be given. The method has to be tested out separately for each different kind of tissue and extraction method employed.

The application of the sulfate method for sulfatide determinations will appear later.

Interference from sulfur-containing amino acids: The sulfur in a lipid extract from mammalian tissue is not derived from sulfate groups only, but also from sulfur-containing amino acids. To free a lipid extract from amino acids is, as a rule, a complicated task. To test whether sulfur-containing amino acids would interfere with the sulfate determination, large amounts, 300–400 μg (*i.e.* about 50–75 μg of sulfur) of cysteine, cystine and methionine were analysed. None of the sulfur in the amino acids was recovered as hydrogen sulfide. The same held true when sulfur-containing amino acids were added to lipid extracts and the mixtures were analysed.

DISCUSSION

The characteristic group of the sulfatides, the sulfate group, can principally be determined in three different ways:

By direct spectrometric determination

WITTMER AND AUSTIN[18] have described a method whereby the sulfuric acid ester group is measured by its infrared absorption. This method cannot be regarded as suited for serial analyses. It is time consuming and cannot be directly applied to a lipid extract without first separating the sulfatide fraction from the bulk of lipids. A correction for phosphate is also necessary.

By precipitation of the sulfate as benzidin sulfate, barium sulfate etc. and determining either the precipitated product, the surplus of the precipitating agent or other products formed

Before precipitation, the sulfate group must be split off. This can easily be achieved when the sulfatides are in the pure state, but when they occur in lipid extracts mixed with other lipids they are rather resistant to hydrolysis. The phospholipids probably exert a protecting influence[5]. Thus it may be difficult to find a simple and suitable hydrolysis method that is strong enough to split off the sulfate group quantitatively without partly oxidising to sulfate the sulfur in the sulfur-containing amino acids. Another disadvantage is that the hydrolysing agent and perhaps even other interfering substances must be neutralized or removed before the precipitation.

The benzidine method has been employed by LEES AND FOLCH[19] for determination of total sulfur in lipid extracts after oxidation with *aqua regia*. The barium chloranilate method of BERTOLACINI AND BARNEY[20] has been tried by the author, but it was impossible to obtain satisfying hydrolysis conditions. It has, however, been employed by GREEN[21] for determination of sulfate in purified sulfatide preparations. LONG AND STAPLES[22] have determined the sulfate in lipid extracts with 4-amino-4-chlorodiphenyl hydrochloride as described by JONES AND LETHAN[23] after hydrolysis with hydrochloric acid.

By reduction of sulfate to sulfide and determination of the hydrogen sulfide formed

This type of reaction was first described by LORANT[11] and modified by ARCHER[9]. It has been used by BAKKE AND CORNATZER[24] in ARCHER's modification for sulfatide-sulfur determination, but after hydrolysis with *aqua regia*, which made it necessary to introduce a series of steps to remove peptide material in the lipid extract. The titrimetric hydrogen sulfide determination in this method was also rather complicated.

In the present method the reducing agent acts without foregoing hydrolysis. Amino acid sulfur does not interfere either. Thus, two important sources of errors are eliminated. At the same time the method becomes simpler and takes less time to perform.

For hydrogen sulfide determination there are several very sensitive both photometric and titrimetric methods described. Among the photometric methods the methylene blue method seems to be the most sensitive one, and probably the one most employed. An attempt was made to utilize it in the modification of GUSTAVSSON[15]. The method was, however, found to be technically difficult to handle, and it was hard to get reproducible results, so it was abandoned. The hydrogen sulfide determination method here adapted is less sensitive. However, its simplicity and the rapidity with which the measurements can be performed makes it very suitable for serial analyses.

For sulfatide sulfate determination other sulfate-containing substances have to be removed. As has been pointed out, the method chosen for this must be tested out for each different kind of tissue and preparation of lipid extract.

To calculate the amount of sulfatide, it is necessary to know the structure and fatty acid composition of the sulfatides in the tissue to be investigated. Thus far sulfatides from brain tissue only have been isolated and analysed for structure. Probably the only sulfatides in brain tissue are sulfuric acid esters of cerebrosides, which here are the dominating neutral glycolipid. Di- and tri-hexosides are, however, known to occur in considerable amounts in erythrocytes[25], spleen[26], liver, and kidney[27]. The possibility that sulfate esters of di- and tri-hexosides exist can therefore not be excluded.

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